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Sex- and Age-Related Differences in Ceramide Dihexosides of Primary Human Brain Tumors

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ABSTRACT: Neutral glycolipids (NGL) are promising diagnostic markers of human gliomas, but differences in NGL with age and sex have not been examined. Previous work demonstrated that ceramide dihexosides (CDH) levels in mouse kidney are age- and sex-dependent, probably due to levels of sex hormones. We quantitated CDH in 181 human gliomas and found significant differences with sex and age, particularly menopause and male puberty. This emphasizes the importance of assessing results of studies on glycolipids in disease states with respect to age and sex in order to avoid erroneous conclusions concerning the relationship of glycolipid composition with diagnosis and pathogenesis.

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Neutral glycolipids (NGL) are ubiquitous within the animal kingdom. Although the specific biological functions of most NGL are unknown, evidence exists that specific ones function as receptors for microorganisms and some bacterial toxins (1–3), and that sialylated glycolipids (gangliosides) can modulate the activities of several receptors (4). Additional evidence for the biological importance of glycolipids is that they undergo major compositional changes with growth, differentiation, and many disease processes including malignant transformation (5–11). The NGL of some tissues also differ between males and females. Such differences have been most extensively studied in murine kidney, in which the most consistent change is that males have more ceramide dihexosides (CDH) than females (12,13), a difference most pronounced after puberty (14). A similar difference occurs in normal human thyroid (15). Although it is well documented that many types of tumors have NGL compositions different from those of the normal tissues from which they were derived (11), we are unaware of reports of a difference in NGL composition within the same tumor type that correlates with patient sex or age. In an ongoing comprehensive study of NGL in human brain tumors, we found a striking sex-related difference in the amount of CDH within these tumors. This novel finding forms the basis of this communication.

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Abbreviations: CDH, ceramide dihexosides; CDH-L, lower band of CDH; CDH-T, total of upper and lower bands of CDH; CDH-U, upper band of CDH; GalCer, galactosylceramide; $GaOse_2Cer$, galabiglycosylceramide, Gal(α1-4)Gal(β1-1)Cer; H and E, hematoxylin and eosin; Hfa, Hydroxy fatty acid; HPTLC, high-performance thin-layer chromatography; LacCer, lactosylceramide; Nfa, nonhydroxy fatty acid; NGL, neutral glycolipids; Sph, sphingosine.

EXPERIMENTAL PROCEDURES

Tissues were removed at surgery and frozen in liquid nitrogen, in most cases within 30 min of excision. To be certain that it consisted of tumor, a small portion of this tissue was processed for microscopy as a frozen section, stained with hematoxylin and eosin (H and E) for histological study by a neuropathologist (AJY). Other portions of the tumor were prepared for diagnostic histology by formalin fixation, paraffin embedding, and staining with H and E and other methods as required. Three neuropathologists (BWS, PCB, and AJY) independently examined representative slides of all tumors. With respect to diagnosis and grade, the majority opinion was accepted.

Frozen tissues were thawed, weighed, lyophilized overnight, desiccated for 1 h, and then weighed again. Dried samples were homogenized in 20 vol of chloroform/methanol/water (1:2:0.75) and insoluble materials removed by centrifugation. The supernatant was removed, the pellet rehomogenized in chloroform/methanol/water (1:1:0.1), and the homogenate centrifuged. The supernatant was pooled with the first supernatant after which the pooled sample was dried under nitrogen and reconstituted in chloroform/methanol/water (30:60:8). Neutral lipids were first separated from acidic lipids on a DEAE-Sephadex column (3-mL bed volume) by eluting them with 30 mL chloroform/methanol/water (30:60:8) and then taken to dryness. NGL were purified using an acetylation technique based on that of Saito and Hakomori (16) with two modifications: neutralization was with 0.01 N HCl; neutralized samples were then taken to dryness, dissolved in chloroform and desalted by centrifugation from chloroform. Total NGL content was estimated using the orcinol method of Neskovic *et al.* (17) with galactose serving as a standard. Individual NGL were separated on silica gel 60 high-performance thin-layer chromatography (HPTLC)

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plates in chloroform/methanol/water (65:25:4), visualized with diphenylamine (18), and quantitated by scanning densitometry at 580 nm in reflectance mode. Values for NGL were corrected based on external standards carried through the entire extraction, purification, and quantitation procedure.

RESULTS

The total number of tumors studied was 181, including 45 oligodendrogliomas, 92 astrocytomas, 15 oligo-astrocytomas, and 29 other primary brain tumor types. The last consisted of 9 primitive neuroectodermal tumors, 4 ependymomas, 4 anaplastic ependymomas, 1 subependymoma, 1 gangliocytoma, 1 ganglioglioma, and 9 unclassifiable primary brain tumors. Of all specimens, 69 arose in females and 112 in males.

On HPTLC the CDH from all tumors ran as a doublet, the more rapidly migrating upper band (CDH-U) frequently constituting more than half of the total CDH (CDH-T) (Fig. 1). Data regarding the amounts of CDH-U (expressed as μg hexose/mg tissue dry weight) for all tumors showed that the median values for males (16.9) were twice those of females (8.3). There were similar differences between median values for the lower band of CDH (CDH-L) in males (17.2) and females (10.3), and for CDH-T in males (35.4) and females (16.4). However, these sex differences vary among different age groups (Table 1). CDH levels are equal in both sexes at ages less than 14 yr. After that, CDH levels increase in males but not in females until after 50 yr of age (Table 1). Expressed on a log scale, values for CDH-T were subjected to an analysis of variance with the least squares difference post hoc test and an experiment-wise error rate of less than 5%. This showed that there was significantly more CDH-T in males over 50 yr than in each of the other age–gender groups. Furthermore, females 14 to 50 yr of age had significantly less CDH-T than either 14- to 50-yr-old males, or females over 50 yr of age. All other pair-wise differences were not statistically significant. A similar pattern of statistically significant differences was seen for CDH-U, CDH-L (Table 1) for all tumors and for CDH-T (Table 2) in astrocytomas. The same trend occurred in the remaining three diagnostic categories (oligodendrogliomas, oligo-astrocytomas, and other tumors), but there were too few patients under 14 and over 50 yr in these groups to permit a meaningful statistical analyses.

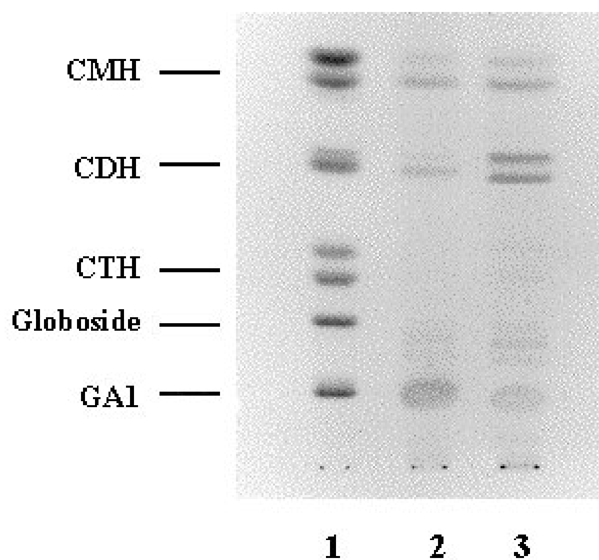

FIG. 1. Total neutral lipids isolated from glioblastoma multiforme tissues separated by high-performance thin-layer chromatography and detected by diphenylamine reagent. Each lane contains a total of 6 μg total hexose. Lane 1, standards; lane 2, female 69 years of age; lane 3, male, 83 years of age. CMH, ceramide monohexoside; CDH, ceramide dihexosides; CTH, ceramide trihexoside; GA1, asialo-GM1.

DISCUSSION

In the TLC solvent system used in our study, galabiglycosylceramide ($GaOse_2Cer$) and lactosylceramide (LacCer) co-migrate (15). Owing to limited amounts of specimens, we were unable to determine the relative contributions of these to the CDH in either the upper or lower bands, which separated on the basis of fatty acid content. However, evidence exists that in some tissues the contents of both types of CDH are greater in postpubertal males than in females either before or after puberty (14). In some mouse strains the CDH in kidney is $GaOse_2Cer$, whereas in others it is LacCer (12,14). Regardless of which type of CDH is present, a sex-related difference exists in the amount of CDH in the kidneys of all mouse strains studied (12–14). Although prior to puberty the CDH content of mouse kidney is the same in both sexes, postpubertal males had considerably larger amounts of CDH than females (14). The amount of galabiglycosylceramide in kidney cells cultured from C57BL/6J male mice is also greater than in cells cultured from females (19). Experimental evidence indicates that these sex-related differences are due to sex hormones. Administration of testosterone to adult female C3H/He, C57/BL, and C57BL/6J mice induced a marked increase in total kidney NGL, especially CDH (13,20). In kidneys of C57BL/6J mice there was a significant increase in $GaOse_2$-Sph-Nfa, a "male-specific glycolipid" (13). In the solvent system we used, this glycolipid migrates in the upper band of CDH (CDH-U), the CDH we found to be present in the largest amounts in tumors from postpubertal males. Estrogen administration to male rats also affected the NGL of kidney, thus indicating that it too can affect glycolipid metabolism (21). Perhaps this explains the increase in CDH in tumors from postmenopausal females (Tables 1 and 2). Whether or not there is a gender-related difference in sex hormone receptors in human gliomas is not known, but there is no gender-related difference in histological appearance of human gliomas.

The sex-related differences in CDH appear to be the result of differences in the activities of glycosyltransferases responsible for the synthesis of GalCer and CDH (22). With respect to our work, it is significant that the following were all elevated in male kidneys: glucosylceramide:UDP-Gal galactosyltransferase; Nfa-Ceramide:UDP-Gal galactosyltrans-

TABLE 1
Sex- and Age-Related Difference in Upper and Lower Bands of CDH

			CDH-U[a]		CDH-L[a]		CDH-T[a]	
Age (yr)	Sex[b]	N[c]	Median	Mean ± SE	Median	Mean ± SE	Median	Mean ± SE
Under 14	F	16	8.2	14.0 ± 4.8	6.8	10.9 ± 3.6	15.6	20.6 ± 5.2
	M	17	8.5	10.8 ± 2.0	5.9	7.8 ± 1.4	14.8	20.2 ± 4.2
14–50	F	33	6.8	12.3 ± 3.2[f]	8.2	11.4 ± 2.6[f]	15.0	22.3 ± 4.8[g]
	M	47	16.0	20.6 ± 3.2	15.5	18.6 ± 2.7	29.6	34.2 ± 4.3
Over 50	F	20	14.4	24.7 ± 6.7	19.1	24.3 ± 6.2	37.2	48.6 ± 11.9
	M	48	29.4	33.2 ± 3.7[d]	26.6	28.0 ± 3.2[e]	55.3	61.9 ± 5.9[d]

[a]CDH-U (probably Nfa-LacCer) and CDH-L (probably Hfa-LacCer) refer to the upper and lower bands, respectively, of ceramide dihexosides (CDH) that separated on thin-layer chromatography in chloroform/methanol/water (65:25:4). CDH-T is the total of these two bands. Values are in mg hexose/mg tissue dry weight.
[b]F and M are female and male, respectively.
[c]N is the number of individual tumors analyzed.
[d]Significantly greater values than all other age–sex groups (experiment-wise $P < 0.05$).
[e]Significantly greater values than all other age–sex groups except females >50 (experiment-wise $P < 0.05$).
[f]Significantly ($P < 0.05$) lower values than males 14–50 yr of age and marginally significantly ($P \approx 0.06$) lower than females >50.
[g]Significantly ($P < 0.05$) lower values than males 14–50 yr of age and females >50.

ferase; Hfa-Galcer: UDP-Gal galactosyltransferase. These differences in enzyme activities would cause an increase in the synthesis of both Hfa-CDH and Nfa-CDH by males, with an even greater synthesis of the latter. A similar sex-related difference in enzyme activities could be responsible for our finding of greater amounts of CDH (more pronounced for CDH-U than CDH-L) in tumors from males than of females. Also consistent with our findings are reported sex-related differences in CDH of human thyroid (15). Male thyroid contains significant amounts of both $GaOse_2Cer$ and LacCer, whereas female thyroid has only small amounts of the former. Although there is very little CDH in normal human brain, most of it is LacCer (23). To our knowledge there has been no study comparing amounts and types of CDH in brains of males and females. Seyfried *et al*. (24) have found that infiltrating inflammatory cells can alter the glycolipid composition of brain tumors implanted into the flank of animals, but they did not report studying differences based on the sexes of the host animals. Nevertheless, attempts to identify the cellular location of glycolipids in tumors growing *in vivo* must take their findings into account.

TABLE 2
Sex- and Age- Related Differences in Total CDH in Astrocytomas[a]

Age (yr)	Sex	N	Median	Mean ± SE
Under 14	F	10	14.7	24.0 ± 8.3
	M	7	14.1	20.9 ± 8.7
14–50	F	12	12.4	22.23 ± 7.0[c]
	M	17	41.8	45.6 ± 7.5
Over 50	F	14	54.2	66.1 ± 14.5[d]
	M	32	67.8	72.8 ± 7.2[b]

[a]Abbreviation and units are as in Table 1.
[b]Significantly greater values than all other age–sex groups except females >50 (experiment-wise $P < 0.05$).
[c]Significantly ($P < 0.05$) lower values than males 14–50 yr of age and females >50.
[d]Significantly ($P < 0.05$) greater values than females <14 and marginally significantly ($P \approx 0.06$) greater than males <14.

There have been few investigations of NGL in primary tumors of the human central nervous system, but those published report that CDH is frequently increased in such neoplasms. Based on small series of astrocytic tumors, Eto and Shinoda (25) and Wiegandt and colleagues (26,27) found CDH to be increased in those of higher histological grade. Singh *et al*. (28) performed glycolipid analyses on 98 primary human brain tumors of several types. The only significant association they found was that anaplastic astrocytomas had higher concentrations of CDH-U, CDH-L, and CDH-T than any of the other tumor types. In none of these studies did the investigators report a difference in the amounts of CDH between tumors from males and females. In contrast, our findings underscore the importance of analyzing such data for sex- and age-related differences in that they may mask important differences in glycolipid composition among tumor types.

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n-3 and n-6 Fatty Acid Enrichment by Dietary Fish Oil and Phospholipid Sources in Brain Cortical Areas and Nonneural Tissues of Formula-Fed Piglets

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ABSTRACT: Sufficient availability of both n-3 and n-6 long-chain polyunsaturated fatty acids (LCPUFA) is required for optimal structural and functional development in infancy. The question has been raised as to whether infant formulae would benefit from enrichment with 20 and 22 carbon fatty acids.To address this issue, we determined the effect of fish oil and phospholipid (LCPUFA) sources on the fatty acid composition of brain cortical areas and nonneural tissues of newborn piglets fed artificially for 2 wk. They were fed sow milk, a control formula, or the formula enriched with n-3 fatty acids from a low-20:5n-3 fish oil added at a high or a low concentration, or the formula enriched with n-3 and n-6 fatty acids from either egg yolk- or pig brain-phospholipids. Both the fish oil- and the phospholipid-enriched formula produced significantly higher plasma phospholipid 22:6n-3 concentrations than did the control formula. The 22:6n-3 levels in the brain, hepatic, and intestinal phospholipids were significantly correlated with plasma values, whereas cardiac 22:6n-3 content appeared to follow a saturable dose-response. Feeding sow milk resulted in a much higher 20:4n-6 content in nonneural tissues than did feeding formula. Supplementation with egg phospholipid increased the 20:4n-6 content in the heart, red blood cells, plasma, and intestine in comparison to the control formula, while pig brain phospholipids exerted this effect in the heart only. The addition of 4.5% fish oil in the formula was associated with a decline in 20:4n-6 in the cortex, cerebellum, heart, liver, and plasma phospholipids, whereas using this source at 1.5% limited the decline to the cerebellum, liver, and plasma. Whatever the dietary treatment, the phosphatidylethanolamine 20:4n-6 level was 10–20% higher in the brain temporal lobe than in the parietal, frontal, and occipital lobes in the temporal lobe by administering the formula enriched with egg or brain phospholipids.

In conclusion, feeding egg phospholipids to neonatal pigs increased both the 22:6n-3 content in the brain and the 20:4n-6 content in the temporal lobe cortex. This source also increased the 22:6n-3 levels in nonneural tissues with only minor alterations of 20:4n-6. These data support the notion that infant formulae should be supplemented with both 22:6n-3 and 20:4n-6 rather than with 22:6n-3 alone.

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Long-chain polyunsaturated fatty acids (LCPUFA) with 20 and 22 carbon atoms, mainly 20:4n-6 and 22:6n-3, are integral constituents of the central nervous system and are thought to play a major role in the development of neural functions during the late fetal and early postnatal periods (1). LCPUFA are necessarily provided by the diet, either directly or in the form of their precursors with 18 carbon atoms, i.e., 18:2n-6 and 18:3n-3. The developing brain prefers preformed 22:6n-3 over 18:3n-3 for fatty acid uptake from blood and brain phospholipid synthesis (2), suggesting that preformed LCPUFA in the diet may be better for securing optimal brain function.

Human milk contains both precursors and LCPUFA (3), whereas conventional infant formulae are almost free of LCPUFA, owing to the fact that their lipid component is essentially constituted by vegetable and oleo oils which naturally contain only fatty acids up to 18 carbons. The need to supplement infant formulae with preformed LCPUFA from other lipid sources continues to be debated vigorously.

Preferential depletion of 22:6n-3 has been found in the cerebral cortex of infants who died from sudden death syndrome fed conventional formulae (4–6), and clinical trials have reported that the addition of preformed LCPUFA in the formula may improve performance on early mental development tests in comparison to conventional formula feeding (7,8). Blood 22:6n-3 content and visual acuity both correlate with the dietary supply of preformed 22:6n-3 (9), although normal visual development also occurs when the formula is fortified solely with 18:3n-3 at the level of at least 2% by weight of total fatty acids (10,11). Both preterm and full-term infants can synthesize LCPUFA from their 18-carbon precursors through the Δ6- and Δ5-desaturation pathways (12–14). However, formula-fed full-term infants are unable to match the n-3 and n-6 LCPUFA status of breast-fed full-term infants until at least 2 mon after birth (15), and adding preformed

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Abbreviations: LCPUFA, long-chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE: phosphatidylethanolamine; PI, phosphatidylinositol; RBC, red blood cells; SM, sow milk.

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22:6n-3 to formulae is undoubtedly more effective than increasing 18:3n-3 to maintain blood phospholipid 22:6n-3 levels similar to those in breast-fed infants (16). Moreover, an adverse effect of lowering the 18:2n-6 to 18:3n-3 ratio on the weight gain of term infants has been recently reported (17), showing that the physiological results of high 18:3n-3 intake in early infancy has to be more accurately evaluated. Dietary LCPUFA may be considered indispensable nutrients for the formula-fed neonate if it is admitted that the lipid status of breast-fed infants is significant.

Enrichment of term infant formulae with preformed LCPUFA at typical breast milk levels has been recently acknowledged as a goal by the Commission of the European Union (18). The question of the nature of the lipid source to use, and whether both 20:4n-6 and 22:6n-3 must be added, remains partly unsolved. Two options may be considered: supplementing either with n-3 LCPUFA only or with both n-6 and n-3 LCPUFA. With this perspective, we studied the effects of adding LCPUFA from different dietary sources, in amounts similar to those found in average human milk, upon the LCPUFA status in the piglet model (19).

We have experimented with two types of LCPUFA enrichment: either by supplementing n-3 without n-6 LCPUFA using fish oil triglycerides, or by supplementing both n-3 and n-6 LCPUFA using animal phospholipids. In the first case, a low-20:5n-3 fish oil was chosen in an attempt to circumvent the competing effect between 20:5n-3 and 20:4n-6 for phospholipid acylation and metabolic conversion. The effect of an excess supply of n-3 LCPUFA was also examined by adding the fish oil in higher amounts than needed. In the second case, we compared two different phospholipid sources purified either from hen egg yolks or from pig brains. The direct supply of the LCPUFA found in natural phospholipids could be particularly beneficial. The developing brain preferentially uses 22:6n-3 from plasma lysophosphatidylcholine (lyso-PC) over unesterified 22:6n-3 for the synthesis of brain phospholipids (20). Some activity of dietary phosphatidylserine on memory retrieval and exploration in mice has been found (21), and the use of this phospholipid has been considered for treating memory loss in later life (22). In infant nutrition, the main advantage of dietary phospholipids would be the simultaneous supply of LCPUFA from both series. Eggs produced by hens reared in free-range conditions have almost equal amounts of 20:4n-6 and 22:6n-3 and are therefore particularly suited to infant feeding (23). The LCPUFA from egg lipids are absorbed as effectively as from human milk (24).

A conventional infant formula was supplemented with the low-20:5n-3 fish oil (ratio of 20:5n-3/22:6n-3 < 1) added either at a low or high concentration (n-3 LCPUFA < 1% and > 1%, respectively), or with the phospholipid concentrate containing similar levels of 20:4n-6 and 22:6n-3 (n-6 LCPUFA < 2%, n-3 LCPUFA < 1%, 20:5n-3/22:6n-3 < 1). After 2 wk, we analyzed the phospholipid fatty acid compositions of the red blood cells (RBC), blood plasma, liver, intestine, heart, cerebellum, whole cerebral cortex, and individual cortical areas of the piglets.

MATERIALS AND METHODS

Animals and milk replacer diet. Official French regulations (Nos. 87 848 and 03 056) for the care and use of laboratory animals were followed. Nonlittermate piglets (Pietrain × Large White) were fed from birth to 14 d of age with either sow milk (group SM), a conventional infant formula (group F), or an LCPUFA-enriched formula, according to the environmental conditions previously described (25). The weight of the newborn piglet brain gains 40% within 2 wk after birth, whereas the same relative gain requires 9 wk in the human term infant. On the basis of this single criterion, one might say that the 2 wk-postpartum period in piglet is comparable to the 9 wk-postpartum period in the term infant, the latter period corresponding to the mean duration of breast-feeding in France.

Seven nonlittermate piglets were fed sow milk (group SM). Five to eight nonlittermate piglets were randomly assigned to one of the five formula-feeding groups after receiving the colostrum for 12 h. Standard sow milk contained LCPUFA of both the n-6 and n-3 series: the major n-6 LCPUFA was 20:4n-6, and 22:5n-3 was the major LCPUFA of the n-3 family (Table 1).

The basic milk-replacer powder was manufactured by Diépal-nsa (Steenvoorde, France). It was similar to an infant formula, but the nutrient formulation was adapted to meet the specific energy requirement of the piglets, i.e., 4200 kJ/L. The formula fat was composed of a blend of palm, coconut, sunflower, and soybean oils. The LCPUFA enrichments were obtained by adding either the low-20:5n-3 fish oil (groups R and R+), egg yolk phospholipids (group E), or pig brain phospholipids (group P). Total lipids in all formulae accounted for 30% by weight of the dry matter. The final fatty acid compositions are given in Table 1. The different formulae supplied similar amounts of 18:2n-6 and 18:3n-3, i.e., 16.5 to 18% and 1.1 to 1.3% of total fatty acids, respectively.

Low-20:5n-3 fish oil supplementation (groups R and R+). A 22:6n-3-rich marine oil product (gift from Produits Roche, Neuilly-sur-Seine, France), with a low 20:5n-3 to 22:6n-3 ratio, was added to the formula in the proportion of either 1.5% by weight of total lipids (group R) or 4.5% (group R+). The final 22:6n-3 and 20:5n-3 contents were 0.3 and 0.1% in formula R, and 0.9 and 0.3% in formula R+, respectively. Arachidonic acid was detectable only in formula R+ (20:4n-6 < 0.1% of total fatty acids).

Egg yolk phospholipid supplementation (group E). Egg yolk lipids have been suggested as a source of LCPUFA in infant feeding (23). Eggs were harvested from hens fed a diet containing 4% by weight of rapeseed oil (26). The yolk phospholipids were prepared by Institut Yves Ponroy (Bailly, France). The phospholipid concentrate was composed of 67% PC, 24% phosphatidylethanolamine (PE), 6% phosphatidylinositol (PI), and 3% sphingomyelin, after class separation using high-performance liquid chromatography and detection by light scattering (see below). It was stored at −80°C under nitrogen until use. The 20:4n-6, 22:6n-3, and 20:5n-3 con-

TABLE 1
Fatty Acid Composition (wt% of total fatty acids) of Sow Milk and Formula Diets

	SM[a]	F[b]	P[c]	E[d]	R[e]	R+[f]
6:0	0	0.2	0.1	0.2	0.1	0.1
8:0	0	2.4	1.8	2.8	1.8	1.9
10:0	0.1–0	2.0	1.5	2.0	1.6	2.3
12:0	0.1–0	14.3	11.8	11.9	12.4	13.3
14:0	1.5–2.0	5.8	5.2	4.9	5.6	5.4
16:0	22.5–22.7	18.7	18.0	20.1	19.5	18.9
18:0	7.5–6.1	5.4	5.3	6.8	3.9	5.3
20:0	0.1–0.1	0.3	0.3	0.3	0.3	0.3
Saturated	31.7–31.0	49.0	44.0	49.0	45.2	47.4
16:1n-9	1.2–0.7	0.1	0.1	0.2	0	0
16:1n-7	3.6–5.2	0	0.2	0.1	0.1	0.4
18:1n-9	44.0–45.1	31.3	34.2	30.7	33.3	31.9
18:1n-7	3.2–2.7	0	1.0	0.2	0	0.1
MUFA ≥ 20 C	0.7–0.8	0	0.4	0.2	0.3	0.4
MUFA	52.8–54.4	31.5	35.9	31.4	33.7	32.8
18:2n-6	12.7–12.0	17.6	17.9	17.1	18.4	16.6
20:2n-6	0.4–0.4	0	0.1	0	0	0.1
20:4n-6	0.9–0.7	0	0.4	0.5	< 0.1	0.1
Σ n-6	14.0–13.1	17.6	18.4	17.6	18.4	16.8
18:3n-3	0.9–1.0	1.3	1.3	1.2	1.3	1.3
20:5n-3	0.1–0.1	0	0	0	< 0.1	0.3
22:5n-3	0.4–0.3	0	0	0.1	0	0.1
22:6n-3	0.2–0.1	0	0.4	0.4	0.3	0.9
Σ n-3	1.6–1.5	1.3	1.7	1.6	1.7	2.5
18:2n-6/18:3n-3	13.6–12.6	13.5	14.0	14.3	14.2	12.8
20:4n-6/22:6n-3	4.0–7.4	0	1.0	1.3	0.1	0.1
20:5n-3/22:6n-3	0.5–0.9	0	0	0	0.3	0.3

[a]SM, sow milk, mean values from colostrum (first value of pair, $n = 8$ samples) to mature milk (second value, $n = 3$ samples).
[b]F, standard infant formula containing a mixture of vegetable oils.
[c]P, formula F enriched with pig brain phospholipids (8.5% of total lipids).
[d]E, formula F enriched with egg phospholipids (17% by weight of total lipids).
[e]R, formula F enriched with low-eicosapentaenoic acid fish oil (1.5% by weight of total lipids).
[f]R+ formula F enriched with low-eicosapentaenoic acid fish oil (4.5% by weight of total lipids). Abbreviation: MUFA, monounsaturated fatty acids.

tents in the phospholipid concentrate were 5.5, 4.9, and 0.1% by weight of total fatty acids, respectively. It was added to the formula fats in the proportion of 17% by weight of total lipids. The egg phospholipid-supplemented formula contained 0.5 and 0.4% by weight of total fatty acids as 20:4n-6 and 22:6n-3, which is close to the mean values generally found in the mature milk of European women (27–29).

Pig brain phospholipid supplementation (group P). Although brain phospholipids cannot currently be considered an edible product acceptable for infant nutrition (owing to prion diseases), we have tested this "endogenous" source as a possible nutritional reference concerning the specific needs of the developing brain for its own structural LCPUFA. Cerebral phospholipids (prepared by the Institut Yves Ponroy) were purified from pig brains and adsorbed onto an aerosyl (silica powder) matrix according to the method previously described (30). The brain phospholipids were composed of PC (43% by weight of total phospholipids), PE (31%), phosphatidylserine (15%), sphingomyelin (8%), and PI (3%). In the supplemented formula, the added brain phospholipids accounted for 8.5% of total dietary fats. Arachidonic acid and 22:6n-3 were present at the same concentration, i.e., 0.4% of total fatty acids.

Collection of tissues. After the 2-wk feeding period, piglets were fasted overnight, with water *ad libitum*, and then killed. Ten milliliters of blood were collected with heparin as anticoagulant and centrifuged. The plasma was drawn off, and the RBC were washed twice with 20 mmol/L Tris HCl, 150 mmol/L NaCl, 1 mmol/L EDTA (pH 7.4). The plasma and RBC were lyophilized.

The whole brain was excised, rinsed with 9 g/L NaCl, drained, and weighed. The whole fresh brain weighed 39.9 ± 1.4 g (mean ± sd) whatever the type of feeding. The brains in groups R and R+ weighed on average 8% less than those of group SM, but this difference was not significant (data not shown). The cerebellum was detached, rinsed, drained, and weighed. The cerebral cortex was entirely excised from the right hemisphere, whereas the left cerebral cortex was dissected into the four main cerebral areas, i.e., frontal, parietal, temporal, and occipital lobes. Preliminary experiments showed that the right and left cerebral hemispheres were not different in terms of phospholipid fatty acid composition (data not shown). Thus, the right hemispheres were used to determine the fatty acid composition in the whole cortex, whereas the left hemispheres from the same animals were used to specify the fatty acid distribution in the four cortical areas. The frontal, parietal, temporal, and occipital lobes represented 32.7 ± 1.0, 33.3 ± 1.9, 16.6 ± 0.8, and 17.4 ± 1.4% (mean ± sd) of the total cortical weight, respectively.

All samples were lyophilized, weighed and thoroughly homogenized before extraction of total lipids. Other organs (liver, heart, and proximal jejunal cells) were collected and prepared as previously described (25) for extraction of total lipids.

Determination of fatty acid composition. Total lipids were extracted from the blood and tissue samples (31). The plasma total phospholipids were separated from neutral lipids by filtration through silica cartridges (Waters, Milford, MA) (32). The plasma phospholipids, composed of 90% PC, were not further purified. The two major phospholipid classes in the different tissue samples, PC and PE, were separated by high-performance liquid chromatography coupled with a light-scattering detector (33,34). The total lipids were loaded onto a 25 cm × 7.5 mm silica column (Lichrosorb SI 60, Merck, Nogent-sur-Marne, France), and the isolation of PC and PE was carried out at a flow rate of 2.5 mL/min using a linear gradient of solvents that began with 100% of the first eluent composed of hexane/isopropanol/chloroform/water (42:44.6:10.4:3, by vol) and ended with 100% of the second eluent, composed of hexane/isopropanol/chloroform/water (32:49.6:10.4:8, by vol).

Fatty acid methyl esters were produced by reacting PC with 10% BF_3 in methanol at 90°C (20 min) (35), and PE with 10% HCl and 4% dimethoxypropane in methanol at 70°C (4 h). After adding 2 mL of distilled water, the methyl esters were extracted twice with 1 mL hexane, washed with distilled water to neutralization, dried under nitrogen, and finally taken up in 100–200 µL isooctane for gas chromatography. An aliquot of 1 µL was injected through the on-column injector of a 9001 gas chromatograph (Chrompack, Middleburg, The Netherlands) equipped with a retention gap and a CP WAX 52 CB bonded fused-silica capillary column of 0.3 mm i.d. and 50-m length. The oven temperature was programmed for 79–140–205°C, at a heating rate of 9°C/min for the first step, and 3°C/min for the second step. The instrument responses attributable to fatty acid methyl esters were automatically integrated, and their factor response and equivalent chain length were compared to standard compounds. The lower limit acceptable for 20:4n-6 and 22:6n-3 factor responses was set at 90%. All compositions were expressed as a percentage by weight of total fatty acids.

Statistical treatment. The significance of fatty acid composition differences between diet groups was evaluated using Statview SE™ (Abacus Concepts Inc., Berkeley, CA) for one-way analysis of variance followed by Fisher's test. The significance was tested at the level of $P < 0.05$ in each tissue (Fig. 1) and in each individual cortical area (Fig. 4). The area effect in the cortical fatty acid composition was specifically tested by one-way analysis of variance and Fisher's test. In this case, the diet groups were confounded and the area location was taken as the grouping factor. An area effect for a given fatty acid was considered as significant when the fatty acid level in one specific area was significantly different from its value in each of the three other areas, at least at the level of $P < 0.05$. When P values lower than 0.05 were found, the data are reported in the text.

RESULTS AND DISCUSSION

Fatty acid composition in SM-fed piglets, general considerations. Tables 2 and 3 give the fatty acid composition in PE and PC from neural and nonneural tissues in group SM. The main observation was that n-3 LCPUFA were three- to fourfold more abundant in the cortical and cerebellum PE than in nonneural tissues (excluding the liver). As already mentioned (25,26) the major n-3 LCPUFA in SM is 22:5n-3 (Table 1). The physiological significance of this particularity of SM is unclear, since 22:5n-3 in SM cannot be considered as equivalent to dietary 22:6n-3 in terms of 22:6n-3 accretion in piglet tissues (26). It should be remembered that achieving a high 22:6n-3 concentration in SM (greater than that of 22:5n-3) required the use of sow rearing diets specially enriched with soybean, canola, and fish oils (36). There was no accumulation of 22:5n-3 in neural tissues from group SM in spite of its substantial incorporation in plasma phospholipids and in nonneural tissues, notably in the heart (Table 2). Thus, n-3 LCPUFA uptake and/or phospholipid esterification was mostly restricted to 22:6n-3 in neural tissues. With respect to n-6 LCPUFA, 20:4n-6 was present in very high concentration (40% of total fatty acids) in the myocardial PE of SM-fed piglets (Table 2). This 20:4n-6 level was the highest that we have found among the different phospholipid fractions examined. In terms of double-bond index, the unsaturation level brought about by n-6 fatty acids in the PE fractions was higher in the heart than in all other tissues (192 vs. 72 to 140, see Table 2), whereas the myocardial n-3 double-bond index (DBI = 55) was among the lowest in comparison to other tissues. On the contrary, the cortical and cerebellum PE presented the highest n-3 double-bond index, essentially generated by their high 22:6n-3 status. Lastly, note that RBC phospholipids had the lowest status in LCPUFA from both n-6 and n-3 series (Tables 2 and 3), the major fatty acids in RBC being 18:1n-9 and 16:0 in PE and PC, respectively. The phospholipid fatty acid composition in piglet RBC was therefore only slightly representative of that of other tissues.

Status of 22:6n-3 in nonneural tissues. The lowest 22:6n-3 level in plasma and nonneural tissues was found in piglets fed the standard formula without preformed LCPUFA (group F). Compared to SM feeding, giving the standard formula lowered the 22:6n-3 status by 27% to 65% in the two phospholipid classes from the different tissues. The major alteration (65%) was observed in the myocardial PE (Fig. 1). Both types of supplementation, fish oil or phospholipids, restored the 22:6n-3 status or significantly increased the 22:6n-3 levels in nonneural tissues and plasma (Fig. 1). Compared to SM feeding, the egg phospholipid supplementation resulted in the significant increase of PE 22:6n-3 levels in all nonneural fractions, whereas pig brain phospholipids caused this level to increase significantly only in the liver (Fig. 1). Overall, formula E can be considered more efficient than formula P, all the 22:6n-3 levels in nonneural tissues being higher in group E than in group P (Fig. 1). This difference between both phospholipid sources could be mainly due to the adsorption of the dietary pig brain phospholipids onto the aerosyl matrix (while egg phospholipids were simply emulsified in the formula) leading to the lower intestinal absorption of dietary 22:6n-3 from formula P than from formula E. In neural tissues (see below), the higher efficiency of formula E compared with formula P was found in the cerebellum PE (Fig. 1). However, the difference between the two phospholipid sources did not reach a significant level in the cortex or the retina [as seen in our previous study (26)]. We also noted that groups E and R did not show a significant difference in their 22:6n-3 status either in plasma phospholipids or in PE from nonneural tissues (Fig. 1). These data suggest that, at equivalent amounts of dietary 22:6n-3, the incorporation of 22:6n-3 into nonneural tissues was not determined by the triglyceride or phospholipid nature of the dietary source. However, the different pattern obtained for 22:6n-3 enrichment in the heart compared to other nonneural tissues (see Fig. 1) led us to test LCPUFA nature (triglycerides or phospholipids) as a statistical factor for determining the 22:6n-3 status in myocardial PE. Two groups were therefore worked out, joining up groups

TABLE 2
Fatty Acid Composition in Plasma Phospholipids and in Phosphatidylethanolamine in Sow Milk-Fed Piglets (wt% total fatty acids, mean ± se)

	Cortex	Cerebellum	Heart	RBC[a]	Intestine	Liver	Plasma
14:0	0.1 ± 0.01	0.2 ± 0.1	3.0 ± 1.2	0.4 ± 0.2	0.4 ± 0.2	0.1 ± 0.1	0.4 ± 0.1
16:0	5.6 ± 0.2	5.4 ± 0.2	4.9 ± 0.5	18.9 ± 0.5	7.1 ± 0.8	8.8 ± 0.4	18.9 ± 0.2
18:0	18.0 ± 0.9	15.1 ± 0.3	18.4 ± 1.5	6.2 ± 0.2	18.6 ± 1.8	27.1 ± 0.3	20.8 ± 0.4
Σ SFA ≥ 20 C	0.4 ± 0.1	0.8 ± 0.2	0.01 ± 0.0	0.4 ± 0.1	2.0 ± 0.2	0.4 ± 0.1	1.2 ± 0.2
Σ Total SFA[a]	24.1 ± 0.9	21.4 ± 0.3	26.4 ± 1.5	25.9 ± 0.5	28.1 ± 1.4	36.4 ± 0.8	41.3 ± 0.3
16:1n-9	0.5 ± 0.1	0.4 ± 0.2	0.5 ± 0.2	0.4 ± 0.2	0.1 ± 0.04	0.4 ± 0.2	0.4 ± 0.1
16:1n-7	0.5 ± 0.1	0.6 ± 0.1	0.4 ± 0.1	2.0 ± 0.2	0.2 ± 0.04	0.4 ± 0.1	0.8 ± 0.2
18:1n-9	12.2 ± 0.3	22.9 ± 0.3	6.5 ± 0.7	33.7 ± 1.0	12.1 ± 0.3	6.5 ± 0.1	13.1 ± 0.2
18:1n-7	2.6 ± 0.2	3.7 ± 0.1	2.2 ± 0.2	3.4 ± 0.2	1.6 ± 0.2	1.3 ± 0.1	2.5 ± 0.1
Σ MUFA ≥ 20 C	1.6 ± 0.1	5.2 ± 0.6	0.3 ± 0.2	0.7 ± 0.1	1.0 ± 0.1	0.6 ± 0.1	1.5 ± 0.2
Σ Total MUFA	17.4 ± 0.4	32.8 ± 0.3	9.9 ± 1.0	40.2 ± 1.0	15.0 ± 0.5	9.2 ± 0.3	18.4 ± 0.3
Σ Total NE PUFA[a]	1.2 ± 0.1	1.0 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	0.5 ± 0.05	0.7 ± 0.1
18:2n-6	0.8 ± 0.1	0.9 ± 0.1	8.4 ± 0.7	12.2 ± 0.3	20.2 ± 0.7	5.7 ± 0.1	12.7 ± 0.3
20:2n-6	0.1 ± 0.1	0.4 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.2 ± 0.1	0.3 ± 0.1	0.4 ± 0.1
20:3n-6	0.6 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	0.5 ± 0.1	1.0 ± 0.1	0.4 ± 0.1	0.6 ± 0.1
20:4n-6	19.8 ± 0.3	15.6 ± 0.4	39.6 ± 2.2	9.5 ± 0.7	20.1 ± 1.3	27.5 ± 0.4	15.2 ± 0.3
22:4n-6	10.5 ± 0.2	7.4 ± 0.2	2.5 ± 0.3	1.0 ± 0.1	1.5 ± 0.2	1.0 ± 0.1	0.6 ± 0.2
22:5n-6	2.8 ± 0.2	1.1 ± 0.2	0.6 ± 0.2	0.5 ± 0.2	0.2 ± 0.1	0.3 ± 0.1	0.2 ± 0.1
Σ Total n-6 PUFA	34.7 ± 0.8	26.1 ± 0.5	52.5 ± 1.9	24.1 ± 0.9	43.2 ± 1.0	35.1 ± 0.5	29.7 ± 0.3
n-6 DBI[b]	139.3 ± 2.2	102.4 ± 2.6	191.8 ± 10.7	71.4 ± 5.2	131.1 ± 8.2	128.4 ± 1.8	92.0 ± 1.5
18:3n-3	0.01 ± 0.0	0.05 ± 0.03	0.2 ± 0.1	0.2 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
20:5n-3	0.1 ± 0.04	0.01 ± 0.0	1.7 ± 0.1	0.4 ± 0.1	1.5 ± 0.1	0.6 ± 0.05	0.6 ± 0.1
22:5n-3	0.9 ± 0.1	1.1 ± 0.2	3.6 ± 0.1	2.0 ± 0.2	3.6 ± 0.2	3.4 ± 0.1	2.2 ± 0.2
22:6n-3	19.2 ± 0.2	15.3 ± 0.4	4.6 ± 0.3	4.8 ± 0.3	6.7 ± 0.3	12.8 ± 0.5	5.9 ± 0.2
Σ Total n-3 PUFA	20.2 ± 0.2	16.5 ± 0.4	10.1 ± 0.1	7.3 ± 0.4	12.3 ± 0.3	16.9 ± 0.6	8.8 ± 0.3
n-3 DBI[c]	120.0 ± 1.4	97.8 ± 2.6	54.6 ± 3.4	41.1 ± 2.8	67.0 ± 3.1	97.0 ± 3.9	49.7 ± 2.1
Other compounds[d]	2.4 ± 0.2	2.1 ± 0.2	0.3 ± 0.03	1.7 ± 0.2	0.7 ± 0.1	1.8 ± 0.2	1.1 ± 0.1

[a]SFA, saturated fatty acids; NE PUFA, nonessential polyunsaturated fatty acids (eicosatrienoic and docosatrienoic acids); RBC, red blood cells; for other abbreviations see Table 1.
[b]n-6 DBI, double-bond index for n-6 PUFA.
[c]n-3 DBI, double-bond index for n-3 PUFA.
[d]Including odd-numbered and branched-chain fatty acids.

SM, R and R+ on the one hand, and groups P and E, on the other, excluding group F which did not receive preformed LCPUFA. The mean 22:6n-3 level was 1.3-fold higher ($P < 0.03$) in the myocardial PE from piglets fed phospholipids than in that from piglets fed triglycerides. Thus, it appears that the 22:6n-3 status in myocardial PE depended on the nature of the lipid source at least as much as on the total amount of dietary 22:6n-3. It has been suggested that dietary fatty acids provided as phospholipids are predominantly incorporated into intestinal very low density particles, whereas fatty acids from dietary triglycerides are incorporated into chylomicrons (37). Differences in the intestinal handling of dietary LCPUFA could influence the bioavailability of dietary LCPUFA, but the reason why the distribution of 22:6n-3 to the heart was specifically affected remains unclear.

Inasmuch as the 22:6n-3 levels in tissue and plasma phospholipids were spread among the different groups (see Fig. 1), it was interesting to relate the changes in whole tissues to those in circulating phospholipids. Plasma phospholipid fatty acid composition reflected what had actually been absorbed and metabolized from the dietary fatty acid pool and, above all, gave an indication of the bioavailability of circulating LCPUFA for their uptake by whole tissues. Plotting the hepatic and intestinal-PE 22:6n-3 levels against those of plasma phospholipids showed that 22:6n-3 changes in liver and intestine were linearly correlated with their plasma counterparts, whereas the myocardial PE responded in a saturable manner (Fig. 2A). Comparison of data from formula F-fed piglets with those from group R+ indicated that 22:6n-3 increased by 152 and 195% in hepatic and intestinal PE at the same time that it increased by 172% in plasma phospholipids. In the myocardial phospholipids from group F, 22:6n-3 accounted for only 0.6 and 2.5% of total fatty acids in PC (not shown) and PE (Fig. 2A), respectively. The myocardial PE

TABLE 3
Fatty Acid Composition in Phosphatidylcholine in Sow Milk-Fed Piglets (wt% total fatty acids, mean ± se)

	Cortex	Cerebellum	Heart	RBC	Intestine	Liver
14:0	1.9 ± 0.1	1.0 ± 0.04	0.6 ± 0.1	0.7 ± 0.03	0.2 ± 0.02	0.5 ± 0.1
16:0	37.4 ± 1.6	29.6 ± 0.3	24.9 ± 0.5	32.8 ± 0.5	21.1 ± 1.8	21.8 ± 0.4
18:0	12.7 ± 0.5	16.1 ± 0.2	10.9 ± 0.8	9.8 ± 0.5	20.6 ± 1.3	20.9 ± 0.8
Σ SFA ≥ 20 C	0.3 ± 0.05	0.5 ± 0.01	0.1 ± 0.0	0.5 ± 0.02	0.2 ± 0.0	0.1 ± 0.0
Σ Total SFA	52.3 ± 1.2	47.2 ± 0.1	36.4 ± 0.3	43.8 ± 0.4	41.8 ± 1.2	43.2 ± 0.6
16:1n-9	2.5 ± 0.1	1.7 ± 0.1	0.7 ± 0.06	0.6 ± 0.1	0.3 ± 0.03	0.6 ± 0.0
16:1n-7	1.5 ± 0.04	1.1 ± 0.01	0.9 ± 0.1	0.8 ± 0.2	0.4 ± 0.05	1.1 ± 0.1
18:1n-9	18.1 ± 0.4	23.1 ± 0.2	19.3 ± 0.6	21.9 ± 0.5	12.8 ± 0.6	11.7 ± 0.2
18:1n-7	5.9 ± 0.3	6.1 ± 0.04	6.1 ± 0.2	3.2 ± 0.07	2.8 ± 0.2	2.7 ± 0.1
Σ MUFA ≥ 20 C	0.8 ± 0.07	1.8 ± 0.07	0.2 ± 0.1	0.2 ± 0.07	0.3 ± 0.1	0.1 ± 0.1
Σ Total MUFA	28.8 ± 0.7	33.9 ± 0.1	27.0 ± 0.4	26.8 ± 0.5	16.2 ± 1.0	16.1 ± 0.3
18:2n-6	1.6 ± 0.1	2.1 ± 0.05	15.8 ± 0.3	17.9 ± 0.4	24.9 ± 1.5	12.1 ± 0.2
20:2n-6	0.2 ± 0.03	0.2 ± 0.0	0.5 ± 0.02	0.2 ± 0.05	0.4 ± 0.04	0.3 ± 0.03
20:3n-6	0.5 ± 0.02	0	1.0 ± 0.2	0.4 ± 0.04	0.7 ± 0.02	0.5 ± 0.03
20:4n-6	9.8 ± 1.1	8.5 ± 0.1	12.8 ± 0.3	6.1 ± 0.2	10.3 ± 0.8	15.2 ± 0.3
22:4n-6	0.8 ± 0.1	0.9 ± 0.0	0.5 ± 0.01	0.2 ± 0.02	0.4 ± 0.1	0.4 ± 0.02
Σ Total n-6 PUFA	13.0 ± 1.3	11.6 ± 0.2	30.7 ± 0.5	24.8 ± 0.4	36.6 ± 0.8	28.5 ± 0.5
n-6 DBI	47.7 ± 5.4	41.9 ± 0.6	89.0 ± 1.6	62.6 ± 1.4	95.3 ± 5.6	88.6 ± 1.8
18:3n-3	0	0.03 ± 0.0	0.4 ± 0.05	0.2 ± 0.04	0.3 ± 0.0	0.2 ± 0.03
20:5n-3	0.1 ± 0.1	0	0.6 ± 0.03	0.3 ± 0.02	0.5 ± 0.1	0.5 ± 0.04
22:5n-3	0.1 ± 0.02	0.2 ± 0.02	1.2 ± 0.06	0.6 ± 0.03	0.9 ± 0.2	2.0 ± 0.03
22:6n-3	3.1 ± 0.3	5.3 ± 0.1	1.7 ± 0.2	1.6 ± 0.1	2.2 ± 0.2	7.7 ± 0.2
Σ Total n-3 PUFA	3.2 ± 0.3	5.5 ± 0.1	3.9 ± 0.2	2.8 ± 0.1	3.9 ± 0.4	10.4 ± 0.2
n-3 DBI	19.3 ± 1.9	33.0 ± 0.5	20.5 ± 2.6	11.9 ± 0.8	21.0 ± 2.2	59.3 ± 1.2
Other compounds[a]	2.6 ± 0.3	1.7 ± 0.2	2.0 ± 0.2	1.8 ± 0.2	1.4 ± 0.1	1.8 ± 0.2

[a]Including odd-numbered and branched-chain fatty acids. See Table 2 for abbreviations.

22:6n-3 level increased twofold from group F to group SM, then plateaued to around 6% in the other groups, independent of the 22:6n-3 levels in plasma (Fig. 2A).

22:6n-3 status in brain. A linear relationship was also found between brain PE-22:6n-3 and plasma 22:6n-3 (Fig. 2B), although the amplitude of variation was 6- to 10-fold lower in cortex and cerebellum than in the PE fractions from nonneural tissues. The changes in brain phospholipids were not large enough to produce significant differences between all the groups (as shown in Fig. 1). However, from group F to group R+, the 22:6n-3 status significantly increased, by 18 and 23% in the cortical- and cerebellum-PE, respectively (Fig. 2B). The mean PE-22:6n-3 levels in the cortex were 1.25 ± 0.04 times those in cerebellum (mean ratio from the six groups ± sd), showing that the 22:6n-3 status in brain cortex and cerebellum developed concurrently whatever the dietary treatment.

The examination of the individual areas (Fig. 3) highlighted some regional particularities. As found in the whole cortex (Fig. 2B), the accretion of 22:6n-3 in the frontal and parietal lobes paralleled that of plasma phospholipids ($P < 0.003$). However, the occipital lobe exhibited a lower response ($P < 0.01$) to the increasing 22:6n-3 plasma levels than did frontal and parietal lobes, and the temporal area appeared to be unrelated to plasma phospholipids. Thus, the linear relationship between cortical and plasma levels of 22:6n-3 exhibited in Figure 1 was mainly generated by the frontal and parietal lobes and, to a lesser extent, by the occipital lobe. The data as a whole could suggest that 22:6n-3 accretion in the different brain structures is regulated through similar pathways, although specific regional requirements cannot be excluded (38).

The main objective of this regional study was to determine whether the occipital lobe, involved in visuospatial processing and in discrimination of color and movement (39), contained higher amounts of 22:6n-3, known to play a role in the process of visual development, than other cortical lobes. Our results do not support this hypothesis, at least not in the piglet model. The temporal lobe, which is involved in the primary organization of sensory input in humans, such as hearing ability and visual and verbal memories (40), was richer in 22:6n-3 than the other areas (except in group SM), but this difference reached statistical significance in groups F and P only. In humans, the frontal lobe controls motor activities, emotional response, and expressive language and is involved in many aspects of social behavior (41), while the parietal lobe

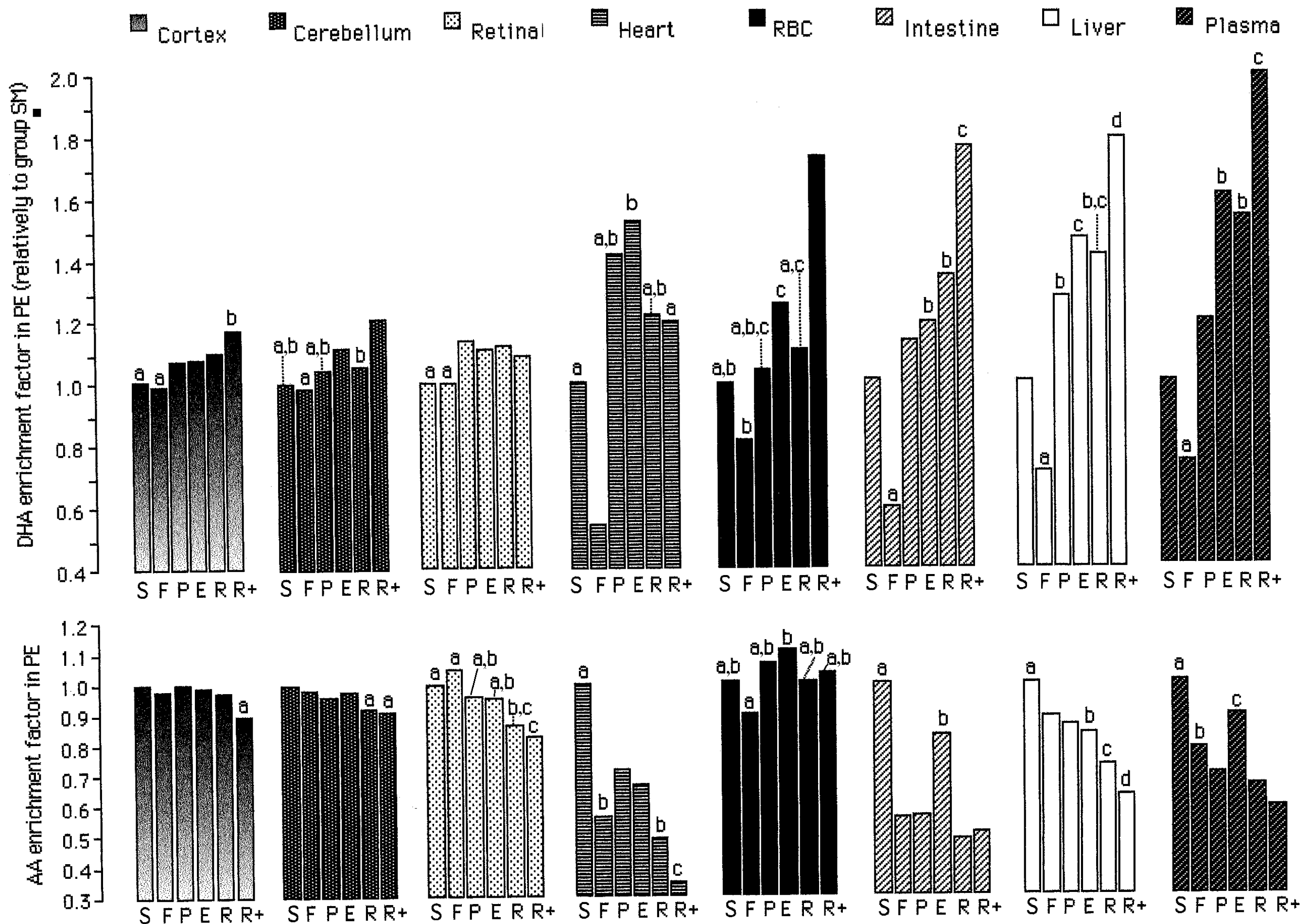

FIG. 1. Enrichment factor for long-chain polyunsaturated fatty acids (LCPUFA) in the phosphatidylethanolamine (PE) fractions from the five formula feeding groups. Upper panel: 22:6n-3 (docosahexaenoic acid, DHA), lower panel: 20:4n-6 (arachidonic acid, AA). The DHA and AA levels were expressed relative to those of the corresponding tissue from the sow milk-fed group, which are indicated in Table 2. The retina values were drawn from the data reported in Reference 26. Differences between diet groups were tested at the level of $P < 0.05$. The histograms with common superscripts are not significantly different. S: sow milk-fed group (0.1% dietary DHA) with an assigned value of 1.0; F: formula-fed group (0% dietary DHA); P: pig brain phospholipid-supplemented group (0.4% dietary DHA); E: egg phospholipid-supplemented group (0.4% dietary DHA); R: fish oil-supplemented group (0.3% dietary DHA); R+: fish oil-supplemented group (0.9% dietary DHA).

is concerned with integrating sensory input and perception of sensory information (39). Behavioral changes, learning impairment, and altered responses to drugs have been described in rodents fed diets deficient in n-3 fatty acids (review in 42). Besides these studies of total deprivation of n-3 fatty acids, the suboptimal provision of n-3 LCPUFA to the rat brain for multiple generations alters spatial learning ability (43). Moreover, breast feeding, or formula supplementation with egg phospholipids, can transiently benefit term infants in neurodevelopmental performance as opposed to standard formula feeding without LCPUFA (8). The question arises whether frontal and parietal lobes, which seem to respond to the bioavailability of dietary 22:6n-3, may be particularly involved in the improvement of some brain functions.

Among the nervous tissues we have examined, the cerebellum, involved in the coordination of voluntary motor movements, equilibrium and muscle tone, had the lowest 22:6n-3 content. These data could suggest that cerebellum functions were poorly affected by dietary supplies of preformed 22:6n-3. Total deprivation of n-3 fatty acids in mice diminished learning capacities without altering motor coordination (44).

20:4n-6 and 20:5n-3 status in nonneural tissues. In comparison to SM feeding, 20:4n-6 levels were significantly reduced in the nonneural phospholipids from piglets receiving any of the formula diets, except in the RBC PE (Fig. 1). Dietary supplementation with egg yolk phospholipids reduced the 20:4n-6-lowering effect in the heart, plasma, and intestine, whereas fish oil used at levels of 4.5% of total lipids (group R+) considerably accentuated the 20:4n-6 decline in the cardiac, plasma, and hepatic phospholipids (Fig. 1). The 20:4n-6 decline was also more marked ($P < 0.0001$) in the plasma and hepatic phospholipids from piglets fed the low concentration of fish oil (group R) than from those fed the standard formula. Thus, in the presence of dietary 22:6n-3 without 20:4n-6, a low 20:5n-3 intake (<0.1% of total fatty

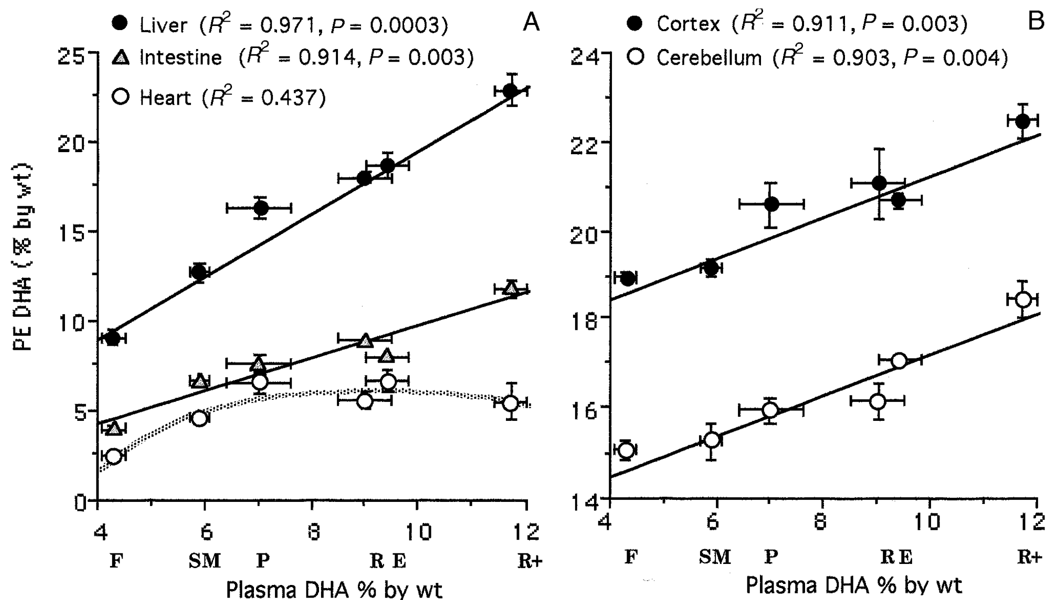

FIG. 2. Correlation between PE DHA levels in nonneural tissues (A) or brain (B) and DHA levels in plasma phospholipids. Each point is the mean value (± se) for DHA levels in the liver, intestine, heart, whole cortex, or cerebellum and plasma from one group assigned to one of the six dietary treatments. Each group is indicated along the abscissa by and positioned according to its plasma DHA value. F: formula-fed group (0% dietary DHA); SM: sow milk-fed group (0.1% dietary DHA), P: pig brain phospholipid-supplemented group (0.4% dietary DHA); R: fish oil-supplemented group (0.3% dietary DHA); E: egg phospholipid-supplemented group (0.4% dietary DHA); R+: fish oil-supplemented group (0.9% dietary DHA). The R^2 and P values testing for linear regression are indicated. For abbreviations see Figure 1.

acids) did not prevent a drop in the 20:4n-6 status in nonneural tissues. The data shows that a decrease in 20:4n-6 contents was not necessarily related to a reciprocal increase in 20:5n-3 contents. Indeed, using the fish oil at a low concentration was effective in maintaining the 20:5n-3 status similar to that in SM-fed piglets (Table 4). In contrast, giving the high concentration of fish oil induced the 20:5n-3 levels to increase significantly (P < 0.0001) in all of the nonneural tissues (Table 4). This resulted in higher levels of 20:5n-3 than 22:5n-3 in group R+, in contrast to what was found in group SM and in all other groups. However, the sum of both fatty acids in group R+ (and in all formula feeding groups) never exceeded the values found in group SM (Table 4).

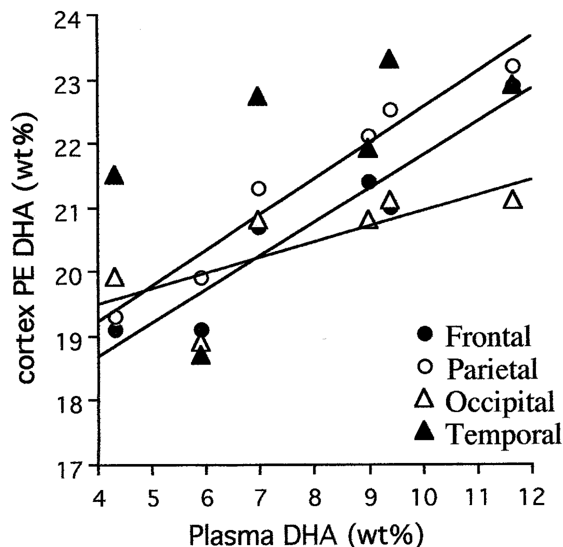

FIG. 3. Correlation between PE DHA levels in the frontal, parietal, occipital, and temporal lobes and DHA levels in plasma phospholipids. Each point is the mean value for DHA levels in cortical lobe and plasma from one group assigned to one of the six dietary treatments (as shown in Fig. 2). The R^2 and P values testing for linear regression were as follows: frontal lobe, R^2 = 0.914, P = 0.003; parietal lobe, R^2 = 0.957, P = 0.0007; occipital lobe, R^2 = 0.546, P = 0.09; temporal lobe, R^2 = 0.34, P = 0.2. For abbreviations see Figure 1.

The 20:5n-3 levels in neural tissues remained very low (<0.1% of total fatty acids) whatever the dietary treatment (data not shown).

The largest fall in 20:4n-6 was observed in myocardial phospholipids, in which the PE- and PC-20:4n-6 levels decreased by 65 and 73%, respectively, in group R+ relative to group SM. It also decreased by 43% in the myocardial PE of group F, and by 33 and 28% in groups E and P (Fig. 1). Decreases in 20:4n-6 of 74 and 44% in cardiac PC and PE were also reported in 16-d-old piglets fed a canola oil-enriched formula as opposed to piglets fed SM (45). In the present study, we found that both phospholipid supplementations improved the cardiac 20:4n-6 status relative to feeding of the control formula, but failed to match the high levels resulting from SM feeding. However, the sum of total n-6 fatty acids in myocardial PE (mainly including 18:2n-6) was not markedly altered by the different dietary treatments (data not shown). This apparent compensation within the n-6 family suggests that the molecular replacement of 20:4n-6 by dietary 18:2n-6 was the main factor responsible for the decrease in 20:4n-6 in the my-

TABLE 4
20:5n-3 and 22:5n-3 Levels in Plasma Phospholipids and in Phosphatidylethanolamine from Nonneural Tissues (wt% of total fatty acids, mean ± se)

	SM	F	P	E	R	R+
Plasma						
20:5n-3	0.6 ± 0.1	0.4 ± 0.1	0.3 ± 0.1	0.4 ± 0.1	0.6 ± 0.1	1.4 ± 0.1[a]
20:5+22:5n-3	2.8 ± 0.1	2.1 ± 0.1[a]	1.3 ± 0.1[b]	1.5 ± 0.1[b]	2.0 ± 0.1[a]	2.6 ± 0.1
Red blood cells						
20:5n-3	0.4 ± 0.05	0.2 ± 0.05	0.4 ± 0.05	0.3 ± 0.05	0.6 ± 0.1	1.1 ± 0.2[a]
20:5+22:5n-3	2.4 ± 0.1[a]	1.5 ± 0.1[b]	1.5 ± 0.1[b]	1.6 ± 0.1[b]	1.9 ± 0.1[a,b]	2.4 ± 0.2[a]
Intestine						
20:5n-3	1.5 ± 0.1[a]	0.4 ± 0.1	0.9 ± 0.2	0.7 ± 0.1	1.2 ± 0.1[a]	3.2 ± 0.2[b]
20:5+22:5n-3	5.1 ± 0.2[a]	1.8 ± 0.1	2.0 ± 0.2	1.6 ± 0.1	2.4 ± 0.1	4.6 ± 0.2[a]
Liver						
20:5n-3	0.6 ± 0.01	0.4 ± 0.1	0.3 ± 0.1	0.3 ± 0.1	0.8 ± 0.1[a]	1.8 ± 0.2[b]
20:5+22:5n-3	4.0 ± 0.1	2.6 ± 0.1[a]	1.6 ± 0.1[b]	1.7 ± 0.1[b]	2.5 ± 0.1[a]	3.3 ± 0.2[c]
Heart						
20:5n-3	1.7 ± 0.1[a]	0.8 ± 0.1	0.8 ± 0.1	0.7 ± 0.1	1.7 ± 0.1[a]	2.8 ± 0.3[b]
20:5+22:5n-3	5.3 ± 0.1	3.2 ± 0.1[a]	2.4 ± 0.1[a,b]	2.3 ± 0.1[b]	3.8 ± 0.1[a]	4.1 ± 0.3[a]

[a–c]Analysis of variance testing for differences between groups: values with different superscripts are significantly different ($P < 0.001$). For abbreviations see Table 1.

ocardial PE of piglets fed milk replacer diets, even though the formula contained substantial amounts of 20:4n-6 from phospholipid sources.

We found that the cardiac 20:4n-6 content was greatly reduced by formula feeding. This alteration could be limited but not totally prevented under our conditions of phospholipid supplementation, and it was further accentuated when the formula contained high amounts of n-3 LCPUFA without 20:4n-6. It can be hypothesized that such modifications in the proportions of 20:4n-6, 20:5n-3 and 22:6n-3 may have the potential to modify the myocardial eicosanoid balance or the state of cardiomyocyte excitability (46).

20:4n-6 status in brain. The global 20:4n-6 decline in nonneural tissues resulting from the different formula diets had low effects on the 20:4n-6 status in brain phospholipids (Fig. 1). Only group R+ had its 20:4n-6 status altered, by around 10%, in the two phospholipid classes of both the whole cortex and the cerebellum (Fig. 1). At the regional level, the decrease was significant in the frontal, parietal, and occipital lobes, the temporal lobe being apparently more preserved (Fig. 4). In addition, the 22:5n-6 level was significantly reduced in the different areas from group R+, including the temporal one (Fig. 4). Thus, alteration of n-6 LCPUFA occurred in neural tissues from piglets fed fish oil from the moment that it accounted for more than 1.5 to 4.5% by weight of total dietary lipids.

Feeding formula without preformed LCPUFA led to the same 20:4n-6 levels in brain phospholipids as SM feeding, whereas the 20:4n-6 contents of all the nonneural tissues from group F were greatly reduced (except in the RBC-PE in which 20:4n-6 levels remained almost unchanged with diets, as in the brain). The data obtained from the piglet brain confirmed its homeostatic 20:4n-6 properties, as shown in humans by postmortem analysis of cortical samples from infants who died from sudden death syndrome, whose 20:4n-6 levels were not altered through feeding with conventional formulae, as opposed to those of 22:6n-3 (5,6). However, our data from the fish oil-fed groups showed that maintaining the 20:4n-6 status in neural tissues was limited to the condition that, in the absence of dietary preformed 20:4n-6, the sum of total n-3 long-chain fatty acids (20 to 22 carbons) remained between 0.6 and 1.2% of total dietary fatty acids. Beyond this threshold, brain 20:4n-6 levels were slightly but significantly reduced, and this alteration could occur even though the dietary 22:6n-3 to 20:5n-3 ratio was greater than 1.

Whatever the dietary treatment, the 20:4n-6 level was 10 to 20% higher (according to groups) in the temporal PE than in the PE from parietal, frontal and occipital lobes ($P < 0.0001$, grouping together the dietary treatments), and the decrease of 20:4n-6 in the temporal lobe PE of group R+ was not significant in comparison to group SM (Fig. 4). Moreover, the temporal PE was the sole fraction to be enriched with significant amounts of 20:4n-6 when both dietary 22:6n-3 and 20:4n-6 were supplied, since piglets fed the phospholipid-supplemented formulae gained 9 and 7% of 20:4n-6 in groups E and P relative to group SM (Fig. 4). There was no other appreciable increase of 20:4n-6 in all the neural tissue samples examined. None of the other n-6 LCPUFA present in the cortical PE samples, i.e., 20:3n-6, 22:4n-6 and 22:5n-6, were found to be preferentially distributed among the different areas, although the 22:5n-6 levels in all groups were slightly lower ($P < 0.05$) in the temporal lobe than in the other lobes (Fig. 4). Higher levels of 20:4n-6 and lower levels of 22:5n-6 resulted in higher 20:4n-6 to 22:5n-6 ratios in temporal lobes ($P < 0.0001$, grouping together the dietary treatments, data not shown). These data suggest that n-6 metabolism in the temporal lobe was preferentially turned to synthesis, accumulation, or sequestration of 20:4n-6 over other n-6 LCPUFA.

In brief, the PE-20:4n-6 content of temporal lobe appeared not to be altered by the high n-3 intake (group R+). Among all the cortical lobes the temporal lobe seemed to be the preferential target for the additional incorporation of 20:4n-6 into PE

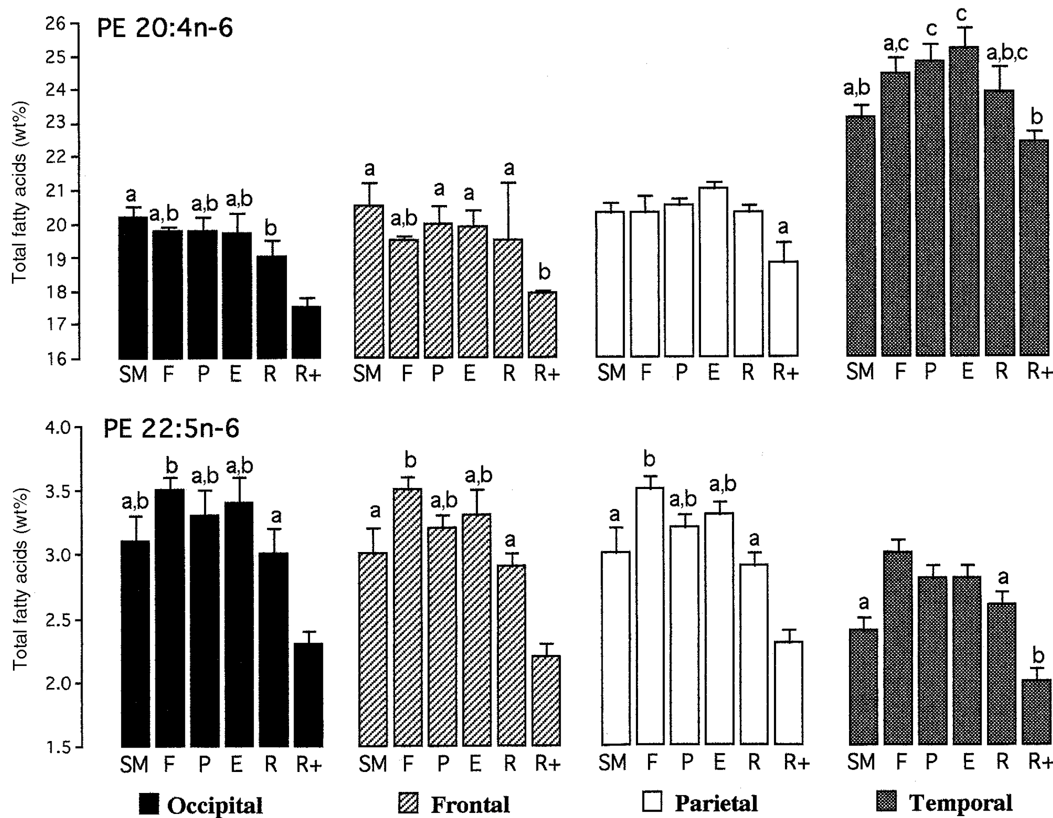

FIG. 4. Levels of n-6 LCPUFA in the brain PE from occipital, frontal, parietal, and temporal lobes (mean ± se). Upper panel: 20:4n-6, lower panel: 22:5n-6. Significant differences between diet groups within the same area are indicated by different superscripts ($P < 0.05$). All areas from group R+ contained less 20:4n-6 and 22:5n-6 than those from group SM, with the exception of the temporal 20:4n-6 level which was reduced only in comparison to groups F, P, and E. Confounding dietary treatments, the mean 20:4n-6 level in the temporal area was significantly higher than the mean value in the other areas ($P < 0.0001$), while the 22:5n-6 level was slightly lower ($P < 0.05$). For abbreviations see Figure 1.

when this fatty acid was provided as a phospholipid (groups E and P). A higher level of 20:4n-6 in the temporal lobe, as compared with frontal or occipital lobes (+21 and +43%, respectively), was also found in the brain microsomal lipids of one macaque monkey (47). It is tempting to suggest that the specific retention of 20:4n-6 in the temporal lobe could be significant from a functional point of view, possibly in terms of signal transduction or eicosanoid synthesis modulation through the cyclooxygenase pathway (reviewed in Ref. 48).

In summary, we evaluated the effects of LCPUFA supplementation upon the fatty acid status in formula-fed piglets. Fortification of formula with a conventional fish oil (with high 20:5n-3 content) has already been shown to increase the 22:6n-3 status in the brain and nonneural tissues of piglets (49). In the present study, we showed that the low-20:5n-3 fish oil, used at concentrations of 1.5% by weight of total lipids, was as efficient as conventional fish oil in increasing the 22:6n-3 status in neural and nonneural tissues; the advantage over conventional fish oils was that, at this concentration, the enhancement of 22:6n-3 occurred without increasing 20:5n-3 levels. However, fish oil supplementation resulted in an overall decrease of 20:4n-6 status, even though the oil contained low amounts of 20:5n-3. The 20:4n-6 decline affected the neural phospholipids, including the retina (26), bearing in mind that the n-3 LCPUFA accounted for more than 0.6% of total fatty acids in the formula deprived of preformed 20:4n-6. Thus, the fortification of formulae with 22:6n-3 without the concomitant incorporation of 20:4n-6 does not result in maximal 20:4n-6 status in neural and nonneural tissues, even though the dietary amount of 22:6n-3 exceeds that of 20:5n-3, the metabolic competitor of 20:4n-6. It may even generate a risk of alteration in the frontal, parietal and occipital lobes of the cortex, the temporal lobe apparently being more efficiently preserved. However, the corresponding short- and long-term effects on cognitive development have yet to be determined. Finally, the heart appeared to be highly sensitive to 20:4n-6 reduction in the absence of dietary preformed 20:4n-6, especially when n-3 LCPUFA from fish oil were added at a higher concentration than 0.6% by weight of total fatty acids. These data also argue in favor of the cosupplementation with both n-6 and n-3 LCPUFA, rather than with n-3 LCPUFA alone. Phospholipid supplementation allowed brain 22:6n-3 to increase and could prevent changes in the 20:4n-6 status to some extent. Nevertheless, richer LCPUFA sources should be used in infant nutrition to imitate the lipid composition of human milk and to circumvent their absence

in vegetable oils. With this in mind, raising both 20:4n-6 and 22:6n-3 levels in the ratio of around 1.3 seems reasonable.

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Abnormal Serum Lysophospholipids in Multiple Myeloma Patients

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ABSTRACT: Lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) mediate various kinds of biological activities and play an important role in cellular signal transduction. We analyzed serum phospholipids obtained from 16 multiple myeloma (MM) patients and observed that serum LPA level was significantly higher in MM patients (5.3 ± 0.5 nmol/mL) than in normal controls (1.7 ± 0.3 nmol/mL). LPC level was also higher than that in normal controls, and it correlated significantly with the concentration of LPA ($r = 0.678$, $P < 0.01$). In MM patients, palmitic acid/linoleic acid ratios in phosphatidylcholine and LPC were higher than those in normal controls. In the 12-mon follow-up study of two patients with the immune globulin G type, we recognized that the increase of LPC, LPA, and arachidonic acid/linoleic acid ratio in phosphatidylinositol corresponded with a decline in the serum albumin level and choline esterase activity.

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Multiple myeloma (MM) is characterized by malignant monoclonal lymphoplasmacytic cells that increase in bone marrow and produce an M-component [a specific immune globulin (Ig) produced by malignant cells in myeloma patients]. It is known that many kinds of malignancies involve abnormal lipid metabolism. Many MM patients have an impaired immune response, which causes a secondary high blood triglyceride level. These lipid-mobilizing activities are caused by cytokine-induced suppression of lipoprotein lipase (1) as well as by suppression of the enzyme related to the alteration of phospholipid compositions.

Lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) mediate various kinds of biological activities and play an important role in cellular signal transduction. LPA activates its own GTP-binding protein (G-protein)-coupled receptor to trigger phospholipase C-mediated Ca^{2+} mobilization and other effector pathways such as induction of smooth muscle contraction (2), cell proliferation mediated by G-protein (3), and mediation of tumor cell invasion (4). LPC in oxidized low density lipoprotein stimulates proliferation of foam cells, which are derived from macrophages and are linked to atherosclerosis progression (5). In producing these lysophospholipids by phospholipase A_2 or C, arachidonate is released from membrane phospholipids and interacts with a G-protein (6). Arachidonate is a precursor of eicosanoids, which promote Ca^{2+} mobilization and activate protein kinase C (7).

Mills *et al.* (8) found that ascites from an ovarian cancer patient strongly induced proliferation of ovarian cancer cells. Recently, Okita *et al.* (9) found higher LPC levels in ascites from ovarian cancer patients. In this study, we analyzed serum phospholipids in MM patients and recognized increased levels of lysophospholipid levels compared to those in normal subjects.

EXPERIMENTAL PROCEDURES

Sera from 16 MM patients were obtained at the Third Department of Internal Medicine of Toyama Medical and Pharmaceutical University (Toyama, Japan). Diagnosis of MM was based on histological increase of myeloid cells on bone marrow biopsy and monoclonal hyperglobulinemia. Clinical data are shown in Table 1. Control subjects were two healthy men and 11 healthy women. Serum lipids were extracted and separated by two-dimensional thin-layer chromatography according to the procedure reported previously (9). Transmethylation of the phospholipids was carried out at 90°C for 2 h in acetylchloride/methanol (5:50 vol/vol). Fatty acid methyl esters were analyzed by gas–liquid chromatography (model GC-14A; Shimadzu, Kyoto, Japan) using margaric acid (17:0) as an internal standard. The results are expressed as the mean ± SE. For comparison of two groups of individuals, the Mann-Whitney U test was used. The coefficient of correlation (r) was calculated by the Spearman R test. P-values were two-tailed and considered significant at less than 0.05.

RESULTS AND DISCUSSION

Serum phospholipids and fatty acid compositions of the phospholipids were analyzed for each type of MM patients and compared with those of controls. No significant differences were

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Abbrevations: BJ type, Bence-Jones type; G-protein, GTP-binding protein; Ig, immune globulin; LPA, lysophosphatidic acid; LPC, lysophosphatidyl choline; MM, multiple myeloma; PC, phosphatidylcholine; PLA_2, phospholipase A_2.

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TABLE 1
Clinical Data of the Study Group[a]

	Control	Myeloma IgG	Myeloma Bence-Jones	Myeloma IgA
Number (sex)	2(M),11(F)	2(M),8(F)	1(M),3(F)	1(M),1(F)
Age (yr)	51 ± 1	65 ± 3	63 ± 9	53
BMI		22.2 ± 1.0	22.1 ± 1.8	21.4
Total protein (g/dL)		9.4 ± 0.5[ab]	6.3 ± 0.2[b]	6.7[a]
Albumin (g/dL)	4.5 ± 0.1	3.6 ± 0.2[a]	4.1 ± 0.1	4
A/G		0.7 ± 0.1	1.9 ± 0.1	1.4
Immune globulin (Ig)				
IgG (mg/dL)		4043 ± 384	1599 ± 1234	415
IgA (mg/dL)		36 ± 14	42 ± 32	1317
IgM (mg/dL)		22 ± 8	16 ± 11	46
RBC (× 10^4/μL)		325 ± 28	266 ± 25	297
WBC (/μL)		4079 ± 831	4563 ± 516	5160
Platelets (× 10^4/μL)		15.7 ± 3.2	22.1 ± 1.4	12

[a]BMI, body mass index; A/G, albumin/globulin ratio; RBC, red blood cells; WBC, white blood cells. Data reported as mean ± SE. [a], $P < 0.01$; [b], $P < 0.05$.

recognized in the serum phospholipid concentrations and fatty acid compositions among the types of disease. Serum LPC levels were significantly higher in MM (193.2 ± 17.3 nmol/mL) than in controls (135.0 ± 8.4, Fig. 1). Serum LPA levels were also significantly higher in MM (5.3 ± 0.5 nmol/mL) than in controls (1.7 ± 0.3 nmol/mL). Serum LPC concentrations correlated with those of LPA ($r = 0.678$, $P < 0.01$, Fig. 2). There were no significant differences in the serum phosphatidylcholine (PC), phosphatidylethanolamine, and phosphatidylinositol concentrations between controls and MM patients.

The Ig type differs with each MM type. Malignancy induces monoclonal Ig production and suppression of other Ig types. The serum albumin level decreases in the IgG and IgA types but not in the Bence-Jones (BJ) type. To indicate the progression of each type of disease, we calculated the albumin/globulin (A/G) ratio. The ratio decreased in the IgG type but increased in the BJ type with disease progression. In the IgG type, LPC concentration and A/G correlated negatively ($r = -0.685$, $P < 0.05$) and positively in the BJ type ($r = 1.000$, $P = 0.05$, Fig. 3).

Fatty acid composition of phospholipids is shown in Table 2. In MM sera, the percentages of the saturated palmitic acid (16:0) in PC and stearic acid (18:0) in phosphatidylethanolamine were higher than those in normal subjects. However, linoleic acid (18:2n-6) in PC and LPC, and arachidonic acid (20:4n-6) in phosphatidylinositol were lower in MM. In comparing the palmitic acid/linoleic acid molar ratio in phospholipids, the ratio in MM patients was significantly higher for PC and LPC than in controls (Fig. 4).

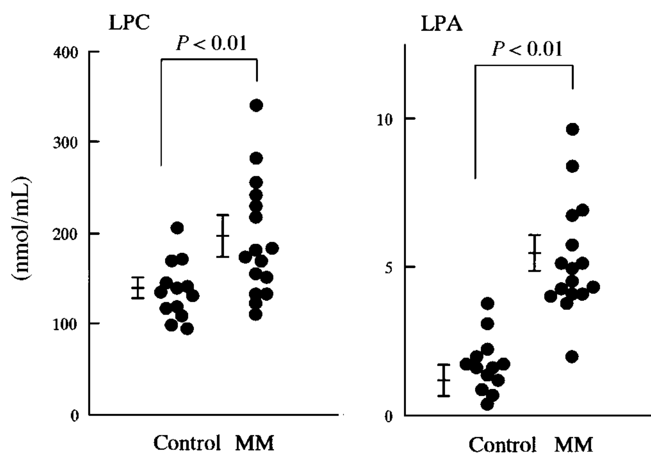

FIG. 1. Serum lysophosphatidylcholine (LPC) and lysophosphatidic acid (LPA) levels in multiple myeloma (MM) patients and control subjects. Significant differences are recognized in LPC ($P < 0.01$) and LPA concentrations ($P < 0.01$). Vertical lines show mean ± SE for 13 controls and 16 MM patients.

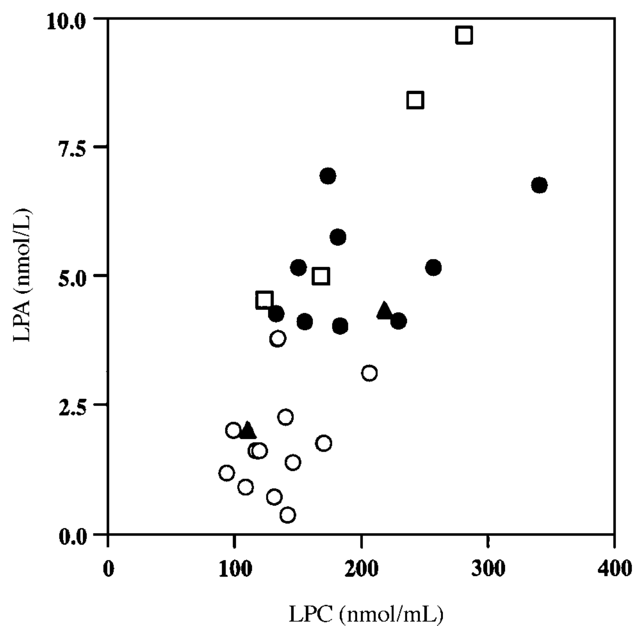

FIG. 2. Correlation of LPC and LPA concentrations. Data points indicated are controls (○), immune globulin G (IgG) type (●), Bence-Jones type (□), and IgA type (▲). Significant correlations are recognized between LPC and LPA concentrations ($r = 0.678$, $P < 0.01$). For abbreviations see Figure 1.

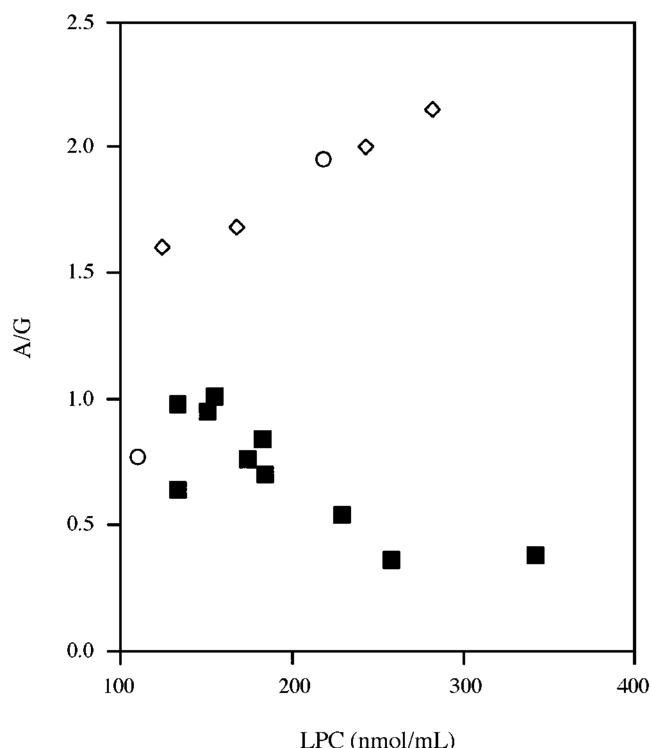

FIG. 3. Correlation of LPC concentration and albumin/globulin (A/G) ratio. Data points indicated are IgG type (■), Bence-Jones type (◇), and IgA type (○). In the IgG type, LPC and A/G ratio correlated negatively ($r = -0.685$, $P < 0.05$) and they correlated positively in the Bence-Jones type ($r = 1.000$, $P = 0.05$). For other abbreviations see Figures 1 and 2.

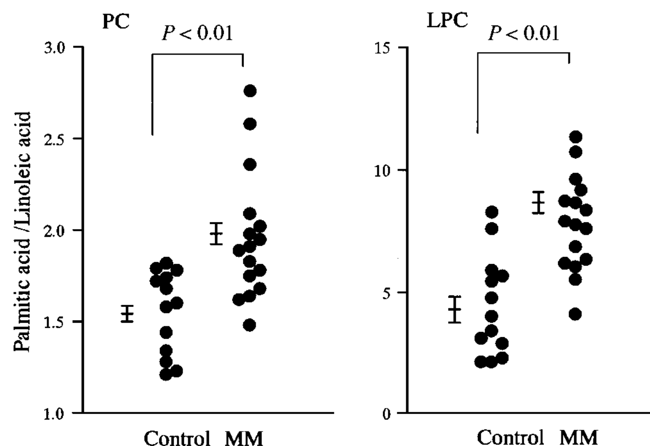

FIG. 4. Palmitic acid/linoleic acid ratio in serum phosphatidylcholine (PC) and LPC. Significant differences are recognized between the palmitic acid/linoleic acid ratio in PC ($P < 0.01$) and LPC concentrations ($P < 0.01$). Vertical lines show mean ± SE for 13 controls and 16 MM patients. For abbreviations see Figure 1.

We observed two patients with the IgG type (61- and 64-yr-old females) for 12 mon. Markedly higher levels of LPC and LPA were observed at the start of follow-up compared with those of normal subjects. These levels increased slightly after 12 mon, corresponding to a decline in serum albumin and choline esterase activity. The arachidonic acid molar ratio in phosphatidylinositol increased with disease progression (Fig. 5).

LPA was produced rapidly in thrombin-activated platelets (10). LPA concentration in heparinized plasma, compared to that in serum, was 3.21 and 6.39 nmol/mL, respectively, in a MM patient with IgG type, whereas these values averaged 0.29 and 1.45 nmol/mL, respectively, in three controls (data not shown). Therefore, LPA production in MM may be higher than that in controls during blood clotting. LPA has been reported to be produced from phosphatidic acid by phospholipase A_2 (PLA_2) (11). LPA plays a role in the growth of ovarian and breast cancer cells (3). LPA stimulates proliferation of Jurkat cells in serum-free medium or in a low concentration of fetal bovine serum. Biological activities differ in each LPA species: oleoyl-LPA has a higher activity, followed by arachidonoyl-LPA, and linoleoyl-LPA which constitute the unsaturated group. LPA can mobilize cellular Ca^{2+}, and palmitoyl-LPA induces maximal activity, on the order of 10^{-6} M (12). LPA concentrations in both serum and plasma in MM are considered sufficient to induce Ca^{2+} mobilization *in vitro*. LPA induces mitogenic responses in fibroblasts, or inhibition of proliferative activity of myeloma cells (13). Imagawa *et al.* (14) reported that LPA induced varying degrees of cell proliferation in different cell types, mediated through pertussis toxin-sensitive or -insensitive G-protein. On the basis of these facts (this research; 3,12–14) we suggest that LPA may be potentially active in the plasma of MM patients.

Okita *et al.* (9) showed that the percentages of palmitoyl- and stearoyl-LPC species in plasma and ascites from ovarian cancer patients were significantly higher than those of con-

TABLE 2
Composition of Selected Fatty Acids in Serum Phospholipids (mol%)[a]

	PC		PE		PI		LPC	
	Control	MM	Control	MM	Control	MM	Control	MM
16:0	33.3 ± 0.8	37.1 ± 0.9[a]	16.8 ± 1.7	20.0 ± 1.4	9.8 ± 0.9	11.0 ± 0.5	43.9 ± 1.9	58.6 ± 0.9[b]
18:0	14.8 ± 0.3	13.3 ± 0.6	16.0 ± 1.0	21.1 ± 1.3[a]	42.3 ± 0.9	43.8 ± 1.0	20.9 ± 0.4	18.8 ± 1.0
18:1	10.6 ± 0.6	12.2 ± 0.4[b]	8.0 ± 0.5	7.1 ± 0.3	8.0 ± 0.6	7.2 ± 0.5	7.6 ± 0.4	8.4 ± 0.4
18:2n-6	21.8 ± 0.8	19.2 ± 0.6[b]	10.8 ± 0.7	9.3 ± 0.6	10.2 ± 0.7	10.2 ± 0.9	12.4 ± 1.3	7.9 ± 0.5[a]
20:4n-6	6.1 ± 0.5	5.9 ± 0.4	16.0 ± 1.1	14.5 ± 0.9	21.7 ± 1.1	18.3 ± 1.2[b]	4.7 ± 0.4	1.6 ± 0.2
20:5n-3	3.2 ± 0.7	1.7 ± 0.2	7.6 ± 1.1	4.2 ± 0.6[b]	0.5 ± 0.1	0.6 ± 0.2	0.7 ± 0.2	0.5 ± 0.1
22:6n-3	5.5 ± 0.6	5.3 ± 0.4	16.4 ± 1.7	17.4 ± 1.5	2.6 ± 0.4	2.6 ± 0.3	1.2 ± 0.3	1.0 ± 0.1

[a]PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; LPC, lysophosphatidylcholine; MM, multiple myeloma. Data presented as mean ± SE, [a], $P < 0.001$; [b], $P < 0.05$, vs. control.

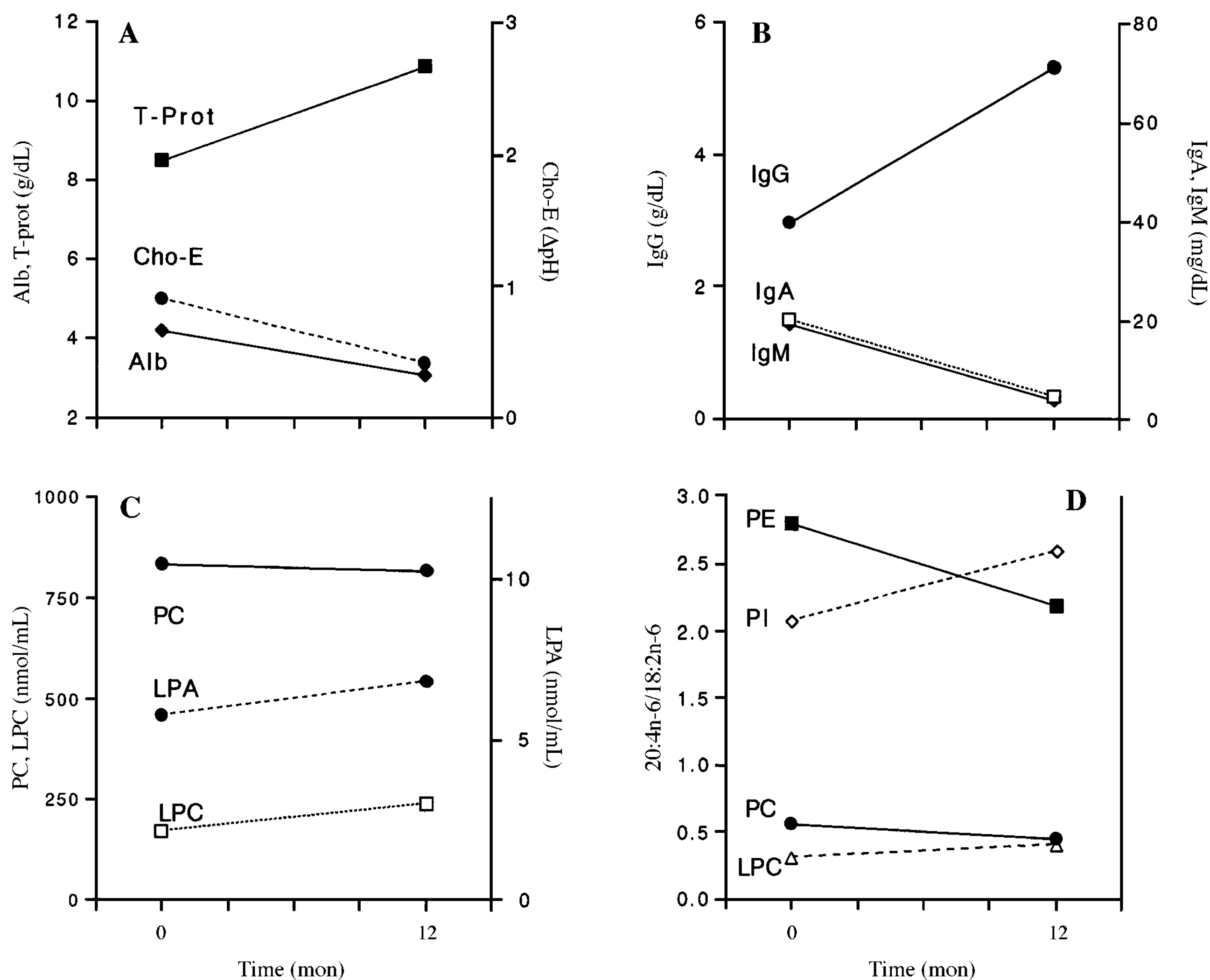

FIG. 5. Follow-up study of two patients of IgG type for 12 mon. Averages of clinical data and serum phospholipid levels are shown at 0 and 12 mon. Abbreviations: T-Prot, total protein; Alb, albumin; Cho-E, choline esterase; PE, phosphatidylethanolamine; PI, phosphatidylinositol. For other abberviations see Figures 1–3.

trols. Our data showed a higher palmitic/linoleic acid ratio of PC and LPC in serum phospholipids of MM patients compared to that in the controls (Fig. 4). Moreover, we recognized an abnormal serum albumin/globulin ratio in MM patients, a decrease in IgG type and an increase in BJ type, and a significant correlation of this ratio with the serum LPC level (Fig. 3). These results may suggest that elevated serum LPC level in MM indicates a progression of the disease. Several biological functions of LPC have been studied. Sakai *et al.* (5) reported that macrophage proliferation occurred following the LPC uptake of oxidized low density lipoprotein through the scavenger receptor. Other investigators (15,16) reported that LPC enhanced T-lymphocyte proliferation and potentiated protein kinase C activation in other cell lineages.

The source of LPC is fatty acid release from PC by PLA_2 and phospholipase A_1. Secretory group II PLA_2 ($sPLA_2$) and cytosolic group IV PLA_2 ($cPLA_2$) regulate extra- or intracellular arachidonic acid release with cross reactivity (17,18). In malignant tissue, phospholipid fatty acid remodeling was regulated through PLA_2 and acyltransferase, and this process leads to increased use of arachidonic acid (18). Another LPC-producing pathway involves transacylation of PC by lecithin cholesterol acyltransferase. Kuliszkiwicz-Janus and Baczynski (19) reported that high density lipoprotein and lecithin cholesterol acyltransferase levels decreased in MM patients, and our data also indicated decrement of these levels. From our present results, a close relation between the formations of two lysophospholipids, LPA and LPC, was observed. It may indicate involvement of lysophospholipase D in the production of LPA (20). Further investigation is needed to determine the relationship between tumor progression and lysophospholipids.

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Differential Effect of *N*-Ethyl Maleimide on Δ6-Desaturase Activity in Human Fetal Liver Toward Fatty Acids of the n-6 and n-3 Series

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ABSTRACT: The effect of *N*-ethyl-maleimide (NEM) on Δ5- and Δ6-desaturase activities and the incorporation of substrates and products into different microsomal lipid classes and phospholipid (PL) subclasses were studied in human fetal liver microsomes, obtained after legally approved therapeutic abortion. Desaturase activities were measured by a radiochemical method using reversed-phase high-performance liquid chromatography (HPLC). After nonphospholipid (NPL) and PL separation on silica cartridges, the radioactivity in different lipids of the NPL group was assessed by two-dimensional thin-layer chromatography, and their fatty acid (FA) composition by gas–liquid chromatography. The PL subclasses were separated, and the distribution of radioactivity between products and substrates was determined in PL subclasses. NEM inhibited the Δ5- and Δ6-desaturase activities in the n-6 series of FA but not the Δ6-desaturase activity in the n-3 series, which suggests the existence of two distinct Δ6-desaturases, one for the n-6 series and another for the n-3 series. Whether NEM was present or absent, most of the radioactivity was recovered in the free FA form (about 80%). The desaturation products, obtained in the presence or absence of NEM, were preferentially incorporated into PL, suggesting a channeling of the newly synthesized FA toward microsomal PL. The comparison of the distribution of substrates and products incorporated into the different PL classes showed that most of the labeled FA were incorporated into phosphatidylcholine and to a lesser degree into phosphatidylethanolamine.

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Polyunsaturated essential fatty acids (PUFA) are required during fetal development as constituents of membrane phospholipids (PL) and precursors of eicosanoids. In addition to autonomous synthesis by the fetal liver, essential fatty acids (EFA) are provided to the fetus from the mother's blood through the placenta (1), the placenta itself being devoid of PUFA synthesis capacity (2). The accretion of PUFA during the fetal period results in a gradient of concentration between maternal plasma and fetal tissues, reported as biomagnification (3). The biosynthesis of PUFA occurs essentially in liver from C_{18} EFA through a sequence of alternating desaturation and chain-elongation reactions.

The desaturation steps are dependent on the Δ5- and Δ6-desaturases, key enzymes localized in the endoplasmic reticulum (4). The Δ6-desaturase catalyzes the conversion of linoleic acid (18:2n-6) into γ-linolenic acid (18:3n-6) and of α-linolenic acid (18:3n-3) into stearidonic acid (18:4n-3). In this pathway, Δ6-desaturation is considered to be the rate-limiting step (5). The last step in the biosynthesis of arachidonic acid (20:4n-6) involves the Δ5-desaturation of eicosatrienoic acid (20:3n-6). Both the Δ5- and Δ6-desaturases are highly dependent on numerous nutritional and hormonal factors (4).

These enzymes were described in fetal rat (6). The data suggested that, close to term, the fetus is capable of supplying some of its arachidonic acid requirements, and thus relies less on the availability from the maternal organism than during early pregnancy. Another study showed that human fetal liver microsomes are able to desaturate fatty acids (FA) (2). However, the desaturase activities observed in the fetal microsomes were low compared to other mammals (7), and they are probably unable to satisfy the PUFA requirements of the fetus, at least between the 17th and 36th gestational weeks (8).

PL are the main lipid components in biological membranes, and their properties are greatly influenced by the types of FA they contain. PL FA composition is known to be one of the major factors in the regulation of membrane stability, fluidity, permeability, and membrane enzyme activity. Both exogenous FA supply and desaturation–elongation processes are responsible for the maintenance of the PUFA membrane status.

In contrast to the growing body of information on EFA desaturation and its regulation, very little is known about the immediate metabolic fate of neosynthesized PUFA *in situ* in microsomal membranes. Yet this is of special interest since neosynthesized FA are supposed to appear in the free form. Indeed, except for one report showing Δ5-desaturation using PL

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Abbreviations : EFA, essential fatty acid; FA, fatty acid; FFA, free fatty acid; HPLC, high-performance liquid chromatography; NEM, *N*-ethyl-maleimide; NPL, nonphospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated essential fatty acid; TLC, two-dimensional thin-layer chromatography.

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as substrate in rat liver (9), it is generally considered that the actual substrate of desaturases in vertebrates is fatty acylCoA. This implies that the FA substrate is released from the PL precursor, then reincorporated after enzymatic modifications.

FA incorporation into membranes is dependent on several enzymatic reactions. As previously shown (10), FA desaturation and FA incorporation into microsomal PL are two simultaneous, but independent, processes. Thus, competition may occur between esterification and desaturation in the endoplasmic reticulum. This may explain in part the weak desaturation rate observed in human fetal liver *in vitro*.

The aim of the present study was to investigate the metabolic fate of neoconverted FA by comparing the distribution and incorporation of substrates and desaturation products into the different lipid classes. To do so, we also studied the influence of *N*-ethyl maleimide (NEM), a sulfhydryl reagent, on both the microsomal desaturase activities and the metabolic fate of desaturated FA, more particularly the PL reacylation by either substrate or desaturation product.

MATERIALS AND METHODS

Biological samples. Biological samples were obtained from the Fetal Pathology Centers of CHU Lapeyronie-Arnaud de Villeneuve, Montpellier, France, and CHU Robert Debré, Paris, France, after legally approved medical abortion according to French law. These therapeutic abortions were obtained by both oral dose of Myfegyne® (mifepristone, 600 mg) and/or Misoprostol® (cytotec, 200 mg/4 h) from Roussel-UCLAF, Paris, France, and Syntocinon® drip from Sandoz, Rueil-Malmaison, France. The abortions were permitted for three reasons: fetal unviability, polymalformations, and 18 or 21 trisomy. The three fetuses were 24-, 24-, and 25-wk old, and the liver samples (4–5 g each) were obtained 1–4 h after fetal death.

Preparation of microsomes. The microsomal suspension was prepared as previously described (8) from liver samples deep-frozen in liquid nitrogen. The microsomal protein concentrations were estimated by the method of Layne (11) with FA-free crystalline bovine serum albumin as standard.

Enzyme assays. Enzyme activities were measured by the radiochemical method previously described (12) using [1-^{14}C]linoleic acid ([1-^{14}C]18:2n-6), [1-^{14}C]α-linolenic acid ([1-^{14}C]18:3n-3), and [1-^{14}C]eicosatrienoic acid ([1-^{14}C]-20:3n-6) (40–60 mCi/mmol, 99% pure; NEN Life Science Products–France S.A., Le Blanc Mesnil, France) as substrates. For each substrate, we used concentrations that allowed the highest desaturase activity, i.e., 28.6, 19, and 57 μM for 18:2n-6, 20:3n-6, and 18:3n-3, respectively. These concentrations were determined in a previous study (12). All reactions were started by the addition of 3 mg of microsomal proteins.

In a second set of experiments, the conditions of incubation were essentially the same, except that we added 0.52 mM NEM to the reaction medium before adding microsomal proteins.

PL and nonphospholipid (NPL) separation. The analytic schema is shown in Figure 1. This separation was performed on pooled lipid extracts obtained from two microsome incubations for enzyme measurement. According to a modified method from Juadena and Rocquelin (13), the PL and NPL fractions of fetal hepatic microsomes were separated using Sep-Pak Plus® silica cartridges (silica cartridges, Waters). The lipid extracts were evaporated to dryness under nitrogen stream and kept in 200 μL of methanol/chloroform (1:2, vol/vol) (Prolabo, Paris, France). The cartridges were rinsed with 8 mL of chloroform, and the samples were injected at the top of the cartridge. After adsorption of sample, 7 mL of chloroform, and then 6 mL of acetone were pushed through the cartridge, and the corresponding eluted fractions containing the NPL fraction were collected. Then, 24 mL of methanol was added, and the fraction containing PL was eluted. Each fraction was evaporated to dryness under nitrogen stream and kept in 300 μL of methanol/chloroform (1:2, vol/vol). The radioactivity of each fraction was measured by liquid scintillation counting (Picofluor; Packard Instruments, Rungis, France) in Packard Tri-Carb Model 2425.

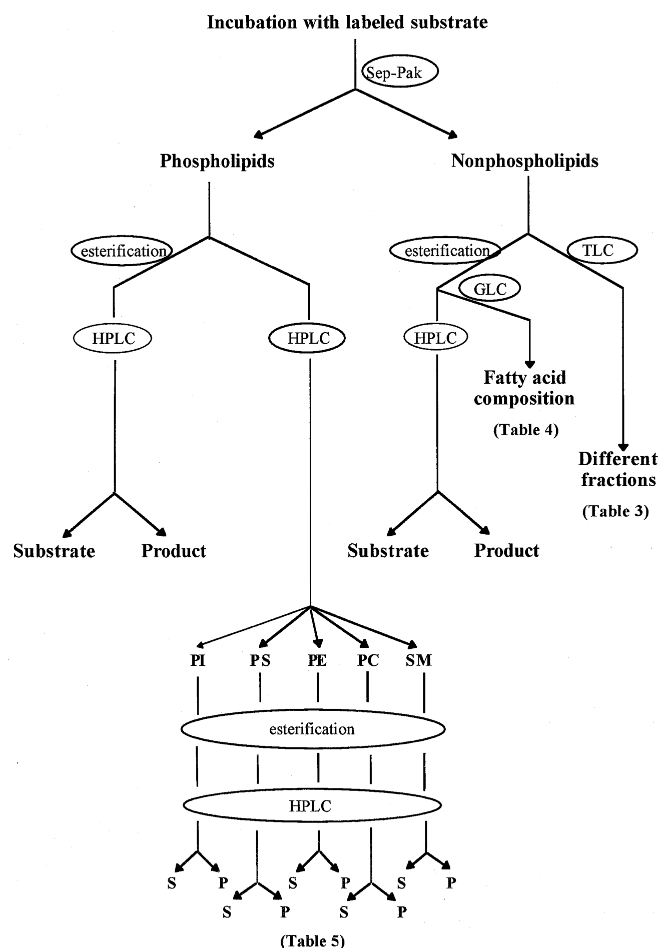

FIG. 1. Analytic scheme: GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; SM, sphingomyelin; TLC, two-dimensional thin-layer chromatography; P, product; S, substrate.

Determination of distribution of radioactivity between substrates and products in the PL and NPL fractions. After

the PL and NPL fractions were transesterified (14), the radioactivity distribution between substrate and desaturation product was determined by a reversed-phase HPLC (15) using a Beckman model 110 A pump (Fullerton, CA) and a Nova-Pack C18 column (60 Å, 4 µm, 3.9 × 150 mm) (Waters, Milford, MA). Activity was expressed as the percentage of converted substrate.

FA composition in the NPL fractions. The FA composition of the NPL fractions was determined by gas–liquid chromatography of methyl esters. After NPL transesterification, the FA methyl esters were analyzed using a DELSI DI 200 chromatograph (Delsi Nermag, Argenteuil, France) and a fused-silica capillary column (50 m × 0.32 mm, C.P. Sil 88; Chrompak, Les Ulis, France). Conditions were as follows: ionization detector, 250°C; injector, 230°C; and oven program, 3.5°C/min from 115 to 230°C. Hydrogen was used as the carrier gas with a flow rate of 1 mL/min^{-1}. Quantitative analysis was achieved with reference to the internal standards using a DELSI ENICA 10. The percentage of total FA was given by weight for the FA of main interest.

Two-dimensional separation of NPL classes. The standard mixture contained equal amounts (80 µg) of triglycerides, cholesterol, and ceramides (Type IV with α-hydroxy FA and Type III with normal FA), equal amounts (90 µg) of 1,2-diacylglycerol and monoglycerides, 70 µg of free fatty acids (FFA), and 60 µg of cholesteryl esters. All standard lipids were purchased from Sigma (St. Louis, MO). Standard lipid mixture was mixed with an aliquot of the microsomal NPL fraction eluted from Sep-Pak. After evaporation to dryness under nitrogen, the samples were kept in 50 µL of methanol/chloroform (1:1, vol/vol). Lipids were applied at the lower left-hand corner of the 20 × 20 cm thin-layer chromatography (TLC) plate (Kieselgel 60 F_{254}; Merck, Darmstadt, Germany). The plate was developed in the first dimension (15 cm) in solvent system I [4 mL of acetic acid (commercial solution at 96%; Merck) and 96 mL of a mixture containing *tert*-butyl-methyl-ether/hexane, 70:30, vol/vol (Prolabo)]. The solvent was allowed to run up to 3 cm from the top. After drying the plate in air, it was developed in the second dimension (15 cm) (see Fig. 2) in solvent system II [ethyl ether/petroleum spirit/acetic acid (commercial solution at 96%), 90:10:1, by vol (Merck, Prolabo)]. The solvent was allowed to run up to 3 cm from the top. The plate was then removed, air-dried, and sprayed with 2′,7′-dichlorofluoroscein (Merck). The separated lipid spots were identified under ultraviolet light and were scraped into counting vials. The gel was mixed with 10 mL of Picofluor 30 scintillation fluid. Radioactivity was determined as indicated above.

Separation of PL classes. The PL classes were separated by HPLC using a Beckman model 110 A pump and a Lichrospher Si-60 column (5 µm, 4 × 125 mm) (Merck), according to the method described by Niessen and Kreysel (16) and adapted as follows: the solvent mixture contained acetonitrile (solvent A) (Carlo Erba, Val de Reuil, France) and H_2O/NH_4OH (0.2%) (commercial solution at 30%) (solvent B) (Carlo Erba), using a flow rate of 1 mL/min^{-1}. The multiphasic elution gradient is shown in Figure 3 (the solvent B percentage varied from 2 to 25%). The separation was in the following order: phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine (PE), phosphatidylcholine (PC), and sphingomyelin. Fractions were collected and stored under nitrogen at −20°C. The fractions obtained during the species separation were dried under a nitrogen stream, saponified, and methylated (14). The distribution of radioactivity between the

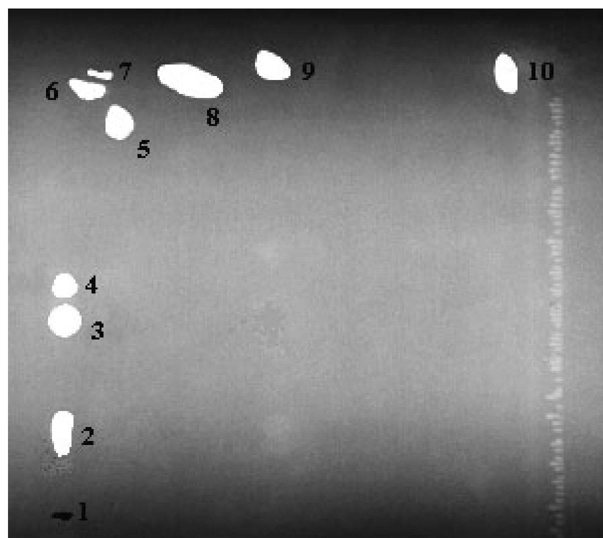

FIG. 2. Representative example of a TLC of radioactive lipids diluted with standard lipids from liver microsomes. 1: origin; 2: ceramide type IV Sigma (St. Louis, MO) with α-hydroxy fatty acids; 3: monoglyceride (MG), 4: ceramide III Sigma with usual fatty acids; 5: cholesterol; 6: 1,2-diacylglycerol (DAG); 7: 1,3-DAG; 8: free fatty acid (FFA); 9: triglyceride (TG); 10: cholesteryl esters (CE). Additionally, 1,3-DAG and 1,2-DAG are well separated from cholesterol. But we have considered the 1,3-DAG and 1,2-DAG together. See Figure 1 for other abbreviation.

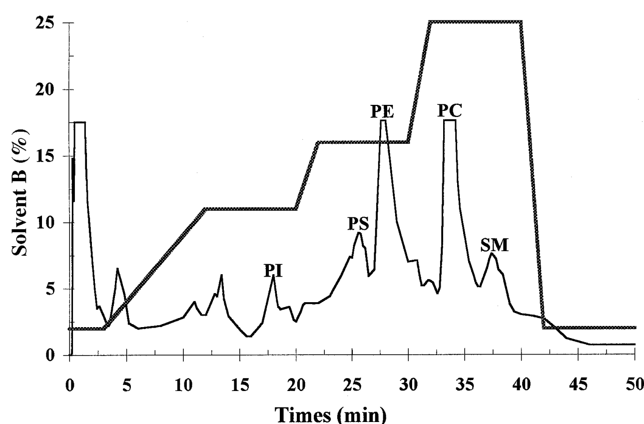

FIG. 3. Separation of phospholipid classes from human fetal liver. The column (4 × 125 mm) was packed with silica (LiChrospher Si 60). Extraction of lipids, application to the column, and elution conditions were as described in the Materials and Methods section. The absorbance of the eluate was measured at 214 nm. PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine, and SM, sphingomyelin. For the compositions of solvents A and B see the Materials and Methods section.

substrate and the desaturation product in each PL class was determined by reversed-phase HPLC as described above for total PL and NPL fractions.

Statistics. Results are expressed as the mean ± SD for n = 3 fetal liver and were analyzed statistically using a Mann-Whitney nonparametric test (StatView™; Alsyd, Meylan, France). The differences between groups were considered significant at 0.05.

RESULTS

Figure 4 compares the effect of NEM on n-6 Δ6-, n-6 Δ5- and n-3 Δ6-desaturase activities of fetal liver microsomes, showing that 0.52 mM NEM significantly decreased (P < 0.05) linoleic and eicosatrienoic acid conversion: 79 and 69.8% for n-6 Δ5- and n-6 Δ6-desaturase activities, respectively. In contrast, n-3 Δ6-desaturase was not inhibited by NEM but, surprisingly, it increased (49.8%). These results suggest that NEM specifically inhibits Δ6-desaturase activity in the n-6 series but not in the n-3 series. When measured in the incubation medium, the endogenous substrate concentrations were 0.21 µM for 18:2n-6; 0.09 µM for 20:3n-6, and ≤0.004 µM for 18:3n-3 (12). Notably the actual endogenous substrate concentrations were only 0.73, 0.47, and ≤0.007% of the exogenous labeled substrate concentrations. In these experimental conditions, the dilution of exogenous substrate with endogenous substrate was negligible and was not able to interfere significantly in enzyme activity measurements.

Table 1 shows the respective incorporation of substrates and desaturation products into the PL and NPL fractions.

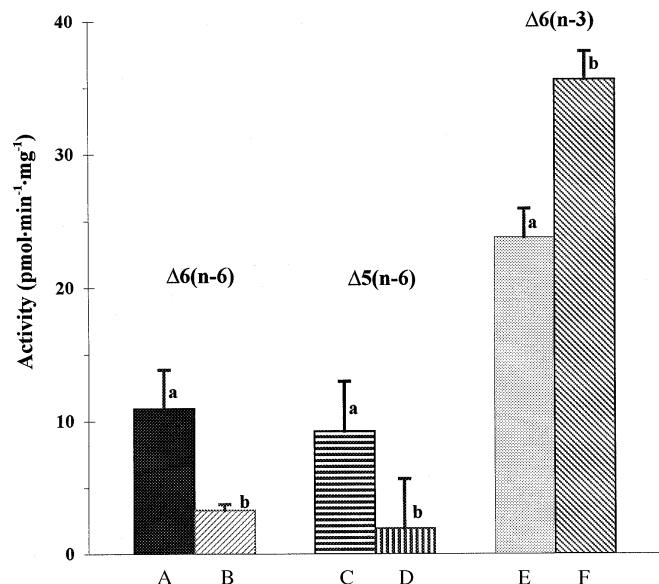

FIG. 4. Comparison of Δ5- and Δ6-desaturase activities in the presence or absence of *N*-ethyl-maleimide (NEM; 0.52 mM). Results are expressed as the mean ± SD for n = 3 fetal liver (age = 24, 24, and 25 wk). The differences between groups were considered significant at P < 0.05 (Mann-Whitney nonparametric test) [A, 18:2n-6 (28.6 µM); B, 18:2n-6 (28.6 µM) + NEM; C, 20:3n-6 (19 µM); D, 20:3n-6 (19 µM) + NEM; E, 18:3n-3 (57 µM); and F, 18:3n-3 (57 µM) + NEM].

TABLE 1
Distribution (%) of Total Radioactivity Between the Phospholipid and Nonphospholipid Fractions from Incubations Carried Out in the Presence and in the Absence of NEM[a]

	A 18:2n-6		B 20:3n-6		C 18:3n-3	
		+NEM		+NEM		+NEM
Phospholipid	11.1	22.2	11.1	8.4	8.6	9.6
Nonphospholipid	88.9	77.8	88.9	91.6	91.4	90.4

[a]The distribution of incorporated radioactivity is expressed as percentage of total radioactivity recovered as phospholipid (PL) plus nonphospholipid (NPL): 30636, 22833, and 66092 cpm in the absence of *N*-ethyl-maleimide (NEM) for A, B, and C incubations, respectively, and 37306, 28564, and 84252 cpm in the presence of 0.52 mM of NEM for A, B, and C incubations, respectively.

Whatever the substrate and in the presence or absence of NEM, most of the radioactivity was recovered in the NPL fraction. When 20:3n-6 and 18:3n-3 were used as substrates (incubations B and C), the distribution varied little in the presence or absence of NEM. When 18:2n-6 was used as substrate, the radioactivity recovered in the PL fraction in the absence of NEM was half that recovered in the presence of NEM.

The radioactivity distribution between substrates and desaturation products in the PL and NPL fractions is shown in Table 2. The incorporation of labeled substrates was clearly higher than that of products in both fractions, in the presence and absence of NEM. In addition, the proportion of recovered products was three- to fivefold higher in PL than in NPL. The percentage of 18:3n-6, 20:4n-6, and 18:4n-3 recovered in microsomal PL was about 3.9-, 5.2- and 3.7-fold higher, respectively, than in microsomal NPL. The highest PL or NPL incorporation was observed for 18:4n-3. This was in accordance with the total enzyme activities observed (Fig. 4). Arachidonic acid was also strongly integrated in the PL fraction despite a lower biosynthesis.

TABLE 2
Distribution (%) of Total Radioactivity Between the Phospholipid and Nonphospholipid Fractions from Incubations Carried Out in the Presence and in the Absence of NEM (0.52 mM)[a]

		Phospholipid		Nonphospholipid	
			+NEM		+NEM
A	18:2n-6	96.1	97.6	99.0	99.2
	18:3n-6	3.9	2.4	1.0	0.8
B	20:3n-6	94.2	96.7	98.9	99.3
	20:4n-6	5.8	3.3	1.1	0.7
C	18:3n-3	90.4	88.4	97.4	97.4
	18:4n-3	9.6	11.6	2.6	2.6

[a]The distribution of incorporated radioactivity is expressed as percentage of total radioactivity recovered as substrate plus product: 5023, 5240, and 6071 cpm in PL and 51622, 36084, and 116684 cpm in NPL in the absence of NEM for A, B, and C incubations, respectively and 7539, 1953, and 5586 cpm in PL and 52017, 36303, and 109380 cpm in NPL in the presence of 0.52 mM NEM for A, B, and C incubations, respectively. See Table 1 for abbreviations.

TABLE 3
Distribution (%) of Total Radioactivity into Different Classes of the Nonphospholipid Fraction from Incubations Carried Out in the Presence and in the Absence of NEM[a]

	A 18:2n-6		B 20:3n-6		C 18:3n-3	
		+NEM		+NEM		+NEM
Origin	4.29	0.89	2.99	0.95	9.62	0.93
Ceramide IV	2.41	0.78	2.40	1.37	3.35	0.25
Ceramide III	2.95	0.56	2.90	1.11	3.75	0.12
Monoglycerides	0.78	ND	1.57	0.72	1.56	ND
Cholesterol	1.07	0.28	2.27	1.37	1.89	0.82
DAG (1,2- + 1,3-)	ND	1.20	7.22	7.52	8.13	3.09
FFA	76.7	90.14	74.38	84.63	63.04	91.79
TG	4.17	0.49	3.83	0.26	5.31	0.22
CE	3.13	0.33	0.52	0.15	0.67	0.18

[a]Data are means of three countings. The distribution of incorporated radioactivity is expressed as percentage of total radioactivity recovered as substrate plus product: 40900, 33600, and 105000 cpm in the absence of NEM for A, B, and C incubations, respectively, and 195500, 140000, and 833239 cpm in the presence of 0.52 mM NEM for A, B, and C incubations, respectively. DAG, diacylglycerol; FFA, free fatty acid; TG, triglycerides; CE, cholesteryl esters. ND, nondetectable. See Table 1 for other abbreviation.

Figure 2 shows a typical two-dimensional TLC of lipids from liver microsomes. Data are reported in Table 3. Whatever the substrate, most of the total radioactivity was found in the FFA fraction. The percentage of total radioactivity (substrate plus product) recovered in the FFA fraction increased in the presence of NEM: 90.2% vs. 76.7%, 84.6% vs. 74.4%, and 91.8% vs. 63% in incubations A, B, and C, respectively. The second-highest radioactivity incorporation was found in diacylglycerol (1, 2- plus 1, 3-) for B and C, but was undetectable in incubation A, in the absence of NEM.

In triglyceride, monoglyceride and ceramide III and IV fractions, the percentage of radioactivity distribution was in the same range whatever the substrate, but decreased with NEM.

Surprisingly, some radioactivity was detected in the cholesterol fraction, but it was also lower when incubations were conducted in the presence of NEM.

The NPL fractions were esterified and analyzed by gas–liquid chromatography (Table 4). The comparison of FA composition in these different fractions obtained from incu-

TABLE 4
Fatty Acid Composition (wt%) in the Nonphospholipid Fractions from Incubations Carried Out in the Presence and in the Absence of NEM[a]

	Exogenous substrate						
	18:2n-6 (A)		20:3n-6 (B)		18:3n-3 (C)		
		+NEM		+NEM		+NEM	means ± SD
14:0	1.23	0.42	0.51	1.04	1.20	0.62	0.84 ± 0.36
16:0	20.15	22.30	19.10	21.07	20.96	21.65	20.87 ± 1.13
18:0	8.95	9.45	8.67	8.74	8.51	9.13	8.91 ± 0.34
24:0	0.63	0.5	1.03	0.60	0.79	0.62	0.70 ± 0.18
16:1n-9	1.84	2.55	1.38	2.20	2.35	1.39	1.95 ± 0.50
18:1n-9	40.43	38.98	43.14	42.92	32.97	35.60	39.01 ± 4.06
16:1n-7	3.77	4.51	3.36	3.45	3.61	4.06	3.79 ± 0.43
18:1n-7	3.59	3.72	3.91	3.75	3.08	3.29	3.56 ± 0.31
18:2n-6			6.15	4.49	5.62	4.60	5.22 ± 0.80
18:2n-6*	**15.97**	**13.74**					
18:3n-6			0.19	0.20	0.13	0.14	0.17 ± 0.04
18:3n-6*	**0.19**	**0.21**					
20:3n-6	0.46	0.5			0.64	0.59	0.55 ± 0.08
20:3n-6*			**8.54**	**8.42**			
20:4n-6	2.13	2.15			2.56	2.41	2.31 ± 0.21
20:4n-6*			**2.98**	**2.02**			
22:4n-6	0.13	0.19	0.21	0.21	0.19	0.13	0.18 ± 0.03
18:3n-3	ND	ND	ND	ND			ND
18:3n-3*					**15.06**	**13.90**	
18:4n-3	ND	ND	ND	ND			ND
18:4n-3*					**1.94**	**1.15**	
20:5n-3	0.21	0.15	0.43	0.58	0.25	0.45	0.34 ± 0.17
22:6n-3	0.29	0.26	0.39	0.30	0.16	0.28	0.34 ± 0.16

[a]Results are expressed as percentage of total identified fatty acids. *The bold characters refer to the fatty acid composition resulting from the addition of exogenous radioactive fatty acid substrates in the medium and their conversion products. Other values report the endogenous microsome fatty acid composition in each incubation condition. For abbreviations see Tables 1 and 3.

TABLE 5
Distribution (%) of Radioactive Precursors and Products in Phospholipid Classes[a]

	PI	PS	PE	PC	SM
18:2n-6	5.6	7.8	12.9	70.1	2.2
18:3n-6	Trace	Trace	Trace	1.4	
20:3n-6	18.0	10.25	21.0	60.2	2.7
20:4n-6	Trace		1.7	4.2	
18:3n-3	9.3	5.8	16.1	65.6	1.9
18:4n-3			Trace	1.2	

[a]The distribution of incorporated radioactivity is expressed as percentage of total radioactivity. Data are means of three countings. PI, phosphatidylinositol; PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; SM, sphingomyelin.

bations in the presence and in the absence of NEM showed that 18:2n-6, 20:3n-6, and 18:3n-3 contents (radioactive plus endogenous substrates in heavy characters) were, respectively, about 3-fold, 15.5-fold, and ≫15-fold higher than the concentration of the corresponding endogenous substrate. Compare for instance 18:2n-6 (heavy characters) in incubation A with 18:2n-6 (endogenous) in incubations B and C. This shows that the FFA of the NPL fraction (the actual substrate of mammalian desaturases) was mainly constituted with exogenous substrate. The desaturation products were distributed among different classes, but at substantially lower levels. The high 18:4n-3 content reflected a high conversion rate.

Table 5 shows the distribution of labeled substrates and products among the different PL classes. They were essentially incorporated into PC and to a lesser extent into PE. The highest percentage of incorporated desaturation product was observed with arachidonic acid.

DISCUSSION

Liver samples were obtained from fetuses having anatomic malformations, but none of the fetuses came from mothers affected with metabolic disorders. No therapeutic interventions were reported in the antecedents except for the expulsion protocol. The low frequency of legally approved medical abortions that provide good conditions for sampling considerably reduced the number of usable livers. In addition, due to the multiplicity of studies on the same human material, the available biological material was limited. To ensure that enzyme activities remained unaltered, livers were immediately frozen in liquid nitrogen and processed as soon as possible. The difference in desaturase activities before and after deep-freezing remained in the range of the experimental variation (17).

The n-6 Δ6-, n-6 Δ5-, and n-3 Δ6-desaturase activities were 10.9, 9.3, and 23.8 $pmol \cdot min^{-1} \cdot mg^{-1}$, respectively (Fig. 4) in the absence of NEM. These values are within the range of the data previously obtained in fetuses of the same gestational age and at the same substrate concentration (8). The Δ6-desaturase activity in the n-3 series was higher than the Δ6- and Δ5-desaturase activities in the n-6 series. All these activities in human fetal liver were lower than in human adults or animals, especially rodents (12).

Some *in vitro* studies suggested that substrate FA incorporation into microsomal lipids may compete with the desaturation reaction (10,18,19). According to this hypothesis, the FA substrate would be preferentially incorporated into PL and only the nonincorporated substrate would be desaturated. In order to verify this hypothesis, we blocked the acyltransferase activity by means of NEM, an SH reagent that was reported to inhibit this activity (20). In this condition, the desaturase activities were expected to increase due to the inhibition of the competing acyltransferase activity. In the presence of NEM, a significant increase in n-3 Δ6-desaturase activity was observed (49%, $P < 0.025$) in accordance with the hypothesis. In contrast, n-6 Δ6- and Δ5-desaturase activities were strongly decreased by 79 and 69.8%, respectively (Fig. 4). It should be noted that this result is consistent with that of Yamaoka *et al*. (21), who showed that NEM inhibited n-6 Δ5-desaturase activity by 46% of control in rat liver. However, this was never reported for Δ6-desaturase. It could be hypothesized that alteration of endogenous substrate concentration under the NEM influence could be responsible for enzyme activity variation by diluting the exogenous substrate. However, notably the actual substrates of desaturases in mammals are not PL FA but only acylCoA. In our experimental conditions, the concentrations of endogenous unesterified FA are much lower than exogenous substrate concentrations and account only for 0.007 to 0.7% of exogenous substrate (cf. Results and Ref. 12). In these conditions, it is very unlikely that endogenous substrate concentration variation could significantly alter the measurement of desaturase activity.

The fact that NEM inhibited the Δ5- and Δ6-desaturase activities of the n-6 series, but not the Δ6-desaturase activities of the n-3 series, may be due to a direct interaction with the enzymes and/or membrane environment modifications. In any case, these results suggest the existence of two distinct Δ6-desaturases, one for the n-6 series and another for the n-3 series. Another study has suggested this by reporting that sesame oil specifically inhibited n-6 Δ5-desaturase of rat liver but not n-3 Δ5-desaturase (22).

We investigated the distribution in the different lipid classes and PL subclasses of [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, and [1-^{14}C]20:3n-6 and their desaturation products [1-^{14}C]-18:3n-6, [1-^{14}C]18:4n-3, and [1-^{14}C]20:4n-6, respectively, after *in vitro* Δ5- or Δ6-desaturation. In the presence or absence of NEM, the substrate and product radioactivity was recovered mainly in the NPL fraction (Table 1). Two hypotheses could explain why NEM did not clearly alter the FA radioactivity distribution between the PL and NPL fractions: either the incubation conditions and/or the microsome preparation that were adapted to desaturase measurement were inadequate for acyltransferase activity, or the acyltransferase system was overloaded by exogenous substrate. The first hypothesis is unlikely since it was recently shown that acyltransferase and Δ6-desaturase activity can be evaluated using the same liver microsome preparation and the same incubation

conditions (23). Overloading of the acyltransferase system is more likely. It should be recalled as indicated above, that in the incubation medium, the FFA concentration resulting mainly from addition of exogenous radioactive substrate was higher than the FA concentration in endogenous membranes and considerably higher than the endogenous FFA concentration. Recently, the activity ratio of acyltransferase/Δ6 n-6 desaturase was estimated to be about 10:1 in rat liver (23). The amount of substrate transformed by the fetal hepatic desaturase, in 15 min in the incubation medium, can be calculated from the specific activity measured for instance for Δ6-linoleic desaturase. If the ratio of acyltransferase/Δ6 n-6 desaturase would be in the same range as in the rat liver, the amount of linoleic acid reacylated would be 10 times higher. When calculated, this theoretical amount of reacylated linoleic acid accounts for only 10% of the exogenous substrate. This interpretation is supported by the observation that about 90% of the exogenous radioactive substrate remained in the NPL fraction, and about 80% was recovered in the FFA form (Tables 1 and 3).

Considering the distribution of the neoconverted FA, it is noteworthy that they tended to be incorporated more into the PL fraction than in the NPL fraction (Table 2), both in the presence and absence of NEM. This preferential PL incorporation of products suggests channeling of the neoconverted PUFA toward the PL of microsomal membrane. This could be of special importance for the control of the desaturation level of the constitutive lipids, a critical parameter for the maintenance of membrane functions.

In the presence of NEM, we observed that the percentage of recovered radioactivity was lower in all lipid fractions, with the exception of FFA, in all incubation conditions (Table 3). This indicates a shift in the radioactivity toward the FFA fraction in the presence of NEM, in agreement with a limited lowering of the acyltransferase activity by this compound. Surprisingly, traces of radioactivity were detected several times in the cholesterol or sterol fraction. Contamination with the radioactivity from another fraction is an unlikely explanation. The liver is a major site of cholesterol biosynthesis, and it should be noted that our incubation medium offered all the conditions for sterol synthesis: liver microsomes had very active HMGCoA reductase; the crude microsomal preparation carried some light mitochondria and/or mitochondrial fragments able to perform β-oxidation; a few cytosols were added to the incubation medium that also contained the cofactors of sterol synthesis, NADPH and ATP; and the incubation duration was 15 min.

The PL-incorporated substrates and products showed that most of the labeled FA were incorporated into PC and to a lesser extent into PE (Table 5). This observation is in agreement with the accepted notion that PC and PE are the main PL classes synthesized at the outer surface of the endoplasmic reticulum (24). Regarding the desaturation products, PC again appears to be the main acceptor. It is interesting to note that the proportion of substrate converted into 20:4n-6 and incorporated into PC and PE was about twice that of 18:4n-3 and 18:3n-6. This result suggests that direct incorporation of arachidonic acid into PC and PE could very efficiently contribute to the maintenance of the high level of this FA in these PL classes. This observation is in agreement with our data obtained in total PL and confirms that a different channeling may be involved in human liver microsomes for the processing of desaturation substrates and products. Such a channeling would give some priority to the neoconverted FA toward the exogenous PUFA.

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Dietary α-Linolenate Suppresses Endotoxin-Induced Platelet-Activating Factor Production in Rat Kidney

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ABSTRACT: In comparison with dietary high-linoleate safflower oil, high α-linolenate perilla oil decreased alkylacyl- and alkenylacyl-glycerophosphocholine (GPC) content in rat kidney by roughly 30 and 25%, respectively. The fatty acid composition was also modified by high α-linolenate oil; arachidonic acid (AA) level in alkylacyl-GPC, a platelet-activating factor (PAF) precursor, decreased by 30% along with concomitant increases in the n-3 fatty acid levels. PAF contents under resting conditions were similarly low in the two dietary groups. Fifteen minutes after endotoxin administration, PAF and lyso-PAF contents increased significantly, and the PAF content in the high α-linolenate group was 60% lower than in the high linoleate group; the lyso-PAF contents also tended to be lower. Lyso-PAF acetyltransferase and CoA-independent transacylase activities in kidney microsomes increased significantly after endotoxin administration, while PAF acetylhydrolase activity in the cytosol was relatively unchanged. The lyso-PAF acetyltransferase and PAF acetylhydrolase activities did not differ between the two dietary groups, but the CoA-independent transacylase activity was roughly 30% lower in the high α-linolenate group. In agreement with *in vitro* study, our present study demonstrates that dietary high α-linolenate suppresses PAF production in rat kidney during systemic endotoxemia, and which is mainly due to the decrease in alkylacyl-GPC content, altered fatty acid compositions of the precursor lipids and lower CoA-independent transacylase activity.

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1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (platelet-activating factor, or PAF) and PAF analogs are potent proinflammatory mediators produced by stimulated phagocytes and endothelial cells. They cause a wide range of biological responses including platelet aggregation, activation of leukocytes, broncho-constriction, increased vascular permeability, and systemic hypotension (1–6). PAF is associated with many pathological states, particularly with endotoxic shock, as noted by increased plasma PAF levels in gram-negative sepsis and experimental endotoxemia. Results of the administration of PAF to experimental animals mimic many pathophysiological features of endotoxic shock, and PAF receptor antagonists have been shown to ameliorate endotoxin-induced hypotension and prolong survival time (7–9).

A number of studies have shown that dietary n-3 fatty acids are effective in reducing arachidonic acid (AA) in membrane phospholipids, attenuating the production of biologically active eicosanoids and PAF, and ameliorating inflammatory diseases (10–14). We have demonstrated that dietary supplementation with perilla oil, having high α-linolenate (18:3n-3) and low linoleate (18:2n-6) contents, suppresses PAF production in rat peritoneal polymorphonuclear leukocytes (PMN) as compared with high linoleate safflower oil, and we have proposed a possible mechanism of the reduction of PAF synthesis in PMN by feeding perilla oil (15,16). In rat kidney, Yeo *et al.* (17) reported that dietary eicosapentaenoate- and docosahexaenoate-rich fish oil reduced the lyso-PAF acetyltransferase activity in microsomes. Furthermore, Park *et al.* (18) showed that dietary high α-linolenate perilla oil decreased the alkylacyl-glycerophosphocholine (GPC) content in rat kidney. However, the dietary effects of these n-3 fatty acids on PAF biosynthesis in kidney *in vivo* have not been evaluated directly by measuring PAF levels because of its presence in low concentrations and rapid hydrolysis to lyso-PAF *in vivo*. Furthermore, we noted no significant differences in alkylacyl-GPC contents in PMN of rats fed perilla oil or safflower oil (15).

The purpose of the present study was (i) to evaluate whether dietary high α-linolenate perilla oil changes the PAF precursor content in rat kidney compared with dietary high linoleate safflower oil and (ii) to quantify and compare PAF biosynthesis *in vivo* using an endotoxemia model.

MATERIALS AND METHODS

Reagents. 1-*O*-Alkyl-2-acetyl-*sn*-glycero-3-phosphocholine (PAF) was purchased from Avanti Polar Lipids Inc. (Birmingham, AL). [^{3}H]PAF was prepared by treating 1-*O*-[^{3}H]octadecyl-2-lyso-*sn*-glycero-3-phosphocholine ([^{3}H]-lyso-PAF, 148 MBq/mmol) (Amersham Co., Buckinghamshire, England) with acetic anhydride/pyridine. [^{14}C]Acetyl-CoA (3.6

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Abbreviations: AA, arachidonic acid; DG, diradylglycerols; DHA; docosahexaenoic acid; DNB, dinitrobenzoyl; DNBC, dinitrobenzoyl chloride; EPA, eicosapentaenoic acid; GPC, glycerophosphocholine; HPLC, high-performance liquid chromatography; PAF, platelet-activating factor; PC, cholinephospholipids; PE, ethanolaminephospholipid; PMN, polymorphonuclear leukocytes; PUFA, polyunsaturated fatty acids; TLC, thin-layer chro-

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MBq/mmol) was purchased from American Radiolabeled Chemicals Inc. (St. Louis, MO). Endotoxin (lipopolysaccharide, *E. coli* 026: B6) and phospholipase C (*Bacillus cereus*) were obtained from Sigma Chemical Co. (St. Louis, MO). Sep-Pak silica cartridges were purchased from Waters Co. (Milford, MA). Silica gel 60 thin-layer chromatography (TLC) plates were obtained from Merck (Darmstadt, Germany). 3,5-Dinitrobenzoyl chloride (DNBC) was obtained from Dojindo (Kumamoto, Japan).

Animals and diets. Male Sprague-Dawley rats (SLC Co., Shizuoka, Japan) at 3 wk of age were divided randomly into two groups and fed a diet supplemented either with linoleate-rich (18:2n-6) safflower oil or with α-linolenate-rich (18:3n-3) perilla oil *ad libitum* for 5 wk. These diets were prepared by supplementing a semipurified fat-free diet (Clea Japan Co., Tokyo, Japan) with 10% (w/w) safflower oil or perilla oil (Ohta Oil Mill Co., Okazaki, Japan). The diets were stored at −20°C. The fatty acid composition of the test diets is shown in Table 1.

Animal treatment. Following anesthesia with sodium pentobarbital (50 mg/kg body weight, i.p.), endotoxin (5 mg/kg) or vehicle (phosphate-buffered saline, 2.5 mL/kg) was injected intravenously. After 15 min, 0.8 mL of whole blood was collected by heart puncture in EDTA and immediately mixed with 3 mL of chloroform/methanol (1:2, vol/vol). Tissue samples (kidney and lung) were harvested, quickly frozen with liquid nitrogen, and stored at −80°C until use.

Measurement of PAF and lyso-PAF. For the PAF and lyso-PAF assays, frozen tissues were thawed and homogenized in 20 mL chloroform/methanol/water (1:2:0.8, by vol). Total lipids were extracted from whole blood and tissue homogenates using the methods of Bligh and Dyer (19). After evaporation of the solvent under N_2 gas, the residue was dissolved in chloroform. The extracted total lipids were applied to a Sep-Pak silica cartridge, which was sequentially washed with 10 mL chloroform, 10 mL acetone, 10 mL acetone/methanol (1:1, vol/vol), and 10 mL chloroform/methanol (7:3, vol/vol) (20). A 10-mL chloroform/methanol/water (1:2:0.8, by vol) eluate was collected, and the lipids were extracted again using the method of Bligh and Dyer. Individual phospholipids were separated by silica gel TLC with chloroform/methanol/water (65:35:6, by vol) as a developing solvent. The areas corresponding to PAF and lyso-PAF were scraped off the plates, and the lipids were extracted as described above. After evaporation of the solvent under N_2 gas, the residue containing PAF was dissolved in Tyrode's solution (3.8 mM HEPES; 2.7 mM KCl; 0.4 mM NaH_2PO_4; 137 mM NaCl; 0.5 mM $MgCl_2 \cdot 6H_2O$; 1.8 mM $CaCl_2$; 5.6 mM glucose; 0.25% gelatin, pH = 7.35) containing 0.25% bovine serum albumin. The residue containing lyso-PAF was chemically acetylated to PAF using acetic anhydride/pyridine (5:1, vol/vol) (21) and dissolved as described above. PAF was quantified by measuring the aggregation of washed rabbit platelets as described previously (22). Briefly, 100 µL of platelet suspension (2.5×10^8/mL) in Tyrode's solution was prewarmed to 37°C and stirred at 1000 rpm. Platelet aggregation was recorded as the percentage change in light transmission after the addition of 10 µL of test sample. PAF concentration was calculated based on a calibration curve obtained with known concentrations of authentic PAF. The recovery of [^{3}H]PAF, which had been spiked into total lipids, in the eluates from Sep-Pak cartridge was more than 95%, and that from TLC was about 75% as estimated using a PAF standard. Total recovery measured by the addition of [^{3}H]PAF to the tissue homogenates was more than 70%. The platelet aggregation-inducing activity of PAF was blocked completely by CV6209, a PAF receptor antagonist (23).

Fatty acid analysis. The total lipids in kidney were extracted as described above. The individual phospholipids were separated by silica gel TLC with chloroform/methanol/acetic acid/water (25:15:4:2, by vol). The area corresponding to cholinephospholipids (PC) was scraped off the plates, and PC were extracted as described above. The fatty acid compositions of PC subclasses were measured according to the method of Nakagawa *et al.* (24). Briefly, PC were hydrolyzed with phospholipase C, and the resultant diradylglycerols (DG) were treated with acetic anhydride and pyridine. The samples were dried under N_2 gas and separated by TLC using petroleum ether/diethyl ether/acetic acid (90:10:1, by vol) and then with toluene. The area corresponding to each DG subclass was scraped from the plates, heptadecanoic acid was added as an internal standard, and the lipids were extracted as described above. Fatty acid methyl esters were obtained by treating the total lipids with 50 mg/mL hydrogen chloride in methanol for 1 h in a boiling water bath, and were analyzed by gas–liquid chromatography as described previously (25). Alternatively, PC subclass contents were determined according to the method of Kito *et al.* (26) with slight modifications. Briefly, alkylacyl-GPC (PAF) was added as an internal standard to the PC residue and hydrolyzed with phospholipase C as described above. The resultant DG were treated with DNBC (25 mg/0.5 mg DG) in pyridine and incubated for 10 min at 60°C to yield the dinitrobenzoyl (DNB) esters of DG. The DNB esters of DG were applied to a Sep-Pak C18 car-

TABLE 1
Fatty Acid Composition of Test Diets[a]

Fatty acid	Safflower oil	Perilla oil
	% (w/w) of total fatty acids	
16:0[b]	6.6	6.4
18:0	2.5	2.0
18:1	14.6	21.0
18:2n-6	75.9	13.4
18:3n-3	0.4	57.2
18:2n-6/18:3n-3 ratio	190	0:23

[a]All components for the preparations of test diets were supplied by Clea Japan Inc. (Tokyo, Japan). The test diets consisted of 44.5% cornstarch, 23.3% casein, 1.9% α-starch, 7.6% cellulose powder, 4.7% sucrose, 1.9% vitamin mixture, 5.7% mineral mixture, 0.4% *dl*-methionine, and 10% each oil. The fatty acid composition of each diet was analyzed by gas–liquid chromatography.
[b]Fatty acids are designated by the carbon chain: the number of double bonds. The position of the first double bond numbered from the methyl terminus is designated as n-6 or n-3.

tridge, and methanol eluates were dried and dissolved in methanol. An aliquot of the solution was applied to high-performance liquid chromatography (HPLC) on a normal-phase HPLC column (Rainin Silica, 4.56 × 250 mm; Rainin Instrument Co. Inc., Woburn, MA) using the mobile phase of hexane/diethyl ether/ethanol (87.5:12.5:0.05, by vol), and the DNB esters of DG were quantitated at 254 nm. The DNB derivatives of alkylacylglycerol, alkenylacylglycerol, alkylacetylglycerol (an internal standard), and diacylglycerol were eluted in order. The phosphorus contents of PC and ethanolaminephospholipid (PE) were determined using the method of Bartlett (27).

Lyso-PAF acetyltransferase, PAF acetylhydrolase and CoA-independent transacylase activities. For lyso-PAF acetyltransferase assay, frozen kidney was thawed and homogenized in 10 vol of 0.25% sucrose, 20 mM Tris-HCl, 10 mM EDTA, 5 mM mercaptoethanol, 50 mM NaF (pH 7.4) using a glass homogenizer (10 strokes). The homogenate was first centrifuged at 450 × *g* for 10 min to remove cellular debris and nuclei. After further centrifugation at 15,000 × *g* for 10 min, membrane pellets were prepared from the supernatant fraction by centrifugation at 100,000 × *g* for 60 min. The microsomes were resuspended in 0.25 M sucrose/1 mM dithiothreitol/20 mM Tris-HCl (pH 7.4). Lyso-PAF acetyltransferase activities were measured by incubating 200 µg of microsomal proteins in 0.8 mL of 0.1 M Tris-HCl (pH 6.9) containing 200 µM [^{14}C]acetyl-CoA and 25 µM lyso-PAF for 10 min at 37°C as described by Lee (28). The total lipids were extracted, and the radioactivity corresponding to PAF on TLC was determined using a liquid scintillation counter. PAF acetylhydrolase was assayed according to the method of Blank *et al.* (29). Briefly, kidney was homogenized in 10 vol of 0.25% sucrose and fractionated as described above. PAF acetylhydrolase activities in cytosolic fractions were measured at 37°C after incubation for 10 min in 0.3 mL 0.1 M phosphate buffer (pH 8.0) containing 25 µg cytosol protein and 10 µM [^{3}H]PAF. After the extraction of total lipids, PAF and lyso-PAF were separated and their radioactivities were counted. For the determination of CoA-independent transacylase activities, incubation was carried out at 37°C for 3 min in 0.4 mL 0.1 M Tris-HCl buffer (pH 7.4) containing 100 µg microsomal protein and 2 µM [^{3}H]lyso-PAF (30). After the extraction of total lipids, PC, lyso-PAF, and neutral lipids were separated and their radioactivities were quantified.

Statistical analysis. Statistical analysis was performed using the Student's *t*-test and two-way analysis of variance (StatView–4.02, Abacus Concepts Inc., Berkeley, CA), and probability (P) values below 0.05 were considered significant.

RESULTS

Fatty acid composition of PC in rat kidney. There were no significant differences in body and kidney weights between the two dietary groups (data not shown). The most abundant phospholipid in kidney was PC, and its content was not different between the high linoleate safflower oil group and the high α-linolenate perilla oil group (9.59 ± 0.25 and 9.35 ± 0.22 µmol/g tissue, respectively).

TABLE 2
Subclass Composition of PC in Rat Kidney[a]

Subclass	Safflower oil group	Perilla oil group
	(mol %)	
Experiment 1		
Diacyl	96.20 ± 0.53	97.40 ± 0.26**
Alkylacyl	1.47 ± 0.09	1.04 ± 0.07*
Alkenylacyl	2.32 ± 0.49	1.60 ± 0.18
Experiment 2		
Diacyl	96.71 ± 0.20	97.48 ± 0.18*
	(9.28 ± 0.25)	(9.11 ± 0.22)
Alkylacyl	1.48 ± 0.11	1.07 ± 0.10*
	(0.14 ± 0.01)	(0.10 ± 0.01)
Alkenylacyl	1.81 ± 0.10	1.45 ± 0.10*
	(0.17 ± 0.01)	(0.13 ± 0.01)

[a]The compositions of cholinephospholipid (PC) subclasses in rat kidney were analyzed by gas–liquid chromatography (Experiment 1) and high-performance liquid chromatography (Experiment 2) as described in the Materials and Methods section. Values are the means ± SEM (n = 5–7). Values in parentheses denote the amounts expressed as µmol/g wet tissue. Statistical analysis was performed by Student's *t*-test; *$P < 0.05$ and **$P < 0.10$.

The molar proportions of PC subclasses are shown in Table 2. When determined using the gas–liquid chromatography method, the alkylacyl and alkenylacyl forms were minor components of the total PC fraction in rat kidney (<3%), in contrast to the reported values of 10–25% (18). However, a significant decrease in alkylacyl-GPC content (by 30%) was reproducibly observed in the perilla oil group as compared with the safflower oil group. In view of the importance of accurately determining the content of PAF precursor, we applied the HPLC method. Again, the alkylacyl-GPC and alkenylacyl-GPC contents in the perilla oil group were significantly lower than in the safflower oil group by 27 and 20%, respectively.

The acyl chain compositions of PC subclasses are presented in Table 3. In regard to alkylacyl-GPC content, the major polyunsaturated fatty acid (PUFA) in the high linoleate safflower oil group was AA, but the AA content was more than 50% lower, which was compensated for by marked increases in eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) contents in the high α-linolenate perilla oil group. The dietary oil-induced changes in fatty acid compositions were qualitatively similar among the three PC subclasses. Despite a very low linoleate/α-linolenate ratio (0.23) in the perilla oil diet, the linoleate content was significantly higher than in the safflower oil group, and α-linolenate was a relatively minor component of the three PC subclasses. The compositions of dimethyl acetal derived from alkenylacyl-GPC did not differ between the two dietary groups (data not shown).

The content of another major phospholipid, PE, did not differ between the two dietary groups, and the differences in the fatty acid compositions of PE between the two dietary groups were also similar to those of the PC subclasses (data not shown).

TABLE 3
Acyl Chain Compositions of PC Subclasses from Rat Kidney[a]

	Diacyl		Alkylacyl		Alkenylacyl	
	SAF	PER	SAF	PER	SAF	PER
	% (w/w) of total fatty acids					
14:0	0.3 ± 0.1	0.3 ± 0.0	Trace	Trace	Trace	Trace
16:0	31.5 ± 0.6	31.5 ± 0.9	34.8 ± 2.0	39.6 ± 0.8	10.5 ± 1.8	12.8 ± 1.1
16:1	0.5 ± 0.1	1.0 ± 0.1*	Trace	Trace	Trace	Trace
18:0	15.0 ± 0.5	14.4 ± 0.5	5.2 ± 1.9	4.9 ± 0.7	5.5 ± 2.1	4.6 ± 2.3
18:1	9.0 ± 0.3	11.8 ± 0.2*	4.5 ± 0.1	8.4 ± 0.8*	4.7 ± 0.6	14.4 ± 0.9
18:2n-6	12.4 ± 0.5	19.7 ± 0.4*	5.5 ± 0.3	8.1 ± 0.3*	4.7 ± 0.6	13.7 ± 0.9*
18:3n-3	N.D.	3.1 ± 0.1*	0.3 ± 0.2	1.3 ± 0.1*	0.1 ± 0.1	1.2 ± 0.1*
20:4n-6	28.9 ± 0.9	8.6 ± 0.3*	43.6 ± 2.9	16.8 ± 0.8*	66.4 ± 5.8	29.8 ± 2.2*
20:5n-3	N.D.	5.8 ± 0.3*	N.D.	9.8 ± 0.6*	N.D.	16.4 ± 2.1*
22:4n-6	0.3 ± 0.1	N.D.*	1.6 ± 0.1	N.D.*	0.6 ± 0.2	N.D.*
22:5n-6	0.5 ± 0.1	N.D.*	2.1 ± 0.1	N.D.*	0.5 ± 0.2	N.D.*
22:5n-3	N.D.	1.2 ± 0.1	N.D.	3.5 ± 0.2*	N.D.	2.1 ± 0.1*
22:6n-3	0.6 ± 0.0	1.6 ± 0.1*	2.2 ± 0.2	6.7 ± 0.3*	0.4 ± 0.2	2.7 ± 0.2*
EPA/AA	0.0 ± 0.0	0.7 ± 0.1*	0.0 ± 0.0	0.6 ± 0.0*	0.0 ± 0.0	0.5 ± 0.0*
n-3/n-6	0.0 ± 0.0	0.4 ± 0.0*	0.0 ± 0.0	0.8 ± 0.0*	0.0 ± 0.23	0.5 ± 0.0*

[a]Values represent means ± SEM (n = 5). Statistical significance of the differences (*P < 0.05) between the safflower (SAF) and perilla (PER) oil groups were evaluated by Student's t-test. EPA, eicosapentaenoic acid; AA, arachidonic acid; for other abbreviation see Table 2.

Endotoxin-induced PAF production in vivo. In preliminary experiments on rats fed a conventional diet, kidneys contained 0.82 ± 0.11 pmol PAF/g tissue (n = 5). The PAF levels 15 min after i.v. injection of endotoxin increased significantly to 14.60 ± 1.70 pmol/g tissue (n = 5), but values 30 min after endotoxin injection decreased markedly, to 2.77 ± 1.01 pmol/g tissue (n = 3) although they were still higher than the basal values. Therefore, we compared the PAF levels in whole kidney homogenates of the two dietary groups at 15 min after endotoxin treatment.

As shown in Figure 1, PAF levels in whole kidney homogenates of endotoxin-treated rats in the high linoleate safflower oil group were significantly higher than in the high α-linolenate perilla oil group. On the other hand, PAF levels in kidney homogenates of vehicle-treated rats were quite low and did not differ between the two dietary groups. In contrast, the lyso-PAF levels in kidney were remarkably higher than the PAF levels even under control conditions, and increased further and significantly 15 min after endotoxin administration. Lyso-PAF levels in the high α-linolenate group were

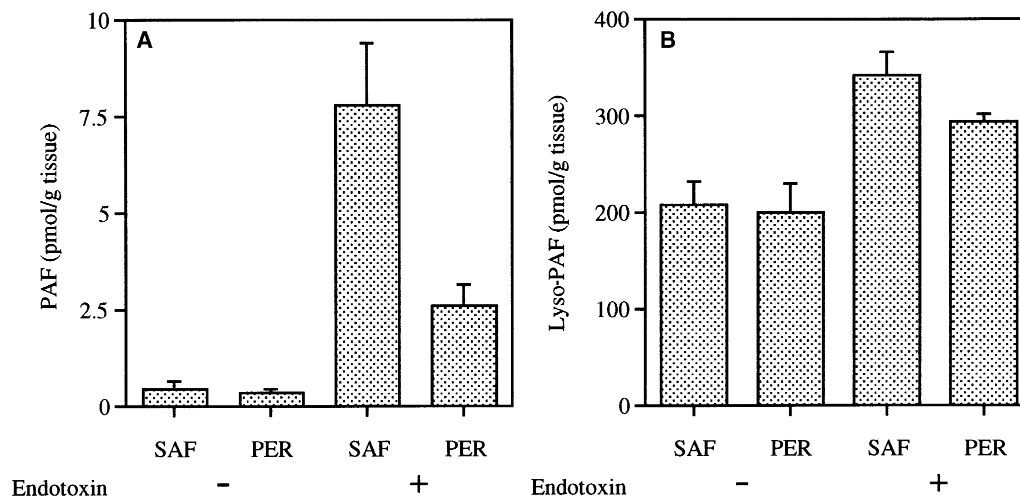

FIG. 1. Effect of dietary fatty acids on platelet-activating factor (PAF) and lyso-PAF contents in rat kidney. Endotoxin (5 mg/kg) or vehicle was injected i.v. into anesthetized rat. Fifteen minutes after injection, kidneys were harvested and stored at −80°C. PAF and lyso-PAF contents were measured as described in the Materials and Methods section. Each value represents the mean ± SEM from 7–8 rats. PAF and lyso-PAF contents in the endotoxin-injected rats were significantly higher than those in the phosphate-buffered saline-injected control rats (P < 0.05). PAF content in rat kidney from the high α-linolenate group (perilla oil, PER) was significantly lower than that from the high-linoleate group (safflower oil, SAF) (P < 0.05). Statistical analysis was performed by two-way analysis of variance.

TABLE 4
Effect of Dietary Fatty Acid on Lyso-PAF Acetyltransferase, PAF Acetylhydrolase and CoA-Independent Transacylase in Rat Kidney[a]

	Control		Endotoxin		ANOVA		
	SAF	PER	SAF	PER	Endotoxin	Diet	Endotoxin × Diet
Lyso-PAF acetyltransferase (nmol/min/mg protein)	0.13 ± 0.00 (5)	0.13 ± 0.00 (5)	0.17 ± 0.01 (6)	0.19 ± 0.02 (6)	$P < 0.01$	N.S.	N.S.
PAF acetylhydrolase (nmol/min/mg protein)	1.76 ± 0.13 (5)	1.68 ± 0.21 (5)	1.82 ± 0.22 (7)	1.53 ± 0.18 (7)	N.S.	N.S.	N.S.
CoA-independent transacylase (pmol/min/mg protein)	20.5 ± 0.7 (5)	15.4 ± 1.1 (5)	24.8 ± 1.0 (5)	18.6 ± 2.0 (5)	$P < 0.02$	$P < 0.01$	N.S.

[a]Endotoxin (5 mg/kg) or phosphate-buffered saline was injected into the tail vein of anesthetized rats. Fifteen minutes after injection, kidneys were harvested and stored at −80°C. Each enzymatic activity was measured as described in the Materials and Methods section. Each value represents the mean ± SEM. Numbers in parentheses represent the number of rats used. Statistical analysis was performed by two-way analysis of variance (ANOVA); N.S., not significant. PAF, platelet-activating factor; for other abbreviations see Table 3.

30% lower (45.3 pmol/g tissue) than those in the high linoleate group following endotoxin administration.

Because Chang *et al.* (9) had reported that blood and lung PAF levels increase within 20 min after intraperitoneal injection of endotoxin, we also measured PAF levels in whole blood and lung homogenates of the two dietary groups. The blood PAF levels in the two dietary groups 15 min after PBS or endotoxin administration were below detection (<0.02 pmol/mL whole blood). The PAF levels in the lung homogenates of both the dietary groups were significantly higher than those of the kidney under resting conditions [2.39 ± 0.29 pmol/g tissue in the high linoleate group (n = 3) and 1.99 ± 0.47 pmol/g tissue in the high α-linolenate group (n = 3)]. PAF levels following endotoxin administration showed no increase over the control values in either dietary group [1.74 ± 0.36 pmol/g tissue in the high linoleate group (n = 5) and 2.26 ± 0.47 pmol/g tissue in the high α-linolenate group (n = 5)].

Lyso-PAF acetyltransferase, PAF acetylhydrolase and CoA-independent transacylase activities in rat kidney. Microsomes of the rats after endotoxin administration showed significantly higher lyso-PAF acetyltransferase activity compared with those of vehicle-treated rats (Table 4). However, the activity of this enzyme was not affected by dietary oils. PAF acetylhydrolase activity in the cytosolic fraction was about 10-fold higher than the lyso-PAF acetyltransferase activity in microsomes. Neither endotoxin treatment nor dietary manipulation affected the PAF acetylhydrolase activity in the cytosolic fraction. The CoA-independent transacylase activity in microsomes of the high α-linolenate group was about 30% lower than that from the high-linoleate group. When the microsomes were prepared from endotoxin-treated rats, the CoA-independent transacylase activity increased slightly but significantly ($P < 0.05$), and the 25% difference was maintained between the two dietary groups.

DISCUSSION

A number of investigations have shown that dietary n-3 fatty acids are effective in reducing the AA content in membrane phospholipids and attenuating PAF production (10–13), but these effects have been evaluated only in isolated cells *in vitro* (14,15). Because the amounts of PAF produced in most tissues are remarkably small and PAF is rapidly degraded by PAF acetylhydrolase *in vivo* (31), it is not clear whether dietary n-3 fatty acids really decrease PAF production in tissues *in vivo*. In the present study, we found that PAF content in rat kidney significantly increases within 15 min after endotoxin administration and that the high α-linolenate diet decreases PAF production in kidney during systemic endotoxemia compared with the dietary high linoleate diet. Among the kidney cells, mesangial cells in glomeruli synthesize PAF in response to several stimuli including endotoxin (32). Pirotzky *et al.* (33) reported that glomerular and medullary cells in the isolated kidney are capable of forming PAF. Recently, we found that dietary high α-linolenate oil suppresses PAF production in rat resident macrophages in peritoneum and lung (Takahashi, T., Oh-hashi, K., Tanabe, A., Watanabe, S., and Okuyama, H., unpublished data) as well as in PMN (15,16). Therefore, mesangial cells may be responsible for the reduced production of PAF in the high α-linolenate group compared with that in the high-linoleate group.

Moreover, we observed that the high α-linolenate diet decreases alkylacyl- and alkenylacyl-GPC contents in rat kidney compared with the high linoleate diet, which is consistent with the previous report of Park *et al.* (18). Yeo *et al.* (34) showed that dietary fish oil decreases the rate of alkylacyl-GPC biosynthesis in rat kidney by using [^{3}H] glycerol, but the precise mechanism remains to be determined. The lower proportion of ether-linked PC in rat kidney in our study (<3%) was consistent with the observation of Blank *et al.* (30), but was much lower than that (10–20%) reported by Park *et al.* (18). We considered that differences in strains, oils, and experimental conditions could be the causes for these inconsistent observations.

As compared with the high linoleate group, the AA content in alkylacyl-GPC in kidney decreased along with concomitant increases in the linoleate (18:1); EPA (20:5n-3), and DHA contents in the high α-linolenate group. The higher content of 18:1 in perilla oil (Table 1) may be the cause of the increased 18:1 content in kidney PC and PE (Table 3). Alternatively, competitive inhibition by α-linolenate and feedback inhibition by EPA and DHA derived from α-linolenate could be the causes for the increased linoleate levels in PC

and PE in the high α-linolenate group (35). Suga *et al*. (36) showed the important role of AA at the *sn*-2 position of alkylacyl-GPC for the PAF biosynthesis *in vitro*. Moreover, cytosolic phospholipase A_2 has been shown to be relatively specific for AA and EPA, but not for DHA (37). Therefore, the decreases in not only AA content but also the sum of AA and EPA content in alkylacyl-GPC in the high α-linolenate group would contribute to the decreased production of PAF.

Some investigators have suggested that the activities of several enzymes responsible for PAF biosynthesis *via* the remodeling pathway were attenuated by dietary n-3 fatty acids. Yeo *et al.* (17) reported that lyso-PAF acetyltransferase activity in kidney microsomes from fish oil-fed rats was lower than that of the high linoleate oil-fed rats. We previously reported that CoA-independent transacylase activity in rat PMN homogenates from the high α-linolenate group was lower than that from the high linoleate group (16). Blank *et al*. (30) also showed that fish oil supplementation decreased CoA-independent transacylase activity in rat spleen microsomes. In our present study, lyso-PAF acetyltransferase and CoA-independent transacylase activities in kidney microsomes, but not PAF acetylhydrolase activity in the cytosolic fraction, increased 15 min after endotoxin injection, implying that the enhanced PAF synthesis in rat kidney during endotoxemia was catalyzed mainly *via* the remodeling pathway. We also found no significant changes in PAF acetyltransferase and acetylhydrolase activities in rat kidney by dietary α-linolenate. Narahara *et al.* (38) reported that endotoxin inhibited the release of PAF acetylhydrolase by macrophages, but PAF acetylhydrolase activity in rat kidney was not affected by endotoxin injection. In the present experiments, quickly frozen samples of rat kidney were used after homogenization to measure PAF acetylhydrolase. Therefore, we cannot exclude the possibility that the endotoxin treatment has some influence on the release of certain kidney cell types as Narahara *et al.* (38) observed in cultured macrophages.

The lower CoA-independent transacylase activities in kidney microsomes in the high α-linolenate group, compared to those in the high linoleate group, would contribute to lower PAF levels in rat kidney. In the present study, we measured the transacylase activity in microsomes following the acylation of the exogenously added acyl acceptor, 1-alkyl-2-lyso-GPC, with endogenous acyl donors in the absence of CoA according to the method of Blank *et al.* (30). Sugiura *et al.* (39) reported that CoA-independent transacylase was relatively specific for C20 and C22 PUFA without discrimination between n-6 and n-3 PUFA. The total C20 and C22 PUFA contents in PC and PE in the high α-linolenate group were significantly lower than in the high linoleate group, which would contribute to the lower CoA-independent transacylase activity in the high α-linolenate group.

In the present study, the alterations of fatty acid composition and subclass composition of PC and CoA-independent transacylase activity in rat kidney by dietary high α-linolenate oil were found to lead to decreased PAF production in rat kidney during systemic endotoxemia, although the observed changes in biochemical parameters may not account for the whole mechanism by which dietary oils affect PAF production in kidney. Because PAF is reported to be associated with renal failure in endotoxemia (40,41), further studies will be required to determine whether high α-linolenate oil would be beneficial for such pathological states.

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Effects of *Pinus pinaster* and *Pinus koraiensis* Seed Oil Supplementation on Lipoprotein Metabolism in the Rat

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ABSTRACT: The aim of the present study was to assess the effect of vegetal oils obtained from *Pinus pinaster* and *P. koraiensis* seeds on plasma lipoprotein levels and apolipoprotein (apo) gene expression in rats. These oils contain two particular fatty acids of the Δ5-unsaturated polymethylene-interrupted fatty acid (Δ5-UPIFA) family: all-*cis*-5,9,12-18:3 (pinolenic) and/or all-*cis*-5,11,14-20:3 (sciadonic) acids. Rats were fed for 28 d a diet containing 5% (w/w) oil supplement. Two control diets were prepared to match the fatty acid composition of *P. pinaster* or *P. koraiensis* oils with the exception of Δ5-UPIFA, which were replaced by oleic acid. *Pinus pinaster* seed oil decreased serum triglycerides by 30% ($P < 0.02$), very low density lipoprotein (VLDL)-triglycerides by 40% ($P < 0.01$), and VLDL-cholesterol by 33% ($P < 0.03$). *Pinus koraiensis* seed oil decreased serum triglycerides by 16% [not statistically significant (ns)] and VLDL-triglycerides by 21% (ns). Gel permeation chromatography and nondenaturating polyacrylamide gel electrophoresis showed a tendency of high density lipoprotein to shift toward larger particles in pine seed oil-supplemented rats. Finally, *P. pinaster* seed oil treatment was associated with a small decrease of liver apoC-III ($P < 0.02$) but not in apoE, apoA-I, or apoA-II mRNA levels. The levels of circulating apo were not affected by pine seed oil supplementation. In conclusion, *P. pinaster* seed oil has a triglyceride-lowering effect in rats, an effect that is due to a reduction in circulating VLDL.

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Fatty acids have unequal lipid-lowering properties (1). Certain classes of plants, marine invertebrates, and insects contain unusual unsaturated polymethylene-interrupted fatty acids (UPIFA). Particularly, UPIFA with a *cis*-5 ethylenic bond, named Δ5-UPIFA, are characteristic and systematic components of Gymnosperm seed oils (2–4). In conifer seeds, some of the following acids may be present, depending on the botanical family considered: all-*cis*-5,9,12-18:3, all-*cis*-5,9,12,15-18:4, all-*cis*-5,11-20:2, all-*cis*-5,11,14-20:3, and all-*cis* 5,11,14,17-20:4. The lipid-lowering potential of oils containing these fatty acids is currently under investigation. *Biota orientalis* (arborvitae, a Chinese Cupressaceae) is used in traditional Chinese medicine. *Biota orientalis* seed oil decreased the concentration of serum cholesterol, high density lipoprotein (HDL)-cholesterol, and phospholipids as compared to a linoleic acid-enriched diet in hypercholesterolemic rats (5). *Pinus koraiensis* (China pine) seeds, another source of Δ5-UPIFA, are consumed in Asia as condiment for various dishes. Supplementation of cholesterol-fed rats with this oil is associated with the lowering of serum triglycerides as compared to linseed and safflower oils (6). It appears from these studies that conifer oils have different lipid-lowering potentials, which may be related in part to differences in their fatty acid composition. Therefore, a detailed analysis of the properties of conifer oils and a comparison of their effects to commercially available oils are necessary before recommending their use in the population at large. *Pinus pinaster* (maritime pine) seeds are harvested in France on a multiton scale for reforestation. The oil extracted from the seeds of this pine contains an average, 16% Δ5-UPIFA that differ in fatty acid composition from previously described pine seed oils (3).

Presently the lipid-lowering potential of *P. pinaster* seed oil is unknown. Moreover, the effect of pine seed oils on circulating lipoprotein has not been explored in detail. Therefore, the aim of our study was to assess the impact of oils extracted from *P. pinaster* and *P. koraiensis* on circulating lipoprotein fractions in rats. Since fatty acids exert part of their effect on lipoprotein levels by modulating the expression of a number of apolipoprotein (apo) genes (7), we evaluated whether any lipid-lowering effect was associated with an effect on apo gene expression and circulating levels.

EXPERIMENTAL PROCEDURES

Animals and diets. All studies were performed with 50-d-old, 200 g, male Wistar rats purchased from IFFA CREDO (L'Arbresle, France). The animals were acclimatized for 1 wk under conditions of controlled temperature (20 ± 1°C) and lighting (dark from 8 P.M. to 8 A.M.) in a room of low background noise. Rats were allowed free access to water and standard rodent chow (Rat and Mouse diet 113; UAR, Villemoisson-sur-Orge, France) during this period. Rats were then separated into

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Abbreviations: apo, apolipoprotein; FAME, fatty acid methyl ester; HDL, high density lipoprotein; ns, not statistically significant; PBS, phosphate buffered saline; UPIFA, unsaturated polymethylene-interrupted fatty acid; VLDL, very low density lipoprotein.

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four groups of six rats fed *ad libitum* different diets prepared from a fat-free semipurified diet (UAR) supplemented with: (i) 5% (w/w) *P. pinaster* seed oil; (ii) 5% (w/w) of a mixture (Control-P) prepared with safflower, oleic acid-enriched sunflower, and linseed oils: 66.5, 31, and 2.5%, respectively; (iii) 5% (w/w) *P. koraiensis* seed oil; and (iv) 5% (w/w) of a mixture (Control-K) prepared with safflower, oleic acid-enriched sunflower, and linseed oils: 55, 44.7, and 0.3%, respectively. *Pinus pinaster* and *P. koraiensis* differed with respect to their fatty acid composition. *Pinus pinaster* contains less 18:1 and more 18:2 and 18:3 than *P. koraiensis*. In order to assess the potential lipid-lowering effect of Δ5 fatty acid, two control diets were prepared to match the fatty acid composition of *P. pinaster* or *P. koraiensis* and to replace the Δ5 fatty acids by oleic acid. Oleic acid was chosen because it is a monounsaturated fatty acid with properties intermediate between those of saturated and polyunsaturated fatty acids. In addition, monounsaturated fatty acids are widely recommended in the diet of dyslipidemic patients (8). The oils were provided by Society BERTIN (Lagny le Sec, France) and by R.L. Wolff for *P. pinaster*. The fatty acid compositions of these diets, as determined by gas–liquid chromatography of the methyl esters, are presented in Table 1. *Pinus pinaster* and *P. koraiensis* seed oils used in these studies contained 15.7 and 17.7%, respectively, of Δ5-UPIFA. Other dietary components were carbohydrate (63%), casein (22.5%), cellulose (6%), salt mixture (7%), and vitamin mixture (1%). Weight gain was monitored throughout the study. After 4 wk, the rats were fasted and then exsanguinated under ether anesthesia. Liver tissue and epididymal adipose tissues were removed immediately and frozen in liquid nitrogen for biochemical analysis.

Extraction of liver lipids, phospholipid isolation, fatty acid methyl ester (FAME) preparation, and analysis. Liver lipids were extracted according to Folch *et al.* (9). Phospholipids were separated by thin-layer chromatography on silica gel plates. FAME from phospholipids were prepared essentially according to Morrison and Smith (10). FAME were analyzed by gas–liquid chromatography using Varian 1400 and 940 chromatographs (Palo Alto, CA) equipped with flame-ionization detectors. Peaks were identified by comparison of their relative retention times to those of commercially available standards. Δ5-UPIFA were identified by their equivalent chain lengths according to Wolff *et al.* (11). The use of equivalent chain lengths was supported by gas–liquid chromatography–mass spectrometry of appropriate derivatives (12).

TABLE 1
Fatty Acid Composition of the Diet Fat (wt%)[a]

Fatty acids[b]	*Pinus pinaster*	Control-P	*P. koraiensis*	Control-K
16:0	3.6	5.9	4.2	5.4
16:1[c]	0.2	0.1	0.1	0.1
17:0	0.1	Trace	Trace	Trace
18:0	2.4	2.6	1.8	2.8
18:1[c]	18.1	32.7	25.5	42.3
9,12-18:2	55.9	56.2	48.4	48.1
11-20:1	1.0	0.2	1.0	0.2
9,12,15-18:3	1.3	1.5	0.2	0.2
11,14-20:2	0.8	n.d.	0.5	n.d.
Δ5 olefinic acids				
5,9-18:2	0.7	n.d.	1.8	n.d.
5,9,12-18:3	7.1	n.d.	14.9	n.d.
5,11-20:2	0.8	n.d.	0.1	n.d.
5,11,14-20:3	7.1	n.d.	0.9	n.d.
Others	0.9	0.8	0.6	0.9

[a]Fatty acid composition of the diet fat was determined by gas–liquid chromatography of the methyl esters. Results are expressed in percentage of total fat.
[b]All ethylenic bonds in the *cis* configuration.
[c]Sum of two isomers. n.d., Not detected.

Lipoprotein separation and measurements. Blood was collected from the carotid artery in dry tubes. Serum was separated by centrifugation (630 × *g*) for 20 min at 4°C. Very low density lipoproteins (VLDL) were separated by ultracentrifugation by using a Beckman TL100 ultracentrifuge (Beckman Instruments France SA, Gagny, France), from 0.5 mL of serum by a single spin at density 1.006 × *g* mL^{-1}. Briefly, 0.5 mL of a 0.9% NaCl solution was added to 0.5 mL of serum and spun in a polycarbonate tube (400,000 × *g*, 10°C) with a Beckman TLA-100.2 rotor (Beckman Instruments France SA) for 2.5 h (13). The tube was sliced, and the remaining 0.5 mL infranate fraction ($d > 1.006$ g mL^{-1}) was analyzed for lipids. Lipids in the VLDL fraction ($d < 1.006$ g mL^{-1}) were determined by subtracting infranate values from total serum values according to the Lipid Research Clinic protocol (14).

Serum $1.019 < d < 1.21$ g mL^{-1} fraction was also separated by sequential ultracentrifugation using a Beckman TLA-100.3 rotor (Beckman Instruments France SA) at 480,000 × *g* and 10°C. Two mL of serum was adjusted at $d = 1.019$ g mL^{-1} with potassium bromide and spun for 4 h. The supernate fraction was discarded, and the infranate fraction was spun at the same density for 3.5 h. Then the infranate fraction was adjusted at $d = 1.21$ g mL^{-1} and spun twice for 4 h. The resulting serum $1.019 < d < 1.21$ g mL^{-1} density fraction was dialyzed against a 10 mM phosphate buffer containing 0.01% EDTA and 0.01% sodium azide.

Lipids were determined enzymatically using commercially available kits for triglycerides (Triglycerides GPO-PAP; Boehringer Mannheim, Mannheim, Germany), cholesterol (Cholesterol C System; Boehringer Mannheim), and phospholipids (Phospholipids PAP 150; BioMérieux, Lyon, France).

Measurements of apo. Rat lipoproteins apoA-I, apoA-II, and apoE were measured by a noncompetitive enzyme-linked immunosorbent assay (sandwich ELISA). Briefly, polystyrene microtiter plates were coated with affinity-purified polyclonal antibodies to rat apoA-I, apoA-II, or apoE (1 mg/mL). Duplicate serum samples were diluted with 100 mM phosphate buffered saline (PBS) containing 1% albumin. The samples were added to the wells along with the standards and controls and incubated for 2 h at 37°C. After incubation, the plates were washed four times with PBS 100 mmol/L, and the corresponding polyclonal antibody conjugated to peroxidase was added. The plates were incubated for 2 h at 37°C and then washed. Color development was performed in 30 min by the addition of peroxidase substrate (*o*-phenylenediamine dichloride; Sigma Chemical Co., St. Louis, MO). The plates were read at 492 nm

on an automated microplate reader model EL340 (Bio-Tek Instruments, Inc., Winooski, VT). ApoC-III was measured by nephelometry using a Behring automated analyzer (Behring Nephelometer Analyser; Behring Diagnostics, Rueil Malmaison, France) and rabbit polyclonal anti apoC-III antibody.

Gel filtration chromatography. The ultracentrifugally isolated serum fraction of density $1.019 < d < 1.21$ g mL^{-1} was further separated using a Superdex 200HR 10/30 column to assess lipoprotein heterogeneity. The gel was allowed to equilibrate with PBS (10 mM) containing 0.01% EDTA and 0.01% sodium azide; 200 µL of serum was eluted with the buffer at room temperature at a flow rate of 0.2 mL min^{-1}. Elution profiles were monitored at 280 nm and recorded with an analog-recorder chart tracing system (Pharmacia LKB Biotechnology, Uppsala, Sweden). The effluents were collected in 0.24-mL fractions. Calibration was carried out with bovine albumin Fraction V (Sigma-Aldrich Chimie SARL, Saint Quentin Fallavier, France). Cholesterol was measured in each collected fraction using commercially available enzymatic kits (Cholesterol C System; Boehringer Mannheim).

Nondenaturing polyacrylamide gel and agarose gel electrophoresis. Lipoprotein sizes were identified by nondenaturing gel electrophoresis using a ready-to-use discontinuous 2–3% polyacrylamide gel LIPOFILM (Sebia, Issy-les-Moulineaux, France). Sera (10 µL) were prestained with Sudan black for 30 min at room temperature. Prestained serum aliquots (5 µL) were applied to the gel, and electrophoresis was run for 45 min at 170 V, 12 mA. The gels were scanned using an appropriate scanner (Intégrateur Lipofilm, Préférence SEBIA, HIT-HR Software 2XX, Issy-les Moulineaux, France). In order to assess whether seed oil supplementation affects lipoprotein electrophoretic mobility, agarose gel electrophoresis was performed according to Noble (15) with a Beckman Paragon system (Beckman Instruments France SA). Briefly, serum (5 mL) was applied on a 0.5% agarose gel (Paragon LIPO lipoprotein electrophoresis; Beckman Instruments France SA). Electrophoresis was performed for 30 min in a barbital buffer (pH 8.6) at 100 V. Gels were stained with Sudan black B.

RNA analysis. Total cellular RNA was isolated from liver tissue by the acid guanidinium thiocyanate/phenol–chloroform method (16). Northern and dot-blot hybridizations were performed exactly as described previously (17). The rat apoA-I, apoA-II, apoC-III, and apoE cDNA probes were described previously (18,19). All probes were labeled by random primed labeling (Boehringer Mannheim). Filters were hybridized to 1×10^6 cpm mL^{-1} of each probe as described (17). They were washed in 300 mL of 75 mM NaCl, 7.5 mM sodium citrate, pH 7.4, and 0.1% sodium dodecyl sulfate for 10 min at room temperature and twice for 30 min at 65°C and subsequently exposed to X-ray film (Kodak X-OMAT-AR; Eastman Kodak Co., Rochester, NY). Autoradiograms were analyzed by quantitative scanning densitometry (Bio-Rad GS670 Densitometer; Bio-Rad Laboratories, Richmond, CA) as described elsewhere (17).

Statistical analysis. Unpaired *t*-tests were used to compare the experimental to the control diets. Whenever a covariable (weight) explained part of the variability of the biological variable, the adjusted error term was calculated for the *t*-test using the LS-Means procedure of SAS software (SAS Institute Inc., Cary, NC).

RESULTS

The body weight, food intake, liver, and epididymal fat tissue weights were not significantly different between rats treated with *P. pinaster* or *P. koraiensis* seed oils and their respective controls (data not shown). *Pinus pinaster* and *P. koraiensis* seed oils ingestion was associated with the appearance of all-*cis*-5,11,14-20:3 and all-*cis*-5,9,12-18:3 fatty acids in liver phospholipids (Table 2). These changes were associated with a concomitant decrease of all-*cis*-9,12-18:2 and all *cis*-5,8,11,14-20:4 in *P. pinaster* seed oil-supplemented rats and with a decrease of all *cis*- 9,12-18:2 in the *P. koraiensis*-fed rats.

The effects of pine seed oil supplementation on serum lipid and lipoprotein levels were assessed after 4 wk of supplementation. The results are presented in Table 3. The levels of serum triglycerides were decreased significantly (by 30%, $P < 0.02$) in the group supplemented with *P. pinaster* seed oil compared to its respective control group. This reduction was accounted for by a decrease in the VLDL fraction, resulting in significantly lower levels of VLDL-triglycerides (–40%,

TABLE 2
Fatty Acid Composition (%) of Liver Phospholipids

Fatty acid	*P. pinaster*	Control-P	P^a	*P. koraiensis*	Control-K	P^a
16:0	17.6 ± 1.5	16.5 ± 1.2	ns	15.8 ± 2.4	16.8 ± 0.8	ns
9-16:1	0.8 ± 0.2	0.6 ± 0.1	ns	0.6 ± 0.2	0.7 ± 0.1	ns
18:0	21.7 ± 0.7	22.0 ± 0.6	ns	23.1 ± 2.5	22.2 ± 0.9	ns
9-18:1	2.9 ± 0.5	2.8 ± 0.1	ns	2.6 ± 0.4	3.3 ± 0.2	0.01
11-18:1	2.7 ± 00.3	2.8 ± 0.2	ns	2.7 ± 0.4	2.8 ± 0.3	ns
9,12-18:2	9.3 ± 1.0	10.5 ± 0.7	0.04	8.5 ± 0.5	9.5 ± 0.8	0.023
5,8,11,14-20:4	29.4 ± 1.1	31.6 ± 1.0	0.004	30.6 ± 1.1	31.8 ± 0.6	ns
Δ5-UPIFA						
5,9,12-18:3	0.6 ± 0.1	n.d.	0.0001	1.2 ± 0.1	n.d.	0.0001
5m11m14-20:3	1.3 ± 0.4	n.d.	0.0001	0.2 ± 0.1	n.d.	0.0008

[a]Unpaired *t*-test: *P* values for pine seed oil vs. control group. ns, not statistically significant; UPIFA, unsaturated polymethylene-interrupted fatty acid. See Table 1 for other abbreviation.

TABLE 3
Serum Lipid Concentrations (mean ± SEM) in Rats Treated with *P. pinaster* and *P. koraiensis* Seed Oils and Their Respective Controls

	P. pinaster (mmol/L)	Control-P (mmol/L)	P^a	*P. koraiensis* (mmol/L)	Control-K (mmol/L)	P^a
Cholesterol	1.86 ± 0.15	2.14 ± 0.14	ns	2.09 ± 0.16	2.06 ± 0.13	ns
Triglycerides[b]	1.24 ± 0.21	1.78 ± 0.26	0.02	1.47 ± 0.21	1.76 ± 0.21	ns
Phospholipids[b]	1.73 ± 0.10	1.87 ± 0.06	ns	1.66 ± 0.08	1.72 ± 0.06	ns
VLDL-cholesterol[b,c]	0.31 ± 0.04	0.46 ± 0.04	0.03	0.46 ± 0.08	0.49 ± 0.01	ns
VLDL-triglycerides[b,c]	0.91 ± 0.20	1.53 ± 0.26	0.01	1.21 ± 0.21	1.53 ± 0.22	ns
Cholesterol in $d > 1.006$ g mL^{-1} fraction	1.55 ± 0.14	1.68 ± 0.12	ns	1.63 ± 0.01	1.57 ± 0.12	ns

[a]Unpaired *t*-test: *P* values for pine seed oil vs. control group.
[b]Values adjusted for weight.
[c]Very low density lipoprotein (VLDL) serum $d < 1.006$ g mL^{-1} density fraction. See Tables 1 and 2 for other abbreviations.

$P < 0.01$) and VLDL-cholesterol (–33%, $P < 0.03$). Total cholesterol, cholesterol in the serum $d > 1.006$ g mL^{-1} serum density fraction, and serum phospholipid levels were not statistically significantly (ns) different between the two groups. Although there was a tendency to lower levels of serum total triglycerides (–16%, ns) and VLDL-triglycerides (–21%, ns) in rats treated with *P. koraiensis* seed oil as compared to its control group, these differences did not reach the level of statistical significance. In order to further assess the potential impact of pine seed oil supplementation on lipoprotein metabolism, the compositions of VLDL and serum $1.019 < d < 1.21$ g mL^{-1} fraction were determined (data not shown). There were no statistically significant differences in lipid composition of the various lipoproteins between treated and control rats with the exception of a moderate increase in VLDL-phospholipid ($P < 0.004$) in *P. koraiensis*-treated rats. Agarose gel electrophoretic patterns of serum lipoproteins were not different among groups (data not shown).

In order to investigate the changes in serum lipid distribution in more detail, lipoproteins were separated by gel permeation chromatography. Cholesterol was determined in the gel filtration elution fractions of the serum $1.019 < d < 1.21$ g mL^{-1} density fraction (Fig. 1). *Pinus pinaster* and *P. koraiensis* supplementation resulted in a slight shift of the HDL peak toward larger particles (Fig. 1A and B). This observation was confirmed by nondenaturing gel electrophoresis that showed a tendency, although ns, to smaller HDL relative mobility (R_f) values in the *P. pinaster* (29.8 ± 2.4 vs. 31.8 ± 2.3, ns) and *P. koraiensis* (30 ± 4 vs. 31.8 ± 4.1, ns) seed oil-supplemented rats than in controls.

Finally, we determined whether pine seed oil treatment has an effect on apo gene expression and apo levels. To this end, the liver mRNA levels of various apo were measured. The results are presented in Table 4. *Pinus pinaster* seed oil-supplemented rats had significantly lower levels of liver apoC-III mRNA than control ($P < 0.04$). This change, however, was not associated with a decrease in the concentration of serum apoC-III (*P. pinaster* 0.038 ± 0.015 vs. control 0.021 ± 0.006 g/L; ns). ApoA-I, apoA-II, and apoE liver mRNA and serum levels (A-I: 0.47 ± 0.08 vs. 0.47 ± 0.08, ns; A-II: 0.37 ± 0.10 vs. 0.45 ± 0.04, ns; E: 0.004 ± 0.003 vs. 0.002 ± 0.001, ns g/L) were not different between animals treated with *P. pinaster* seed oil and control regimen-treated rats. *Pinus koraiensis*

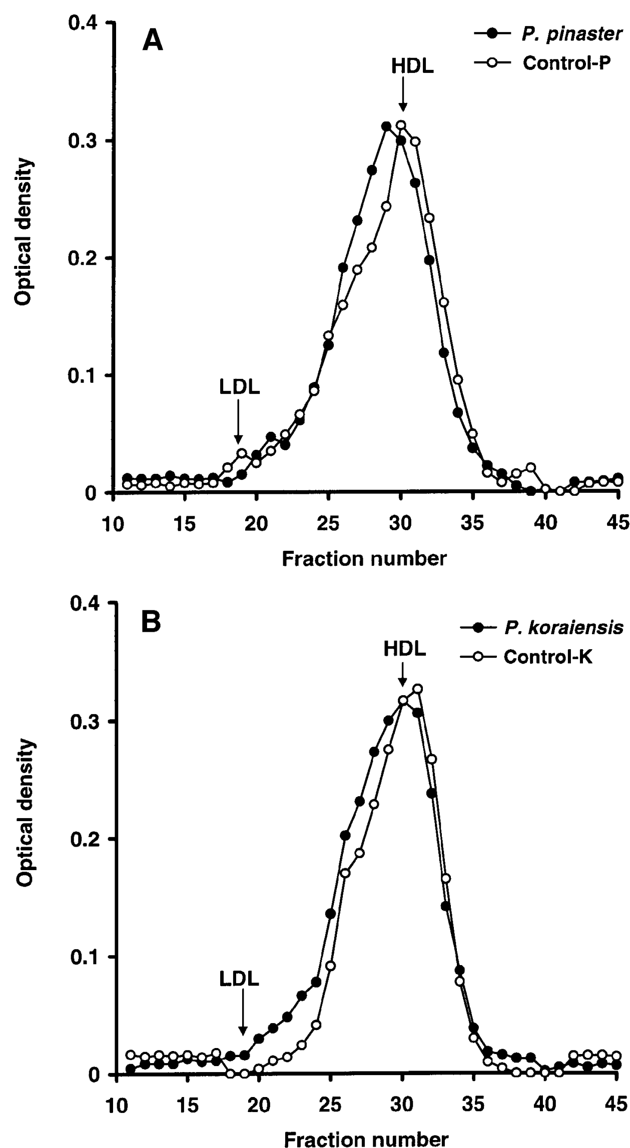


FIG. 1. Cholesterol profile of serum density fraction $1.019 < d < 1.21$ g mL^{-1} of *Pinus pinaster* (A) and *P. koraiensis* (B) supplemented rats and their respective controls. Values are medians of four profiles. The $1.019 < d < 1.21$ g mL^{-1} serum fraction was fractionated on a Superdex 200HR 10/30 column (Pharmacia LKB Biotechnology, Uppsala, Sweden). Pine seed oil and control treatments are presented with closed and open circles, respectively. HDL, high density lipoprotein; LDL, low density lipoprotein.

TABLE 4
Liver Apolipoprotein mRNA Levels (mean ± SEM) in Treated and Control Rats[a]

	P. pinaster	Control-P	P^b	*P. koraiensis*	Control-K	P^b
ApoA-I	100 ± 8	100 ± 15	ns	88 ± 9	100 ± 3	ns
ApoA-II	92 ± 3	100 ± 5	ns	105 ± 6	100 ± 5	ns
ApoC-III	85 ± 4	100 ± 5	0.04	103 ± 5	100 ± 5	ns
ApoE	100 ± 2	100 ± 6	ns	100 ± 4	100 ± 6	ns

[a]Units are percentage of controls.
[b]Unpaired *t*-test: *P* values for pine seed vs. control group; apo, apolipoprotein. See Tables 1 and 2 for other abbreviations.

seed oil supplementation had ns effect on any apo liver mRNA (apo A-I, A-II, C-III, and E) and apo levels (A-I: 0.51 ± 0.07 vs. 0.44 ± 0.07, ns; A-II: 0.37 ± 0.06 vs. 0.39 ± 0.07, ns; C-III: 0.024 ± 0.004 vs. 0.024 ± 0.005, ns; E: 0.002 ± 0.001 vs. 0.003 ± 0.003, ns g/L) compared to control diet.

DISCUSSION

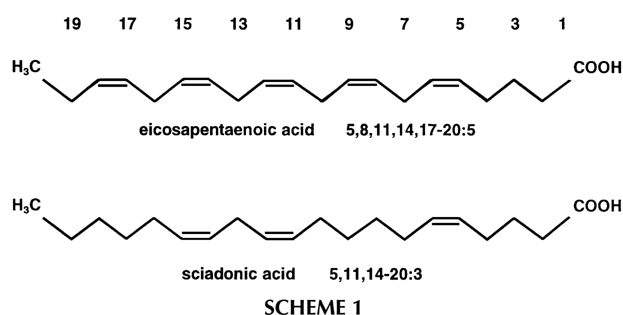
The major finding of the present study is the lowering of triglycerides, VLDL-triglycerides, and VLDL-cholesterol levels in rats treated with *P. pinaster* seed oil compared to rats treated with control regimen, in which oleic acid replaced Δ5-UPIFA. In contrast, *P. koraiensis* seed oil treatment had ns effect on lipid or on lipoprotein and apo levels compared to oleic acid-enriched diet.

Recent studies analyzed the effects of two conifer seed oils on circulating total cholesterol and triglyceride levels in rats. Ikeda *et al*. (5) found lower levels of serum cholesterol and triglycerides in hypercholesterolemic rats treated for 24 d with a diet supplemented with *B. orientalis* oil compared with a diet supplemented with linoleic acid. Similarly, Sugano *et al*. (6) observed lower levels of serum triglycerides in rats supplemented with *P. koraiensis* seed oil compared with linseed and safflower oils. In the latter studies, however, the results were confounded by the addition of 0.5% cholesterol and 0.125% sodium cholate to the diet. In the present study, all-*cis* 5,11,14-20:3 and/or all-*cis* 5,9,12-18:3 fatty acids were replaced by 18:1 in the control regimens, and no cholesterol or cholate was added to the diet, suggesting that Δ5-UPIFA has a triglyceride- and VLDL-lowering effect as compared to oleic acid. This effect is more pronounced for *P. pinaster* than for *P. koraiensis* seed oil, suggesting that sciadonic acid (all-*cis*-5,11,14-20:3), which is more abundant in *P. pinaster* seed oil, has a greater VLDL-lowering potential than pinolenic acid (all-*cis*-5,9,12-18:3). However, notably oils, not fatty acids, were used in the present study. This fact has potential implications in the interpretation of the data since factors other than fatty acids may be present in the oil and could interfere with lipoprotein metabolism.

Eicosapentaenoic acid (5,8,11,14,17-20:5) is present in marine oils and, similar to sciadonic acid (5,11,14-20:3) of *P. pinaster* seed oil, has 20 carbon atoms and *cis* double bonds in positions Δ5, Δ11, and Δ14 (Scheme 1). Eicosapentaenoic acid has a major impact on triglyceride levels (20). It is difficult to compare the results of the present study with those of previous publications on marine oils (21–24) because of differences among studies such as the amount of fat or cholesterol consumed, the length of treatment, and the type of control diet. Although the effects of *P. pinaster* seed oil on lipid and lipoprotein levels appear to resemble those of fish oils, no evidence exists that proves that *P. pinaster* oil, like fish oils, could be used safely and efficiently in man.

Clearly, evidence exists that apo have a crucial role in lipid metabolism. ApoA-I and apoA-II are key proteins of HDL metabolism (25). ApoE is necessary for lipoprotein remnant clearance (26). ApoC-III is a major component of triglyceride-rich lipoparticles and interferes with VLDL lipolysis (27–30) and uptake by cellular receptor (31–34). Evidence also exists that fatty acids exert part of their lipid-lowering effects by altering the expression of various apo genes (35). As a first indication of a possible effect of pine seed oil on apo gene expression, the mRNA levels of apoA-I, apoA-II, apoC-III, and apoE were measured in the liver of supplemented rats. Although a decreased expression of apoC-III gene in the *P. pinaster*-supplemented rats existed, this effect was modest and not associated with a decrease in serum apoC-III levels, suggesting a marginal impact of *P. pinaster* on apoC-III metabolism. Therefore, mechanisms other than a direct effect of *P. pinaster* on apo metabolism may have contributed to the triglyceride-lowering properties of *P. pinaster* seed oil, such as (i) decreased *de novo* lipid synthesis, (ii) reduced substrate availability for lipoprotein formation, or (iii) changes in VLDL physicochemical properties. Further studies are necessary to assess the mechanism of triglyceride reduction by *P. pinaster* in rats.

In the present study, *P. pinaster* seed oil supplementation reduced triglycerides and VLDL levels in rats, suggesting a potential benefit for *P. pinaster* in lowering high blood triglyceride levels. However, additional investigations in other animal models as well as controlled studies in humans are necessary before recommending the use of *P. pinaster* oil as an alternative source of oil in patients with dyslipidemia.



SCHEME 1

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Differences in the Sterol Composition of Dominant Antarctic Zooplankton

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ABSTRACT: The composition of free sterols was determined in Antarctic zooplankton species with various feeding behaviors. In the Southern Ocean, the dominant calanoid copepods *Calanoides acutus, Calanus propinquus, Metridia gerlachei*, and *Euchaeta antarctica* were investigated during different seasons and compared with the euphausiids *Euphausia superba, E. crystallorophias*, and *Thysanoessa macrura*. In addition, the Arctic copepods *Calanus hyperboreus, C. glacialis*, and *C. finmarchicus* were studied for comparison. Analyses were performed using gas chromatography and mass spectrometry. The zooplankton species exhibited a simple sterol content of up to six sterols. In the copepods, cholest-5-en-3β-ol (22.1 to 60.5%, range of sample means), cholesta-5,24-dien-3β-ol (22.3 to 45.2%), and cholesta-5,22*E*-dien-3β-ol (4.3 to 33.4%) contributed most, while in euphausiids the sterol composition was less complex with cholest-5-en-3β-ol always accounting for more than 75% of the total. Although sterols are membrane constituents and are expected not to vary considerably, differences in the abundance of sterols were observed between the species and the seasons. In herbivorous copepods, cholesta-5,24-dien-3β-ol increased by a factor of 1.5 to about 45% during the main feeding period in summer; this sterol is a metabolic precursor of cholest-5-en-3β-ol in the process of the dealkylation of dietary C-24 alkylated phytosterols. Cholest-5-en-3β-ol decreased by the same proportion. Omnivorous and carnivorous copepods showed average levels of cholesta-5,24-dien-3β-ol below 25%. These changes in sterol composition between copepod species seem to reflect their different feeding modes.

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In high Antarctic waters, primary production is extremely seasonal. Zooplankters generally accumulate large lipid deposits as energy reserves to survive the periods of low productivity and to reproduce (1,2). Apart from lipids stored as reserves, lipids such as phospholipids and sterols are major components of the membranes. Sterols are especially abundant in the plasma membranes of eukaryotic organisms. It is generally believed that they modulate membrane fluidity by interaction with the phospholipid components (3). These structural elements maintain the functionality of membranes even in extreme environments such as the cold polar oceans.

Sterols are present in the form of cholest-5-en-3β-ol in nearly all animals. However, marine invertebrates, whether or not they are sterol auxotrophs, discriminate much less among their dietary sterols than do mammals. Therefore, these marine animals may exhibit compositions reflecting the general availability of these compounds in their food (3). Among marine invertebrates, the crustaceans have the simplest sterol compositions (4–6). It is well established that crustaceans are incapable of *de novo* sterol biosynthesis from acetate or mevalonic acid. Their sterols originate either directly from the diet or by dealkylating dietary C-24 alkyl-substituted phytosterols (6–9). However, we know little about the content and the variation of sterols in zooplankton from the polar oceans (10,11).

The herbivorous copepods *Calanoides acutus, Calanus propinquus*, the omnivorous *Metridia gerlachei*, and the carnivorous *Euchaeta antarctica* contribute substantially to zooplankton biomass in the Southern Ocean (12–14). Among the euphausiids, the herbivorous *Euphausia superba* is the key species with biomass estimates of hundreds of millions of tons (15). *Euphausia crystallorophias* and *Thysanoessa macrura* are also major components of the Antarctic macrozooplankton biomass (12). The dominant herbivorous Arctic copepods *Calanus glacialis, C. hyperboreus*, and *C. finmarchicus* were considered for comparison, since they exhibit life strategies similar to those of the Antarctic *C. acutus*.

In this study, the dominant zooplankton species of the polar oceans were investigated for sterols in order to gain information about species-specific compositions and possible metabolic processes. In the Antarctic copepods, seasonal changes in the sterol compositions were studied to elucidate the influence of the feeding behavior on sterols in organisms coping with the extreme environment of the high latitudes.

MATERIALS AND METHODS

Zooplankton sampling was carried out in the Antarctic Weddell Sea during several expeditions of RV *Polarstern* (Table 1). Samples were collected during austral spring (November/December 1986 and November/December 1997), summer (January/February 1985), and fall (April/May 1992 and

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Abbreviation: GC–MS, gas chromatography–mass spectrometry.

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TABLE 1
Zooplankton Samples from Various *Polarstern* Cruises to the Southern (ANT) and to the Arctic Ocean (ARK)

Cruise	Region	Season	Species
ANT III/3 Jan./Feb. 1985	Southern Weddell Sea	Summer	*Calanoides acutus, Calanus propinquus, Metridia gerlachei, Euchaeta antarctica Thysanoessa macrura, Euphausia crystallorophias, Euphausia superba*
ANT V/3 Nov./Dec. 1986	Central Weddell Sea	Spring	*C. acutus, C. propinquus*
ANT X/3 April/May 1992	Eastern Weddell Sea	Fall	*T. macrura, E. crystallorophias, E. superba*
ANT XIII/4 March/April 1996	Central Weddell Sea	Fall	*C. acutus, E. superba, T. macrura*
ANT XIV/2 Nov./Dec. 1997	South Shetland Region	Spring	*C. acutus, M. gerlachei*
ARK XI/2 Sep./Oct. 1995	Greenland Sea	Fall	*Calanus hyperboreus, Calanus glacialis, Calanus finmarchicus*

March/April 1996). For comparison, some Arctic copepods were obtained from the Greenland Sea (September/October 1995). According to species, stage, and season, the copepods and euphausiids were sampled by vertical bongo net hauls and various plankton and nekton net hauls, respectively. The specimens were sorted in a cooled container-laboratory and stored deep-frozen in glass vials at −80°C. Arctic samples were kept in dichloromethane/methanol (2:1, vol/vol) at −30°C. Samples of euphausiids consisted of single individuals; up to 200 copepods were pooled for each sample. Sterols in the gut content are regarded as negligible. With respect to gut pigments in copepods during spring (16), the ratio of sterols to pigments in algae (17), and the sterol content in copepods (18), the sterols in the gut were estimated to contribute less than 2% to total sterols.

Total lipid was extracted as described by Folch *et al.* (19). Preparative separation of lipid classes was performed by thin-layer chromatography on silicic acid (60H; Merck, Darmstadt, Germany). A mixture of *n*-hexane/diethyl ether/formic acid (65:35:0.04, by vol) was used as eluting solvent. According to their R_f values, the bands of the different lipid classes were identified, scraped off, and eluted with dichloromethane/methanol (2:1, by vol). Steryl esters contributed less than 1% to the total of sterols in copepods (Albers, C., unpublished data).

The solutions containing free sterols were evaporated under a stream of nitrogen and dried over silica gel for 15 h. Sterols were converted into their corresponding trimethylsilyl ethers applying *N,O*-bis-(trimethylsilyl)-trifluoroacetamide (BSTFA; Macherey-Nagel, Düren, Germany) at 60°C for 2 h. After the evaporation of excess BSTFA, the steryl ethers were redissolved in *n*-hexane and analyzed by a Chrompack 9002 gas chromatograph equipped with a fused-silica capillary column (HP5-MS, 30 m × 0.25 mm I.D.) and a flame-ionization detector. Helium was used as carrier gas. A splitless injection mode was employed. The oven temperature was raised from 55 (1.6 min) to 200°C at 30°C/min, then to 325°C at 6°C/min, and held for 15 min. Gas chromatography–mass spectrometry (GC–MS) analyses were performed with an HP 5890II gas chromatograph directly coupled to a VG AutoSpec mass spectrometer. Electron impact mass spectra were acquired at an ionization energy of 70 eV and a mass resolution of 1000. A range from *m/z* 600 to *m/z* 35 was monitored at a rate of 1 scan/s. Sterols were identified by comparison of GC retention indices, mass spectra, and the use of authentic reference substances (20).

Cholesta-5,24-dien-3β-ol and 24-methylcholesta-5,22*E*-dien-3β-ol have only slightly different GC retention times. Cholesta-5,24-dien-3β-ol always dominated in the samples; quantification by GC–MS showed that even in summer the amount of 24-methylcholesta-5,22*E*-dien-3β-ol was at least 10 times smaller than that of cholesta-5,24-dien-3β-ol. For species that were sampled during the same season, but not analyzed by GC–MS, a similar ratio of cholesta-5,24-dien-3β-ol to 24-methylcholesta-5,22*E*-dien-3β-ol was assumed. The stereochemistry of C-24 epimers is not discussed in this work, since they could not be separated.

RESULTS

The zooplankton investigated never contained more than six sterols (Fig. 1). The percentage distribution of these sterols in copepods showed species-specific (Fig. 2) and seasonal variabilities (Table 1). Sterol patterns of euphausiids were simpler than those of the copepods and were strongly dominated by cholest-5-en-3β-ol. No difference occurred between copepodite stages CIV–CV and adult females; thus, these samples were discussed together.

Antarctic Copepoda. In *C. acutus* cholest-5-en-3β-ol was the major sterol observed in spring (49.4% of total free sterols); cholesta-5,24-dien-3β-ol and cholesta-5,22*E*-dien-3β-ol followed next in abundance. In summer, an increased abundance of cholesta-5,24-dien-3β-ol of up to 45.2% was

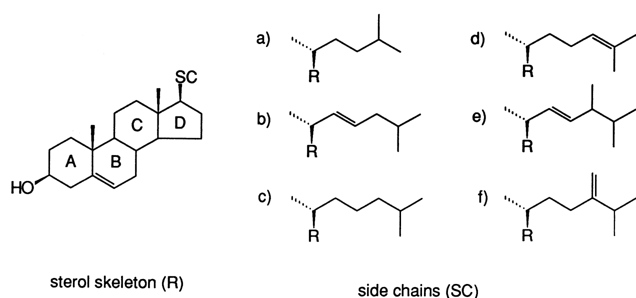

FIG. 1. Structure of sterols found in Antarctic crustaceans (R ring skeleton, SC side chain). (a) 24-Norcholesta-5,22*E*-dien-3β-ol, (b) cholesta-5,22*E*-dien-3β-ol, (c) cholest-5-en-3β-ol, (d) cholesta-5,24-dien-3β-ol, (e) 24-methylcholesta-5,22*E*-dien-3β-ol, and (f) 24-methylcholesta-5,24(28)-dien-3β-ol.

striking, while levels of cholest-5-en-3β-ol exhibited a respective decrease. Cholesta-5,22*E*-dien-3β-ol contributed considerably to the sum of sterols both in spring and in summer. The very low level of 24-methylcholesta-5,24(28)-dien-3β-ol had increased to 2.5% in summer. During fall, 60.5% of cholest-5-en-3β-ol was measured, a proportion higher than during spring and summer. The abundance of cholesta-5,24-dien-3β-ol was comparable to that found during spring, whereas the proportion of cholesta-5,22*E*-dien-3β-ol (6.6%) was least in the fall (Table 2).

In *C. propinquus* cholest-5-en-3β-ol constituted 40.5% of the total in spring; cholesta-5,24-dien-3β-ol and cholesta-5,22*E*-dien-3β-ol yielded about 30 and 20%, respectively. As observed for *C. acutus*, cholesta-5,24-dien-3β-ol was the major sterol in the summer specimens; its level had increased by a factor of about 1.5 in comparison with the spring animals. In summer, nearly equal proportions of cholest-5-en-3β-ol and cholesta-5,22*E*-dien-3β-ol were measured (Table 2).

In *M. gerlachei* collected during spring, cholest-5-en-3β-ol was most abundant, followed by cholesta-5,22*E*-dien-3β-ol and cholesta-5,24-dien-3β-ol. Cholesta-5,24-dien-3β-ol exhibited a stronger variation than cholesta-5,22*E*-dien-3β-ol (relative standard deviation of 40 vs. 20%). Besides, 24-nor-cholesta-5,22*E*-dien-3β-ol contributed to the sum of sterols with a proportion (9.4%) considerably higher than in *C. acutus* and *C. propinquus*. During summer, the main sterol found in *M. gerlachei* was cholesta-5,22*E*-dien-3β-ol (33.4%). This observation was in contrast to the summer specimens of *C. acutus* and *C. propinquus*, which contained cholesta-5,24-dien-3β-ol as main component. Cholest-5-en-3β-ol and cholesta-5,24-dien-3β-ol were detected in similar percentages of about 25%. 24-Methylcholesta-5,24(28)-dien-3β-ol showed an increase in abundance by a factor of about 5.5 (Table 2).

In summer, the predatory *E. antarctica* exhibited about 1.5–2 times more cholest-5-en-3β-ol than the herbivorous and omnivorous species; *E. antarctica* contained cholesta-5,24-dien-3β-ol and cholesta-5,22*E*-dien-3β-ol in similar proportions (Table 2).

Arctic Copepoda. In the fall, *C. hyperboreus* and *C. glacialis* contained similar proportions of cholest-5-en-3β-ol and cholesta-5,24-dien-3β-ol (about 40%). *Calanus finmarchicus* exhibited a higher abundance of cholest-5-en-3β-ol (56.7%), but less cholesta-5,24-dien-3β-ol (24.4%). In contrast

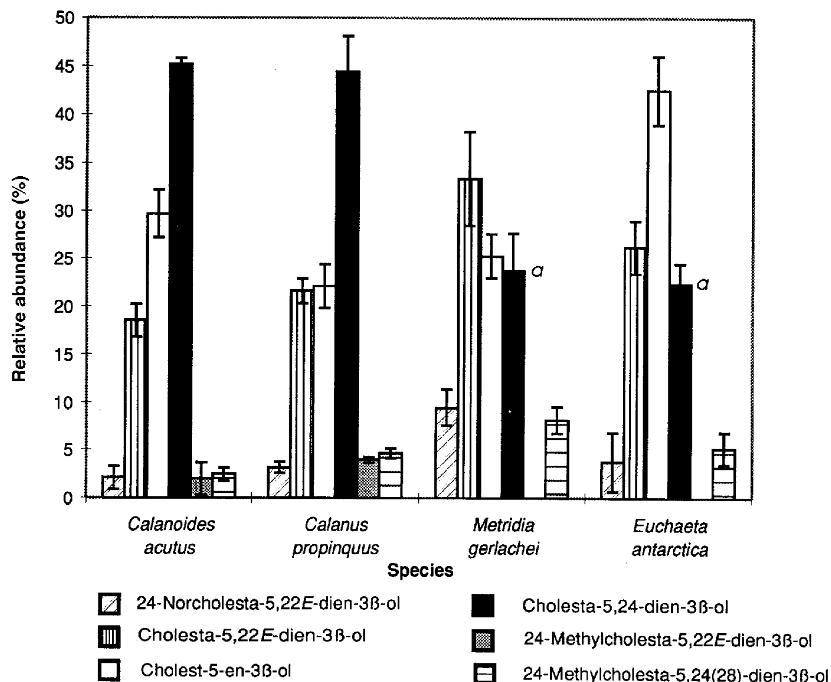

FIG. 2. Mean abundance of free sterols (%) in Antarctic copepods during summer. [a]Coeluting cholesta-5,4-dien-3β-ol and 24-methylcholesta-5,22*E*-dien-3β-ol; error bars indicate standard deviation.

TABLE 2
Sterol Compositions of Antarctic Copepods

Sterol		Percentage composition (of total free sterols)[a]							
		Calanoides acutus			*Calanus propinquus*		*Metridia gerlachei*		*Euchaeta antarctica*
		Spring (9)	Summer (3)	Fall (2)	Spring (3)	Summer (3)	Spring (8)	Summer (5)	Summer (5)
24-Norcholesta-	Range	N.D.–2.2	1.1–3.4	N.D.	3.3–4.0	2.7–3.8	2.9–14.5	7.4–12.1	0.5–7.1
5,22*E*-dien-3β-ol	Mean ± sd	0.8 ± 1.0	2.1 ± 1.2		3.7 ± 0.4	3.2 ± 0.6	9.4 ± 4.5	9.5 ± 1.9	3.8 ± 3.1
Cholesta-	Range	12.8–22.6	17.5–20.5	6.6–6.6	20.3–22.9	20.7–23.2	18.9–36.7	28.4–40.3	22.5–30.0
5,22*E*-dien-3β-ol	Mean ± sd	19.6 ± 3.5	18.5 ± 1.7	6.6	21.1 ± 1.5	21.6 ± 1.3	26.7 ± 5.4	33.4 ± 4.9	26.2 ± 2.8
Cholest-	Range	39.4–59.1	26.9–31.6	59.3–61.6	37.8–43.2	20.0–24.5	21.5–51.2	22.5–28.3	37.1–46.1
5-en-3β-ol	Mean ± sd	49.4 ± 5.7	29.7 ± 2.5	60.5	40.5 ± 2.7	22.1 ± 2.3	39.9 ± 9.5	25.2 ± 2.3	42.5 ± 3.5
Cholesta-	Range	22.4–37.7[b]	44.6–45.7	29.1–31.0	29.6–33.1[b]	40.2–47.3	11.6–38.9[b]	19.3–28.3[b]	18.4–23.3[b]
5,24-dien-3β-ol	Mean ± sd	29.2 ± 5.8	45.2 ± 0.6	30.1	31.6 ± 1.8	44.4 ± 3.7	22.5 ± 9.1	23.7 ± 4.0	22.3 ± 2.2
24-Methylcholesta-	Range		0.5–3.9	2.7–3.1		3.6–4.1			
5,22*E*-dien-3β-ol	Mean ± sd		2.0 ± 1.7	2.8		4.0 ± 0.3			
24-Methylcholesta-	Range	N.D.–3.1	1.9–2.3	N.D.	2.6–3.9	4.2–5.3	0.1–3.7	6.0–9.4	3.0–7.2
5,24(28)-dien-3β-ol	Mean ± sd	1.0 ± 1.3	2.5 ± 0.7		3.1 ± 0.7	4.7 ± 0.5	1.5 ± 1.2	8.2 ± 1.4	5.2 ± 1.7

[a](*n*), number of samples; sd, sample standard deviation; N.D., not detectable.
[b]Coeluting cholesta-5,24-dien-3β-ol and 24-methylcholesta-5,22*E*-dien 3β-ol; the proportion of 24-methylcholesta-5,22*E*-dien-3β-ol is suspected not to exceed 10% of the sum.

to the Antarctic copepod species, the abundance of cholesta-5,22*E*-dien-3β-ol was low (4.3–10.2%) in all Arctic species, but comparable to that of *C. acutus* in the fall (6.6%) (Table 3).

Antarctic Euphausiacea. All euphausiid species showed a similar sterol pattern with cholest-5-en-3β-ol as the dominant sterol followed by cholesta-5,24-dien-3β-ol and cholesta-5,22*E*-dien-3β-ol (Table 4). Proportions of cholest-5-en-3β-ol were always higher than in the copepods. In all euphausiids, 24-norcholesta-5,22*E*-dien-3β-ol and C_{28} phytosterols were only found in traces or were below the detection limit. Summer and fall samples exhibited very similar sterol compositions. Because of the limited number of samples we did not discriminate between seasons.

DISCUSSION

Polar zooplankton have developed various solutions for adaptation to the cold environment. The biosynthesis and storage of lipids with a large variety of fatty acids and alcohols is especially pronounced in these species (21). Lipid levels and compositions depend on life cycle strategies and trophic preferences. It is well known that not only herbivorous polar copepods but also euphausiids are capable of accumulating large amounts of lipid deposits (1,22,23). In contrast to lipids accumulated as energy reserves, membrane lipids such as phospholipids and sterols have to fulfill basic physiological demands. They guarantee the functionality of membranes and modulate the fluidity. The hydroxy function at position C-3 of ring A of the ring skeleton (Fig. 1) permits the orientation of the planar and rigid sterol molecule in the bilayer. Sterol molecules are able to separate or laterally displace both the acyl chains and polar head groups of membrane phospholipids (3).

All copepods and euphausiids analyzed in this study exhibited simple sterol patterns. The dominant sterol in vertebrates, cholest-5-en-3β-ol, is also a major sterol in copepods

TABLE 3
Sterol Compositions of Arctic Copepods (fall)[a]

Sterol		Percentage composition (of total free sterols)		
		Calanus hyperboreus (7)	*Calanus glacialis* (7)	*Calanus finmarchicus* (6)
24-Norcholesta-5,22*E*-dien-3β-ol	Range	1.4–2.4	1.7–3.8	2.2–4.6
	Mean ± sd	2.0 ± 0.4	2.8 ± 0.9	2.8 ± 0.9
Cholesta-5,22*E*-dien-3β-ol	Range	2.8–6.1	4.5–9.5	8.2–11.2
	Mean ± sd	4.3 ± 1.4	6.5 ± 1.8	10.2 ± 2.2
Cholest-5-en-3β-ol	Range	34.5–52.1	36.8–58.9	54.0–64.5
	Mean ± sd	41.9 ± 6.1	44.1 ± 10.1	56.7 ± 3.9
Cholesta-5,24-dien-3β–ol	Range	32.9–52.5	21.8–49.4	15.3–27.8
	Mean ± sd	44.2 ± 7.3	36.7 ± 10.3	24.4 ± 4.7
24-Methylcholesta-5,22*E*-dien-3β-ol	Range	2.7–4.4	3.0–4.7	1.5–2.8
	Mean ± sd	3.7 ± 0.6	3.5 ± 0.9	2.4 ± 0.5
24-Methylcholesta-5,24(28)-dien-3β-ol	Range	0.9–11.2	2.9–9.3	2.2–5.4
	Mean ± sd	3.9 ± 3.5	6.4 ± 2.0	3.5 ± 1.2

[a]For abbreviations see Table 2.

TABLE 4
Sterol Compositions of Antarctic Euphausiids (summer and fall)[a]

		Percentage composition (of total free sterols)		
Sterol		*Euphausia superba* (4)	*Thysanoessa macrura* (4)	*Euphausia crystallorophias* (3)
24-Norcholesta-5,22*E*-dien-3β-ol	Range	N.D.	N.D.	N.D.
	Mean ± sd			
Cholesta-5,22*E*-dien-3β-ol	Range	0.7–2.8	N.D.–4.9	1.6–4.6
	Mean ± sd	1.9 ± 0.9	3.0 ± 2.2	3.2 ± 1.5
Cholest-5-en-3β-ol	Range	76.7–88.5	80.5–93.3	76.2–82.4
	Mean ± sd	82.1 ± 5.6	86.4 ± 5.3	78.7 ± 3.3
Cholesta-5,24-dien-3β-ol	Range	9.9–18.7	6.4–13.8	13.1–20.3
	Mean ± sd	14.6 ± 4.1	9.9 ± 2.8	16.3 ± 3.5
24-Methylcholesta-5,22*E*-dien-3β-ol	Range	1.4–2.1	0.3–0.7	1.2–1.8
	Mean ± sd	1.8 ± 0.4	0.5 ± 0.2	1.5 ± 0.3
24-Methylcholesta-5,24(28)-dien-3β-ol	Range	N.D.–0.1	N.D.	N.D.
	Mean ± sd	0.1 ± 0.1		

[a]For abbreviations see Table 2.

and euphausiids. This is in accordance with observations of Goad (4) and Teshima (6) and references therein. It is well established that crustaceans must obtain sterols either directly from their diet or by dealkylating dietary C-24 alkyl-substituted phytosterols (6–8). The production of cholesta-5,24-dien-3β-ol as an intermediate in this dealkylation process provides an attractive explanation for the occurrence of considerable amounts of this sterol in many crustaceans (4,24). The mechanisms of the dealkylation of 24-ethylcholest-5-en-3β-ol operating in insects—like the crustaceans, they belong to the Arthropoda—have been studied in detail; 24-ethylcholesta-5,24(28)-dien-3β-ol, its 24,28-epoxide, and cholesta-5,24-dien-3β-ol have been identified as intermediates (4,25). Baker and Kerr (9) described the dealkylation of 24-methylcholesta-5,24(28)-dien-3β-ol and 24-ethylcholesta-5,24(28)-dien-3β-ol to cholesta-5,24-dien-3β-ol and cholest-5-en-3β-ol in marine sponges, indicating a mechanism parallel to that observed in phytophagous insects.

Thus, cholesta-5,24-dien-3β-ol may be accumulated during summer, when phytoplanktonic food is abundant. Indeed, an increased proportion of cholesta-5,24-dien-3β-ol was observed in the herbivorous *C. acutus* and in *C. propinquus* during summer, whereas the abundance of cholesta-5,24-dien-3β-ol in the fall was similar to that in spring; cholesta-5,24-dien-3β-ol may have been converted to cholest-5-en-3β-ol. The North Atlantic *C. finmarchicus* and the Arctic *C. glacialis* also exhibited a higher content of cholest-5-en-3β-ol than cholesta-5,24-dien-3β-ol in the fall. A higher abundance of cholesta-5,24-dien-3β-ol in the Arctic *C. hyperboreus* may be explained by differences in the food availability.

Direct uptake of cholesta-5,24-dien-3β-ol from the diet is regarded as negligible since this sterol is not a common major component in algal sterol profiles (26). This compound was a minor constituent of the particulate sterols found in the surface of the Weddell Sea in spring (27); in the fall, its mean abundance was only 3.1% of all free sterols in particulate matter of surface waters (28).

Findings similar to this study were described by Serrazanetti *et al.* (29) for mixed zooplankters from the Adriatic Sea. These authors observed that levels of cholesta-5,24-dien-3β-ol were higher by a factor of about three during algal blooms than later in the year. Our data revealed a decrease of cholest-5-en-3β-ol compared to cholesta-5,24-dien-3β-ol, whereas they also found increased abundances for cholest-5-en-3β-ol in bloom situations. In zooplankters from the Gulf of Trieste cholesta-5,24-dien-3β-ol exhibited the lowest proportion in winter when sterols from phytoplankton are scarce (30). However, the higher percentage of cholest-5-en-3β-ol in Adriatic zooplankters during bloom situations is difficult to evaluate, since sterols in mixed zooplankton were analyzed.

In contrast to the herbivorous copepods, the sterol pattern of the omnivorous *M. gerlachei* changed to a higher proportion of cholesta-5,22*E*-dien-3β-ol in summer. This finding may be attributed to the deviating feeding behavior and to differences in metabolic pathways. *Metridia gerlachei* feeds not only on phytoplankton but also on zooplankton and detritus (31) containing this sterol. Cholesta-5,22*E*-dien-3β-ol is a frequent sterol in marine particulate matter (27,28). In *Fragilariopsis (Nitzschia) cylindrus*, a diatom common in the Weddell Sea, cholesta-5,22*E*-dien-3β-ol contributed to about 70% to the total sterols (32). Virtue *et al.* (10) demonstrated that the proportion of cholesta-5,22*E*-dien-3β-ol in the digestive gland of *E. superba* decreased by an order of magnitude during short-term starvation, suggesting an algal origin of this sterol. Thus, high abundances may be indicative of a high dietary input. However, the herbivorous species did not exhibit enhanced levels of cholesta-5,22*E*-dien-3β-ol. A difference in the metabolic pathway cannot be excluded. It has been noted, that some arthropods are not able to convert cholesta-5,22*E*-dien-3β-ol to cholest-5-en-3β-ol (33).

In *M. gerlachei* a distinct increase of the C_{28} phytosterol 24-methylcholesta-5,24(28)-dien-3β-ol was observed in summer, which may be explained by a higher uptake. Other copepods also exhibited a slightly higher proportion of this sterol in summer. However, even in summer levels were still low; thus, dealkylation probably takes place soon after ingestion.

Nearly all copepods contained 24-norcholesta-5,22*E*-dien-3β-ol, it being most abundant in *M. gerlachei* with almost 10%.

Since this sterol is common in marine invertebrates, it seems likely that the primary producer may be an organism early in the food chain (4). According to Minale and Sodano (34), 24-norcholesta-5,22*E*-dien-3β-ol may be formed from 24-methylcholesta-5,22*E*-dien-3β-ol by side-chain dealkylation. Boutry and Barbier (35) suggested that C_{26} sterols can be produced through degradation of C_{28} precursors.

Euchaeta antarctica is a planktonic predator. In contrast to the herbivorous and omnivorous species, its major sterol was the zoosterol cholest-5-en-3β-ol in summer. The proportion of cholesta-5,24-dien-3β-ol was only half of that observed for copepods feeding on phytoplankton. Changes in the sterol composition may reflect the trophic preference of *E. antarctica*.

All euphausiid species showed a similar and very simple sterol composition. Although the species investigated have been described to feed omnivorously or even herbivorously (36,37), cholest-5-en-3β-ol was more dominant than in the copepods. For example, *E. superba* is known to feed mainly as an herbivore (1); nevertheless, the content of cholesta-5,24-dien-3β-ol was almost nine times less than that of cholest-5-en-3β-ol and was always less than in the copepods. Although copepods contained C-24 alkylated phytosterols in abundances up to 8%, their proportions were even smaller in euphausiids with levels below 2%. In contrast to copepods, 24-norcholesta-5,22*E*-dien-3β-ol could not be detected in euphausiids. These observations suggest a faster and/or more complete metabolic conversion of phytosterols to cholest-5-en-3β-ol in euphausiids than in copepods.

Not only the sterol compositions of copepods and euphausiids differed but also the compositions of their phospholipids. The Antarctic krill *E. superba*, *T. macrura,* and *E. crystallorophias* exhibited higher proportions of 18:1n-9 and 20:5n-3 and lower levels of 22:6n-3 fatty acids in their phospholipids (38–40) than are reported in copepods (41). Farkas *et al.* (42) found that the molecular species composition of membrane lipids plays a decisive role in membrane adaptation. In accordance with these observations the deviating sterol compositions suggest different ways of cold adaptation in the membranes of copepods and euphausiids attained by specific interactions of the various membrane components.

In summary, the zooplankters investigated exhibited simple sterol compositions. The copepods showed clear species-specific differences. The patterns observed in euphausiids were dominated to a greater extent by cholest-5-en-3β-ol than those in the copepods, suggesting a faster or more complete metabolic conversion of phytosterols in euphausiids. In Antarctic copepods, differences in the abundance of sterols were noticed during the seasons in which samples were collected, which may have been caused by the trophic preferences of these animals. While herbivorous copepods contained enhanced levels of cholesta-5,24-dien-3β-ol during the phytoplankton blooms in summer, the carnivorous *E. antarctica* demonstrated a high percentage of the zoosterol cholest-5-en-3β-ol. The characteristic sterol composition of the zooplankton may be accounted for by the existence of specific physiological mechanisms of accumulation and elimination.

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Mitochondrial Membrane Composition of Two Arctic Marine Bivalve Mollusks, *Serripes groenlandicus* and *Mya truncata*

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ABSTRACT: The phospholipid and fatty acid composition of gill mitochondria membranes from two Arctic marine bivalve mollusks, *Mya truncata* and *Serripes groenlandicus*, were examined. These animals were collected from the Arctic Ocean, where waters remain below 0°C throughout the year. In both species, the primary membrane phospholipids were phosphatidylcholine, and phosphatidylethanolamine. Although a low ratio of bilayer-stabilizing phospholipids to bilayer-destabilizing phospholipids is frequently associated with cold acclimation in temperate species, this ratio is very different between the two species. The monounsaturated fatty acid 20:1 was abundant in the membranes of both Arctic species equaling 13.0% of the fatty acid composition in *S. groenlandicus*, and 17.7% in *M. truncata*. Polyunsaturated fatty acids were relatively low in the Arctic species, equaling 35.9% of total membrane fatty acids compared to that of temperate zone mollusks. It is suggested that monoenes are common in the tissues of Arctic species since they play a role in maintaining membrane function at subzero temperatures.

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Polar marine environments are among the most thermally stable regions of the world. Such stability resulted in many organisms becoming highly specialized to function at subzero temperatures (1). The properties of biological membranes in particular must be adapted to function under these conditions. The composition of cellular membranes can be manipulated to maintain membrane properties and function. Membrane phospholipid and fatty acid composition are two of the components that can be altered to maintain membrane function (2–4). Study of temperate, ectothermic species demonstrated that the bilayer-destabilizing phospholipid phosphatidylethanolamine (PE), as well as long-chain polyunsaturated fatty acids (PUFA) accumulate within cellular membranes upon acclimation to low temperatures (2,3,5–9). Such manipulation of membrane composition aids in maintaining membrane properties (2–4,6,9).

Several studies indicate that the lipids of polar organisms differ from those of temperate zone organisms. In particular, whole tissue lipids of Arctic and Antarctic copepods contain unusually high amounts of monounsaturated fatty acids (monoenes) (10). It is not known if these differences apply to membrane lipids. Chronic exposure to very low temperature such as occurs in polar organisms may require membrane adaptational strategies previously unidentified by thermal acclimation studies of temperate species.

The purpose of this study is to characterize the mitochondrial membranes of the two Arctic bivalve mollusk species *Serripes groenlandicus* and *Mya truncata*. As a result of their limited ability for locomotion, these species remain in subzero waters throughout the year in the Canadian Arctic. By comparing the membrane composition of these animals to those of cold-adapted temperate species, it may be possible to establish if mollusk species from the Arctic and Temperate zones utilize similar methods of membrane modification in overcoming the challenge of living at low temperatures.

MATERIALS AND METHODS

Experimental animals. Serripes groenlandicus and *M. truncata* were collected by scuba divers in depths of 7–18 m from the waters of the Arctic Ocean surrounding Igloolik Island (81°45′ W, 69°23′ N), Northwest Territories. The water temperature was −1.5–0.0°C, and the animals were observed to be feeding at time of capture. The animals were held in an aerated marine aquarium, maintained below 0°C for a maximum of 24 h prior to tissue processing.

Mitochondrial isolation. The gills were excised and placed in ice-cold isolation medium consisting of 400 mM sucrose, 1 mM EDTA, 2 mM EGTA, 10 mM HEPES pH 7.4 at 20°C, and 1% bovine serum albumin (essentially fatty acid-free). The gills of two animals were pooled to obtain a sufficient mass of tissue. The tissue was then weighed, minced, and suspended in ice-cold isolation medium in a ratio of 1 g of tissue to 5 mL of isolation medium. This mixture was homogenized

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Abbreviations: CL, cardiolipin; monoene, monounsaturated fatty acid; PC, phosphatidylcholine; PC/(PE + CL), phosphatidylcholine to (phosphatidylethanolamine + cardiolipin) ratio; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SPH, sphingomyelin.

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with three passes of a loosely-fitting Teflon pestle in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 15 min at 1200 × *g*. The resulting supernatant was centrifuged for 15 min at 13600 × *g*, the pellet was removed and resuspended in 1 mL isolation medium, stored at −20°C for 3–7 d, and transported to the laboratory at the University of Guelph where it was stored at −80.0°C until further processing.

Measurement of marker enzymes. Marker enzymes were measured in both the initial homogenate and in the mitochondrial preparation. The following were used as markers for cellular membranes: cytochrome C oxidase (mitochondrial membrane), glucose 6-phosphatase (endoplasmic reticulum), Na^+,K^+-ATPase (plasma membrane), and peroxidase (peroxisomes). The activities of these marker enzymes were measured as described by Gillis and Ballantyne (11).

Analysis of mitochondrial membrane phospholipid composition. The mitochondrial membrane phospholipids were extracted, separated, and analyzed as described by Gillis and Ballantyne (11)

Protein determination. Protein was determined by the method of Bradford (12) using bovine serum albumin as a standard. Mitochondrial and tissue protein was determined by measuring the difference between the protein concentration in the isolation medium and the mitochondrial suspension.

Chemicals. The lipid standard used (Nu-Chek-Prep., Inc. Elysian, MN) was augmented by the addition of menhaden oil extract. Menhaden oil contains fatty acids not found in the commercial standard, but which are present in the lipids of most marine and freshwater organisms. Solvents were obtained from Fisher Scientific Ltd. (Whitby, Ontario) and were of American Chemical Society-certified grade. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

RESULTS

Characterization of mitochondrial membrane purity. The isolation procedure used results in a substantial enrichment of the mitochondrial membrane marker cytochrome C oxidase in relation to the other marker enzymes for plasma membrane, peroxisomes, and endoplasmic reticulum (Table 1), indicating little contamination of the mitochondria with these cellular membranes. A second indication of membrane purity was the almost complete absence of sphingomyelin (SPH) (<1.0%). This phospholipid is found in trace amounts in the mitochondrial membrane (1%) while it is prevalent in lysosomes, plasma membranes, and Golgi membranes, representing as much as 20, 16, and 8%, respectively, of total phospholipid in these membranes (13). The very low proportion of this phospholipid is further evidence that there was minimal contamination of the membrane preparations by these organelles in the present study.

Phospholipid composition. In *M. truncata,* PE was the dominant phospholipid, representing 47.8% of the total membrane phospholipids, while phosphatidylcholine (PC) and phosphatidylinositol (PI) made up 22.4 and 19.5%, respectively, of the membrane phospholipid composition (Table 2). In *S. groenlandicus*, PE and PC were the two most abundant phospholipids, equaling 38.4 and 40.4% of the total membrane phospholipids, while PI represented 13.3% (Table 2). Cardiolipin (CL) and phosphatidylserine (PS) were minor components in the membrane phospholipids of both bivalve species. The ratio of bilayer-stabilizing (PC) to bilayer-destabilizing phospholipids (PE, CL) equaled 1.01 in *S. groenlandicus* and 0.45 in *M. truncata*. The phospholipid SPH was present in trace amounts in the mitochondrial membranes of both species but in all preparations represented less than 1% of total membrane phospholipid.

Gill mitochondrial membrane fatty acid composition. The predominant fatty acids in the cumulative fatty acid composition of membrane phospholipids in both bivalve species were 16:0, 20:1, and 20:5n-3 (Table 3), equaling a combined total of 45.9% in *S. groenlandicus* and 56.1% in *M. truncata*. Saturated fatty acids (SFA) were the most prevalent class of fatty acids equaling 42% in *S. groenlandicus* and 38% in *M. truncata* (Table 3).

In both species of mollusk, SFA, monoenes, and PUFA represented similar proportions of the fatty acids in the phospholipid PE (Table 4). The main individual fatty acids in this phospholipid from both species were 20:0 and 20:1. In *S. groenlandicus,* 20:0 equaled 24.3 ± 3.2% of total fatty acid content while 20:1 equaled 23.1 ± 3.3%. In *M. truncata* these fatty acids equaled 18.5 ± 4.7 and 29.4 ± 3.6%, respectively.

In mitochondrial PC of *S. groenlandicus*, SFA and PUFA were the prevalent classes of fatty acids (Table 4); this was

TABLE 1
Fold Purification of Marker Enzymes in Mitochondrial Fraction Isolated from Gill Tissue of *Mya truncata* and *Serripes groenlandicus*[a]

	M. truncata	*S. groenlandicus*
Na^+/K^+-ATPase	0.074 ± 0.030	0.080 ± 0.028
Cytochrome C oxidase	13.62 ± 3.62	15.22 ± 4.09
Peroxidase	0.060 ± 0.028	0.059 ± 0.007
Glucose 6-phosphatase	0.30 ± 0.053	0.22 ± 0.057

[a]Values are means ± SE for $n = 4$. Fold purification = (activity in mitochondrial pellet/mg protein)/(activity in homogenate/mg protein).

TABLE 2
Percentage of Phospholipids in Gill Mitochondria from *Serripes groenlandicus* and *Mya truncata*[a]

Phospholipid	*S. groenlandicus* ($n = 8$) (mol %)	*M. truncata* ($n = 8$) (mol %)
Cardiolipin	2.82 ± 0.40	4.90 ± 0.74
Phosphatidylethanolamine	38.48 ± 1.50	47.78 ± 2.43
Phosphatidylinositol	13.34 ± 0.66	19.49 ± 1.84
Phosphatidylserine	4.87 ± 0.83	5.93 ± 1.63
Phosphatidylcholine	40.48 ± 1.63	22.37 ± 0.76
PC/(PE + CL)[b]	1.01 ± 0.098	0.45 ± 0.038

[a]Values are presented as means ± SEM.
[b]PC = phosphatidylcholine, PE = phosphatidylethanolamine, CL = cardiolipin.

TABLE 3
Cumulative Percentages of Individual Fatty Acids in Gill Mitochondria from *Serripes groenlandicus* and *Mya truncata*[a]

Fatty acid	*S. groenlandicus* (*n*=8) (mol %)	*M. truncata* (*n* = 8) (mol %)
14:0	7.70 ± 0.57	0.95 ± 0.12
14:1	0.53 ± 0.15	0.23 ± 0.08
16:0	17.14 ± 0.99	22.14 ± 0.93
16:1	4.48 ± 0.32	4.97 ± 0.30
18:0	7.90 ± 0.32	4.29 ± 0.26
18:1n-9	3.36 ± 0.38	3.05 ± 0.29
18:2n-6	0.21 ± 0.06	n.d.[b]
18:3n-3	0.40 ± 0.09	0.69 ± 0.10
18:4n-3	0.56 ± 0.15	0.36 ± 0.03
20:0	9.20 ± 1.15	9.90 ± 2.14
20:1	13.02 ± 1.18	17.68 ± 1.28
20:2n-6	0.16 ± 0.04	0.08 ± 0.03[c]
20:3n-6	0.93 ± 0.29	1.55 ± 0.13
20:4n-6	1.66 ± 0.28	1.09 ± 0.15
20:3n-3	0.08 ± 0.04[c]	0.02 ± 0.01[c]
20:4n-3	0.31 ± 0.05	0.34 ± 0.07
20:5n-3	15.86 ± 1.71	16.32 ± 0.66
22:0	0.24 ± 0.06	0.20 ± 0.04
22:1	0.10 ± 0.03	0.10 ± 0.02
22:2n-6	4.83 ± 0.13	1.94 ± 0.11
23:0	0.39 ± 0.08	0.48 ± 0.02
22:4n-6	0.88 ± 0.09	1.92 ± 0.09
22:5n-6	0.09 ± 0.06[c]	0.04 ± 0.03[c]
22:5n-3	2.77 ± 0.18	2.83 ± 0.16
22:6n-3	7.10 ± 0.25	8.72 ± 0.43
Saturated fatty acids	42.59 ± 1.25	37.99 ± 1.36
Monounsaturated fatty acids	21.51 ± 1.15	26.05 ± 1.11
Polyunsaturated fatty acids	35.90 ± 1.74	35.96 ± 1.34
n-3 Polyunsaturated fatty acids	27.10 ± 1.69	29.31 ± 1.16
n-6 Polyunsaturated fatty acids	8.79 ± 0.37	6.64 ± 0.32
n-3/n-6 Polyunsaturated fatty acids	3.12 ± 0.24	4.44 ± 0.19
Unsaturation index[d]	185.60 ± 8.04	199.50 ± 6.33
Chain length[e]	18.61 ± 0.10	18.87 ± 0.05

[a]Values are presented as means ± SEM.
[b]Not detectable.
[c]Three or more values expressed in mean were not detectable therefore included as zeroes.
[d]Unsaturation index = $\Sigma m_i \pm n_i$, where m_i is the mole percentage and n_i is the number of C-C double bonds of the fatty acid$_i$.
[e]Mean chain length = $\Sigma f_i \pm c_i$, where f_i is the mole fraction and c_i is the number of carbon atoms of the fatty acid $_i$.

due to the large proportions of 14:0, 16:0, and 20:5n-3, equaling 17.8 ± 1.3, 21.0 ± 1.1, and 18.4 ± 4.4%, respectively, of the fatty acids in this phospholipid. In *M. truncata* PUFA represented the largest class of fatty acids in PC due to the high levels of 20:5n-3 (33.7 ± 0.6%) in this phospholipid (Table 4).

PUFA was the largest class of fatty acids in CL from *S. groenlandicus* (Table 4). This was due to 20:5n-3, equaling 28.7 ± 3.9% of the fatty acids in this phospholipid. The monoene 20:1 was also prominent in this phospholipid, equaling 19.9 ± 6.5% of the fatty acid content. SFA and PUFA were the most common fatty acids in CL from *M. truncata* due to the high levels of 16:0 and 20:5n-3. These two fatty acids equaled 26.6 ± 1.4 and 24.9 ± 1.2%, respectively.

In PS from *S. groenlandicus*, SFA and PUFA were the dominant fatty acids (Table 4) as a result of the levels of 18:0 and 20:5n-3. These two fatty acids equaled 30.5 ± 2.2 and 17.9 ± 2.5%, respectively. In *M. truncata*, SFA was the most common class of fatty acids in this phospholipid due to the levels of 16:0 and 20:0. These two fatty acids equaled 19.6 ± 3.8 and 13.3 ± 1.2%, respectively. The monoene 20:1 was also prominent in PS, equaling 16.0 ± 2.6% of the fatty acid content.

In the phospholipid PI, SFA was the prevalent class of fatty acids in both species (Table 4). This result is due to the high levels of 16:0 in this phospholipid, equaling 53.7 ± 1.5% in *S. groenlandicus* and 71.2 ± 3.8% in *M. truncata*.

DISCUSSION

In the mitochondrial membranes of both *S. groenlandicus* and *M. truncata,* PE and PC were the main phospholipids, typical of most mitochondrial membranes (13). These two phospholipids play an important role in regulating membrane properties in the biological membranes of temperate zone organisms (2,6,14). The ratio of PC to PE decreases in many ectothermic animals acclimated to lower temperatures (2). PC stabilizes the bilayer as it favors the formation of a laminar bilayer, while PE destabilizes the bilayer by keeping the membrane close to the phase transition between laminar and hexagonal (H_{II}) phase conformations (15). For example, acclimation of trout *Salmo gairdneri* from 20 to 5°C lowered the ratio of PC/PE from 1.71 to 0.78 (16). Mitochondria contain a second bilayer-destabilizing phospholipid, CL. This phospholipid adopts the H_{II} phase conformation in the presence of Ca^{2+} (17,18) and the membrane protein cytochrome C oxidase (19), both of which are associated with the inner mitochondrial membrane. The ratio of PC/(PE + CL) in *M. truncata* was about half that of *S. groenlandicus.* This difference is as a result of twofold higher proportion of PC in the membranes of *S. groenlandicus*. Such interspecific differences in this ratio are not uncommon in temperate marine bivalves. In the oyster, *Crassostrea virginica,* this ratio is twice that of the quahog, *Mercenaria mercenaria* (11,20), and similar to that of *S. groenlandicus* in the present study. These results suggest that a low PC/(PE + CL) ratio is not a unique characteristic of Arctic marine mollusks.

The monounsaturated fatty acid (monoene) 20:1 was one of the three primary fatty acids in the cumulative totals of the mitochondrial membrane phospholipids in both bivalve species. Accordingly, this fatty acid was dominant in the phospholipids PE, CL, and PI in both species as well as in PS of *M. truncata*. This is different from the fatty acid composition of temperate marine bivalves where the predominant fatty acids are SFA and PUFA (11,20). Other cold-water organisms have high levels of monoenes. The monoene 18:1 is reported to be prevalent in red muscle mitochondrial membranes of Arctic char *Salvelinus alpinus* sampled from 4°C waters of the Arctic Ocean (21). The monoenes 20:1 and 22:1 were also reported in unusually high proportion in the whole tissue lipids of Arctic and Antarctic copepods (10).

The combination of the monoene 18:1 in the *sn*-1 position and a long-chain PUFA in the *sn*-2 position in the phospho-

TABLE 4
Percentages of Fatty Acid Classes in the Membrane Phospholipids from the Gill Mitochondria of *Serripes groenlandicus* and *Mya truncata*[a]

Species	Class	PE	PC	PS	PI	CL
S. groenlandicus (*n* = 8)	SFA	37.6 ± 1.3	43.6 ± 2.5	48.9 ± 3.7	64.9 ± 1.7	20.0 ± 4.6
	Monoenes	28.8 ± 2.2	15.9 ± 1.5	8.7 ± 2.5	16.5 ± 2.2	28.3 ± 6.7
	PUFA	33.7 ± 2.9	40.5 ± 3.6	42.4 ± 3.8	18.6 ± 0.9	51.6 ± 5.2
	n-3 PUFA	21.8 ± 2.2	34.3 ± 3.8	26.1 ± 2.8	10.4 ± 0.6	41.5 ± 6.7
	n-6 PUFA	11.8 ± 0.9	6.2 ± 0.4	16.3 ± 1.4	8.2 ± 0.4	10.1 ± 2.2
	Unsat ind	171.1 ± 10.8	212.8 ± 17.0	182.8 ± 16.6	94.5 ± 3.2	270.8 ± 27.3
M. truncata (*n* = 8)	SFA	28.2 ± 4.0	22.1 ± 0.7	42.2 ± 2.8	77.8 ± 2.8	35.9 ± 1.9
	Monoenes	35.2 ± 3.0	15.3 ± 0.6	28.3 ± 3.6	11.6 ± 1.5	19.1 ± 1.5
	PUFA	36.5 ± 3.9	62.6 ± 0.8	29.5 ± 4.6	10.7 ± 1.4	45.1± 2.7
	n-3 PUFA	28.2 ± 3.1	55.8 ± 0.7	24.7 ± 5.0	6.8 ± 1.3	39.6 ± 2.6
	n-6 PUFA	8.3 ± 0.8	6.7 ± 0.4	4.8 ± 0.9	3.9 ± 0.3	5.5 ± 1.1
	Unsat. Ind[b]	206.2 ± 17.7	324.9 ± 3.4	179.6 ± 22.2	59.2 ± 8.9	248.5 ± 13.7

[a]Values are presented as means ± SEM. [b]PS, phosphatidylserine; PI, phosphatidylinositol; SFA, saturated fatty acids; monoenes, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See Table 2 for other abbreviations.
[b](Unsat) index = $\Sigma m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C-C double bonds of the fatty acid $_i$.

lipid PE in cold-adapted and acclimated teleosts was suggested to play a role in fluidizing the biological membrane (13,22). These authors suggest that the presence of these specific fatty acids in PE is at least partially responsible for the increased disorder and lower transition temperatures of membranes of fish either evolutionarily or seasonally adapted to low temperatures. In PE of *S. groenlandicus,* the monoene 20:1 was the predominant fatty acid while in *M. truncata* it and 20:0 were of equally high proportion. Compared to the levels in PE of the temperate marine bivalve *C. virginica*, 20:1 was at least five times as abundant while PUFA content was one-half in this phospholipid in the Arctic species (20). This result suggests that this monoene is of particular importance to this phospholipid in the membranes of Arctic organisms.

While monoenes are in abundance in the mitochondrial membranes of the Arctic bivalves, PUFA are not. The PUFA content in the mitochondrial membranes of the Arctic animals is at least one-third less than that reported for the mitochondrial membranes of the temperate marine bivalve species acclimated to either −1, 10, or 12°C (11,20). It is the comparatively low levels of the fatty acid 22:6n-3 in a number of the membrane phospholipids of the Arctic bivalve species that can account for this difference. This PUFA was demonstrated to be accumulated in the membranes of both cold-adapted and cold-acclimated teleost fish (6,14,22) and is the primary fatty acid in the mitochondrial membranes of *S. alpinus* caught from 4°C water of the Arctic Ocean (21). The low level of 22:6n-3 in the Arctic bivalves is not due to a limited supply of its n-3 precursors as the level of the fatty acid 20:5n-3 is similar to that of temperate species (20). This implies that high levels of 22:6n-3 are not required for optimal membrane function at low temperatures.

The SFA content in the mitochondrial membranes of the Arctic bivalves is higher than any reported values for the mitochondrial membranes of marine and freshwater teleost fish (8,21,23), or of tissue phospholipids of marine and freshwater crustaceans (5,7). However, this finding is similar to what has been reported for the gill mitochondrial membranes of the temperate marine bivalves *C. virginica* and *M. mercenaria* acclimated to either −1.5, 10, or 12°C (4,11). This suggests that high concentrations of SFA, in particular 16:0, in mitochondrial membranes may be a common characteristic of marine mollusks. Having high proportions of SFA in the membranes of animals from very cold temperatures does not conform to what is predicted by acclimation studies of teleost fish (2,8, 9,22). As membrane fluidity was not measured in our study, the thermotropic phase transition temperatures of the membranes are unknown. However, as the Arctic species were active and feeding at time of capture, their membranes must have been sufficiently fluid to be functioning.

Diet plays a role in determining the fatty acid composition of biological membranes (24). The prevalence of monoenes in the tissues of Arctic copepod plankton (10) indicates that these fatty acids are common in the Arctic food chain. These fatty acids would be of adaptive value to the copepods, and when transferred to the mollusks feeding on them would require little modification to confer optimal membrane properties of the mollusks.

Overall the membranes of the Arctic species are very different from what would be expected based on previously published studies of animals acclimated to the cold. This may be due to the chronic lower temperatures faced by Arctic organisms. High levels of monoenes appear to be characteristic of polar animals and may play a functional role in the maintenance of membrane fluidity. The high proportion of 20:1 in PE may support the fluidizing effect of this phospholipid on the membrane. The low levels of PUFA, specifically 22:6n-3, compared to temperate species in the Arctic bivalves suggest that these fatty acids are not essential to membrane function at low temperature.

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Influences of Subzero Thermal Acclimation on Mitochondrial Membrane Composition of Temperate Zone Marine Bivalve Mollusks

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ABSTRACT: The phospholipid and phospholipid fatty acid composition of gill mitochondrial membranes from two temperate zone marine bivalve mollusks, the quahog, *Mercenaria mercenaria,* and the American oyster, *Crassostrea virginica,* were examined after acclimation to 12 and −1°C. Cardiolipin (CL) was the only phospholipid with proportions altered upon acclimation to −1°C, increasing 188% in the mitochondrial membranes of *M. mercenaria*. Although the ratio of bilayer stabilizing to destabilizing lipids is frequently associated with cold acclimation in ectothermic species, no change was found in this ratio in either of the species. Polyunsaturated fatty acids (PUFA) were found only to increase in *C. virginica* with cold acclimation, with total n-3 PUFA increasing in the phospholipid phosphatidylethanolamine, total n-6 PUFA increasing in CL, and total PUFA increasing in phosphatidylinositol. Monounsaturated fatty acids, not PUFA, were found to have increased in *M. mercenaria,* with 18:1n-9 increasing by 150% in CL, and 20:1 increasing in both CL and phosphatidylcholine, by 146 and 192%, respectively. These manipulations of membrane phospholipid and fatty acid composition may represent an attempt by these species to help maintain membrane function at low temperatures.

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Ectothermic animals alter the phospholipid and fatty acid composition of their cellular membranes in order to defend membrane function from the effects of lowered environmental temperature. This is accomplished by strategies which include alterations in the proportions of membrane phospholipids and the fatty acid composition of membrane phospholipids (1,2).

While there are numerous studies of the effects of thermal acclimation on marine fishes, to date, no study has been made of membrane acclimation in ectothermic species below 5°C. Adaptation to subzero temperatures may demand previously unidentified changes to membrane composition. Changes in the physical properties of subzero water, such as pH, density, as well as the large impact of very low temperature on the kinetic properties of biological membranes may pose a significant challenge to the maintenance of membrane function. Many temperate zone species are exposed to subzero temperatures during the winter. Marine mollusks, owing to their limited mobility, are unable to migrate to warmer temperatures and are therefore seasonally exposed to such low temperature. Only limited study has been done of thermal effects on the fatty acid composition of bivalve mollusks (3–5).

To determine how marine mollusks utilize phospholipid composition to help maintain membrane function at subzero temperatures, we examined the mitochondrial membranes of two common species of temperate zone mollusk: the American oyster, *Crassostrea virginica*, and the quahog, *Mercenaria mercenaria* with acclimation to 12 and −1°C. These two species demonstrate tissue growth during the winter (4,6), suggesting that cellular activity and therefore membrane function are maintained during this time. The animals used in this study were obtained from waters which range in temperature from 12°C in the summer to −1.5°C in the winter. The acclimation temperatures used therefore resemble those to which the animals are accustomed.

MATERIALS AND METHODS

Quahogs, *M. mercenaria,* and American oysters, *C. virginica,* cultured in Prince Edward Island, were obtained from a local seafood supplier in September. The animals were held at 12.0 ± 0.7°C in a flow-through seawater system (32‰) at the University of Guelph for a period of 2 mon prior to any temperature manipulation in 250-L tanks. A blended mixture of frozen krill was fed to the animals three times a week prior to and during the experimental period. The photoperiod of the holding facilities in which the bivalves were kept was 12 h dark/12 h light. Individuals of each species were randomly assigned to one of two experimental tanks. One tank was maintained at 12.0°C for the entire experimental period, while the temperature of the other tank was lowered to −1.0°C over a period of 26 d by decreasing the temperature by 1°C every 2 d using a water chiller (Ramco Inc., Columbus, OH). Once −1.0°C was achieved in the cold tank, both tanks were kept at

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Abbreviations: CL, cardiolipin; monoene, monounsaturated fatty acid; PC, phosphatidylcholine; PC/(PE + CL), phosphatidylcholine to (phosphatidylethanolamine + cardiolipin) ratio; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; SPH, sphingomyelin.

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their respective temperatures for 65 d. Both acclimation tanks remained in the flow-through biological filtration system throughout the experimental period.

Mitochondrial isolation. The gills were excised and placed in ice-cold isolation medium consisting of 400 mM sucrose, 1 mM EDTA, 2 mM EGTA, 10 mM HEPES pH 7.4 at 20°C, and 1% bovine serum albumin (essentially fatty-acid free). The gills of two animals were pooled to obtain a sufficient mass of tissue, which was then weighed, minced, and suspended in ice-cold isolation medium in a ratio of 1 g of tissue to 5 mL of isolation medium. This mixture was homogenized with three passes of a loosely fitting Teflon pestle in a Potter-Elvehjem homogenizer. The homogenate was then centrifuged for 15 min at $1,200 \times g$. The resulting supernatant was centrifuged for 15 min at $13,600 \times g$; the pellet was removed and resuspended in 1 mL isolation medium, frozen in liquid nitrogen, and then stored at −80°C.

Analysis of mitochondrial membrane phospholipid composition. Mitochondrial lipids were extracted by a modification of the method of Bligh and Dyer (7) as described by Glemet and Ballantyne (8). The thin-layer chromatography method used for the separation of phospholipids from each other and from neutral lipids is essentially that of Holub and Skeaff (9). The lipid samples dissolved in 25 µL of a 2:1 mixture of chloroform/methanol were run on Merck silica gel 60 glass precoated 20×20 cm plates with a 0.25-mm layer thickness (British Drug House, Toronto, Canada). Phospholipid standards were run in separate lanes on the plates with the membrane lipids in separate lanes for reference. The solvent system used to develop the plates consisted of chloroform/methanol/acetic acid/water in a 50:37.5:3.5:2 (by vol) mixture. Once the solvent system had run within 0.5 cm of the top of the plate, the plate was removed and allowed to air dry then sprayed with a saturated solution of 2,7-dichlorofluorescein in methanol/water, 1:1 (vol/vol). The plate was then placed in a raised-bottom developing chamber containing 50–100 mL of 25% ammonium hydroxide for 5 min. The phospholipid bands were then visualized under ultraviolet light at a wavelength of 366 nm. This method is effective in completely separating the phospholipids: phosphatidylethanolamine (PE), phosphatidylcholine (PC), cardiolipin (CL), phosphatidylinositol (PI), phosphatidylserine (PS), and sphingomyelin (SPH) (9).

The preparation of fatty acid methyl esters from the separated phospholipids followed the method of Holub and Skeaff (9). The individual phospholipid bands were scraped into glass Kimax tubes containing 2 mL of 6% H_2SO_4 in methanol and 10 µg of heptadecanoic acid (17:0) as an internal standard. The sealed tubes were then mixed and incubated at 80°C for 2 h. Upon completion, the tubes were cooled to room temperature, 2 mL of petroleum ether was added, and the tubes were then vortexed for 60 s. Double-distilled H_2O (1 mL) was added, and the tubes were vortexed for a further 30 s. The upper petroleum ether phase containing the eluted fatty acid methyl esters was removed and transferred to a glass vial and stored at −20°C until further processing. These samples were than analyzed as described by Glemet and Ballantyne (8), using a Hewlett-Packard (Mississauga, Ontario, Canada), HP5890 series II gas chromatograph fitted with a flame-ionization detector, an automatic injector (7673A; Hewlett-Packard) and an electronic pressure control program. Fatty acid methyl esters were analyzed on DB 225 megabore fused-silica column (Chromatographic Specialties Inc., Brockville, Ontario, Canada). Chain lengths shorter than C:14 were not resolved and therefore were not reported. Phospholipid content was calculated by summing the concentrations of fatty acids for each phospholipid, taking into account the number of fatty acids esterified to each phospholipid. The quantitative recovery of a known PI standard following these methods of thin-layer chromatography, methylation, and gas chromatography is 95% (9).

Extraction and quantification of total lipids from frozen krill. Total lipids of the frozen krill fed to the animals throughout the thermal acclimation study were extracted and quantified by the same methods used for the mitochondrial preparations.

Measurement of marker enzymes. Marker enzymes were measured in both the initial homogenate and in the mitochondrial preparation. The following marker enzymes were used: cytochrome C oxidase (mitochondrial membrane), glucose 6-phosphatase (endoplasmic reticulum), Na^+,K^+-ATPase (plasma membrane), and peroxidase (peroxisomes). A temperature-controlled Hewlett-Packard HP8452 diode array spectrophotometer maintained at 10°C by a Haake D8 circulating water bath (Haake Buchler Instruments Inc., Saddlebrook, NJ) was used for all enzyme assays. The methods for the specific enzyme measurements were as follows:

(i) Cytochrome C oxidase. The oxidation of fully reduced cytochrome C (50 µM) by the sample in 50 mM imidazole buffer (pH 7.5) at $\lambda = 550$ nm was measured by the method as described by Stuart and Ballantyne (10).

(ii) Glucose 6-phosphatase. The method used to measure the activity of the sample was modified from that of Aronson and Touster (11). The assay conditions were as follows: 50 mM imidazole (pH 6.5), 2 mM NAD, 20 mM glucose-6-phosphate, 9.6 units glutamate dehydrogenase, and 0.2 units malate dehydrogenase. Phosphorus production after 1 h incubation was measured at $\lambda = 820$ nm.

(iii) Na^+,K^+-ATPase. The method used to measure the activity of the sample was modified from that of Chen *et al*. (12). The sample was suspended in an environment of: 50 mM imidazole buffer (pH 7.5), 80 mM NaCl, 20 mM KCl, 6 mM $MgCl_2$, 1.5 mM EGTA, 10 mM NaF, and 3 mM ATP. Phosphorus production after 1 h of incubation was measured at $\lambda =$ 820 nm. Background ATPase activity was measured by comparing the rates of the enzyme in the presence to that in the absence of ouabain (10 mM). As ouabain acts to inhibit Na^+,K^+-ATPase, the difference in activity between measurement with and without ouabian equals the activity of Na^+,K^+-ATPase.

(iv) Peroxidase. Activity of the sample in 50 mM imidazole buffer (pH 7.5) was measured after the addition of 3 mM H_2O_2 at $\lambda = 240$ nm (10).

Protein determination. Protein content was determined by the method of Bradford (13) using bovine serum albumin as a standard. Mitochondrial and tissue protein was determined by measuring the difference between the protein concentration in the isolation medium and the mitochondria suspension.

Chemicals. The lipid standard used (Nu-Chek-Prep., Inc., Elysian, MN) was augmented by the addition of menhaden oil extract. Menhaden oil contains fatty acids not found in the commercial standard but which are present in the lipids of most marine and freshwater organisms. Solvents were obtained from Fisher Scientific Ltd. (Whitby, Ontario, Canada) and were of American Chemical Society-certified grade. All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO) and were of the highest purity available.

Statistical analysis. Differences between the percentages of individual phospholipids and fatty acids from phospholipids were tested using a one-factor analysis of variance followed by Bonferroni's *posthoc* test. Statistical comparisons were only made with fatty acids which represented greater than 1.0% of the total. In all cases, the assumptions of parametric tests were verified and a $\log_{10}$ or square root transformation was used when necessary to normalize the data (14).

RESULTS

Characterization of mitochondrial membrane purity. The isolation procedure used resulted in a substantial enrichment of the mitochondrial membrane marker cytochrome-C-oxidase in relation to the other marker enzymes for plasma membrane, peroxisomes, and endoplasmic reticulum (Table 1), indicating little contamination of the mitochondria with these cellular membranes. A second indication that the membranes recovered were not significantly contaminated by other membranes was the almost complete absence of SPH (<1.0%). This phospholipid is found only in trace amounts in the mitochondrial membrane (1%) while it is prevalent in lysosomes, plasma membrane, and Golgi membrane, representing as much as 20, 16, and 8%, respectively, of total phospholipid in these membranes (15). The very low proportion of this phospholipid in these membranes is evidence that there was minimal contamination of the membrane preparations by these organelles in the present study.

Phospholipid composition. The most prevalent phospholipid in the mitochondrial membranes of both acclimation groups of *M. mercenaria* was PE equaling 41–42% of total membrane phospholipid (Table 2). In *C. virginica* PE and PC were the most prevalent phospholipids, equaling a cumulative 75% of membrane phospholipid in both acclimation groups (Table 2). The phospholipid SPH was present in trace amounts in the mitochondrial membranes of both species but in all preparations represented less than 1% of total membrane phospholipid. The proportion of CL increased in *M. mercenaria* with cold acclimation but not in *C. virginica* (Table 2). There was no change in the ratio of bilayer stabilizing- (PC) to bilayer-destabilizing phospholipids (PE, CL) in either of the two species with cold acclimation.

Gill mitochondrial phospholipid fatty acids. Polyunsaturated fatty acids (PUFA) were the predominant fatty acids in the mitochondrial membranes of both species from either acclimation group (Table 3), resulting from the high proportions of 22:2n-6 and 22:6n-3 in the membranes of *M. mercenaria* and 20:5n-3 and 22:6n-3 in *C. virginica* (Table 3). The saturated fatty acid (SFA) 16:0 represented the largest proportion of individual fatty acids in the membranes of both species (Table 3). The only effect of cold acclimation on the cumulative fatty profile of either species was a decrease in 22:4n-6 in *M. mercenaria* by 33.6% (Table 3).

Acclimation of *M. mercenaria* to −1°C caused alteration in the fatty acid composition of mitochondrial membrane PE, PC, and CL while PE, CL, PS, and PI were altered in *C. virginica*. In mitochondrial membrane PE of both species, PUFA were the dominant class of fatty acid constituting from 67.0–71.4% of the fatty acids in this phospholipid in both species (Table 4). With cold acclimation there was a decrease in the n-6 PUFA content in PE from the mitochondrial membranes of *M. mercenaria.* SFA in PE were found to increase with cold acclimation in *M. mercenaria* caused by a compositional increase in the fatty acid 16:0 (Fig. 1). In *C. virginica* there was an increase in n-3 PUFA content and unsaturation index in PE caused by increased proportions of the fatty acid 22:6n-3 (Fig. 1).

PUFA were the most prevalent fatty acids in mitochondrial membrane PC in both species ranging from 52.5–60.3% (Table 4). Cold acclimation of *M. mercenaria* increased the proportion of monounsaturated fatty acids (monoenes) due to an increase in the fatty acid 20:1 (Fig. 2); 22:4n-6 was found to decrease in this phospholipid. The fatty acid composition of PC from the mitochondrial membranes of *C. virginica* was not affected by cold acclimation.

TABLE 1
Assessment of Purity (fold purification) of Mitochondrial Membranes from Gill Tissue of *Crassostrea virginica* and *Mercenaria mercenaria* Acclimated to Either –1 or 12°C[a]

	C. virginica		*M. mercenaria*	
	–1°C	12°C	–1°C	12°C
Na^+,K^+-ATPase	0.054 ± 0.016	0.041 ± 0.017	0.069 ± 0.019	0.062 ± 0.015
Cytochrome C oxidase	24.84 ± 3.01	21.22 ± 4.56	30.09 ± 7.11	27.72 ± 4.35
Peroxidase	0.059 ± 0.007	0.046 ± 0.015	0.038 ± 0.012	0.030 ± 0.008
Glucose 6-phosphatase	0.29 ± 0.044	0.27 ± 0.076	0.16 ± 0.037	0.11 ± 0.046

[a]Values are means ± SE for n = 4. Fold purification = (activity in mitochondrial pellet/mg protein)/(activity in homogenate/mg protein).

TABLE 2
Percentage by mol of Phospholipids in Gill Mitochondria from *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either –1.0 or 12.0°C[a]

	M. mercenaria		*C. virginica*	
Phospholipid	–1.0 ± 0.5°C (*n* = 8)	12.0 ± 0.7°C (*n* = 8)	–1.0 ± 0.5°C (*n* = 8)	12.0 ± 0.7°C (*n* = 8)
Phosphatidylethanolamine	40.60 ± 1.22	41.80 ± 1.02	30.70 ± 0.59	32.58 ± 2.01
Phosphatidylcholine	20.11 ± 0.82	23.08 ± 1.09	44.10 ± 0.78	42.41 ± 1.92
Cardiolipin[b]	4.92 ± 1.42	1.70 ± 0.22	1.80 ± 0.39	3.14 ± 0.73
Phosphatidylserine	14.85 ± 1.12	12.77 ± 1.60	9.58 ± 0.58	8.61 ± 1.32
Phosphatidylinositol	19.50 ± 0.76	20.62 ± 0.84	13.70 ± 0.57	13.36 ± 0.73
PC/(PE + CL)[c]	0.44 ± 0.021	0.54 ± 0.030	1.35 ± 0.04	1.21 ± 0.15

[a]Values are presented as means ± SEM.
[b]Statistically significant difference between *M. mercenaria* acclimated to –1.0 and 12.0°C.
[c]PC = phosphatidylcholine, PE = phosphatidylethanolamine, CL = cardiolipin.

TABLE 3
Cumulative Percentages of Individual Fatty Acids in Gill Mitochondria from *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either –1.0 or 12.0°C[a]

	M. mercenaria		*C. virginica*	
Fatty acid	–1.0 ± 0.5°C (*n* = 8)	12.0 ± 0.7°C (*n* = 8)	–1.0 ± 0.5°C (*n* = 8)	12.0 ± 0.7°C (*n* = 8)
14:0	0.4 ± 0.1	0.2 ± 0.1	1.1 ± 0.1	0.9 ± 0.1
14:1	0.5 ± 0.2	0.3 ± 0.2	0.6 ± 0.2	0.7 ± 0.4
16:0	22.6 ± 0.8	23.9 ± 1.6	20.6 ± 0.5	20.5 ± 1.0
16:1	4.6 ± 0.8	6.6 ± 0.7	3.7 ± 0.3	5.0 ± 0.5
18:0	8.1 ± 1.5	6.5 ± 0.4	5.2 ± 0.2	5.2 ± 0.3
18:1n-9	3.9 ± 0.4	3.1 ± 0.4	6.2 ± 0.3	6.1 ± 0.5
18:2n-6	n.d.[b]	n.d.	n.d.	n.d.
18:3n-3	1.0 ± 0.2	0.9 ± 0.3	1.0 ± 0.1	1.6 ± 0.3
18:4n-3	0.9 ± 0.3	0.4 ± 0.2	0.1 ± 0.1c	1.1 ± 0.4
20:0	1.6 ± 0.2	1.5 ± 0.2	1.7 ± 0.2	2.0 ± 0.3
20:1	7.3 ± 0.6	6.6 ± 0.7	10.6 ± 0.5	10.2 ± 0.9
20:2n-6	0.9 ± 0.1	1.0 ± 0.1	0.1 ± 0.02[c]	0.1 ± 0.03[c]
20:3n-6	2.4 ± 0.1	3.3 ± 0.2	5.2 ± 0.4	5.2 ± 0.3
20:4n-6	1.6 ± 0.4	0.7 ± 0.1	1.6 ± 0.3	1.0 ± 0.2
20:3n-3	0.2 ± 0.1[c]	n.d.	0.1 ± 0.02[c]	0.2 ± 0.2[c]
20:4n-3	0.2 ± 0.1	0.1 ± 0.1[c]	0.3 ± 0.1	0.2 ± 0.1[c]
20:5n-3	6.7 ± 0.5	6.2 ± 0.5	13.4 ± 0.3	12.0 ± 0.6
22:0	0.3 ± 0.1	n.d.	0.4 ± 0.2	0.4 ± 0.3[c]
22:1*iso*	0.1 ± 0.1[c]	0.1 ± 0.2[c]	0.02 ± 0.02[c]	0.2 ± 0.1
22:2n-6	14.7 ± 0.6	15.2 ± 1.2	8.2 ± 0.2	9.9 ± 0.6
23:0	1.1 ± 0.1	1.8± 0.1	0.5 ± 0.03	0.6 ± 0.1
22:4n-6	3.4 ± 0.1	4.5 ± 0.3[d]	1.2 ± 0.1	1.2 ± 0.1
22:5n-6	n.d.	n.d.	n.d.	n.d.
22:5n-3	4.0 ± 0.3	3.4 ± 0.3	2.0 ± 0.1	2.3 ± 0.4
22:6n-3	13.1 ± 0.8	13.7 ± 0.8	16.3 ± 0.7	13.7 ± 0.5
24:0	n.d.	n.d.	n.d.	n.d.
24:1	n.d.	n.d.	n.d.	n.d.
Total	100	100	100	100
Saturated fatty acids	34.3 ± 1.3	33.9 ± 1.2	29.5 ±0.7	29.7 ± 0.9
Monounsaturated fatty acids	16.5 ± 1.1	16.7 ± 0.9	21.1 ± 0.8	22.3 ± 1.5
Polyunsaturated fatty acids	49.2 ± 1.1	49.4 ± 1.4	49.5 ± 0.9	48.0 ± 1.1
n-3 Polyunsaturated fatty acids	26.2 ± 1.0	24.8 ± 1.1	33.2 ± 0.8	30.7 ± 0.6
n-6 Polyunsaturated fatty acids	23.0 ± 0.5	24.7 ± 1.4	16.3 ± 0.5	17.3 ± 0.8
n-3/n-6 Polyunsaturated fatty acids	1.1 ± 0.1	1.0 ± 0.1	2.0 ± 0.1	1.8 ± 0.1
Unsaturation index[e]	215.0 ± 5.8	214.5 ± 6.1	243.4 ± 4.3	229.3 ± 3.7

[a]Values are presented as means ± SEM.
[b]Not detectable.
[c]Three or more values expressed in mean were not detectable, therefore included as zeroes.
[d]Statistically significant difference between treatment groups of the species in this column.
[e]Unsaturation index = $\sum m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.

TABLE 4
Percentages of Fatty Acid Classes in the Membrane Phospholipids from the Gill Mitochondria of *Mercenaria mercenaria* and *Crassostrea virginica* Acclimated to Either 12.0 or –1.0°C[a]

		PE		PC		CL		PS		PI	
Species	Class	12°C	–1°C	12°C	–1°C	12°C	–1°C	12°C	–1°C	12°C	–1°C
M. mercenaria	SFA	11.7 ± 0.6[b]	16.0 ± 0.7	24.4 ± 1.8	20.3 ± 1.1	32.1 ± 8.7	39.5 ± 4.6	44.9 ± 8.6	28.8 ± 3.3	86.9 ± 2.3	90.9 ± 1.5
(*n* = 8)	Monoenes	18.5 ± 2.1	14.4 ± 1.7	15.3 ± 1.1[b]	21.1 ± 1.9	7.3 ± 3.2[b]	13.5 ± 1.1	19.4 ± 2.8	17.4 ± 3.4	9.2 ± 2.1	5.3 ± 1.5
	PUFA	69.8 ± 1.7	69.5 ± 1.2	60.3 ± 2.2	58.6 ± 1.9	60.6 ± 8.8	47.0 ± 5.0	35.7 ± 9.2	53.8 ± 4.8	3.9 ± 0.4	3.8 ± 0.5
	n-3 PUFA	27.6 ± 1.6	33.7 ± 1.8	44.2 ± 1.9	45.0 ± 1.3	43.0 ± 7.8	35.6 ± 4.1	14.2 ± 4.0	14.5 ± 1.2	1.1 ± 0.2	1.5 ± 0.2
	n-6 PUFA	42.2 ± 1.5[b]	35.9 ± 0.9	16.1 ± 0.8	13.6 ± 1.0	17.6 ± 5.1[b]	11.4 ± 1.0	21.5 ± 8.2	39.3 ± 5.3	2.8 ± 0.3	2.4 ± 0.3
	Unsat. ind.[c]	273.0 ± 7.3	288.0 ± 8.2	308.4 ± 10.5	301.7 ± 8.1	289.6 ± 42.2	238.4 ± 24.5	148.9 ± 29.4	181.9 ± 6.8	23.0 ± 3.1	19.4 ± 2.1
C. virginica	SFA	17.4 ± 0.8	16.3 ± 0.6	24.8 ± 1.0	26.1 ± 0.8	24.8 ± 1.8	26.2 ± 4.0	28.5 ± 1.4	29.2 ± 1.5	83.9 ± 4.2[b]	70.1 ± 1.1
(*n* = 8)	Monoenes	15.6 ± 2.4	12.2 ± 0.7	22.8 ± 1.6	20.8 ± 0.3	26.9 ± 4.3	25.4 ± 7.7	20.9 ± 0.9	20.1 ± 2.8	12.8 ± 3.0	17.6 ± 1.0
	PUFA	67.0 ± 2.8	71.5 ± 1.0	52.5 ± 1.0	53.0 ± 0.9	48.3 ± 4.9	48.4 ± 9.4	50.7 ± 1.5	50.8 ± 2.7	3.3 ± 1.4[b]	12.4 ± 0.9
	n-3 PUFA	36.7 ± 1.3[b]	43.6 ± 1.4	39.3 ± 0.5	40.1 ± 0.7	43.0 ± 4.2	37.3 ± 8.6	15.3 ± 0.9	17.4 ± 0.9	2.1 ± 1.2[b]	5.8 ± 0.4
	n-6 PUFA	30.3 ±1.6	27.9 ± 0.8	13.2 ± 0.6	12.9 ± 0.5	5.4 ± 1.3[b]	11.1 ± 1.5	35.3 ± 1.1	33.3 ± 2.2	1.2 ± 0.3[b]	6.6 ± 0.6
	Unsat. ind.[c]	286.2 ± 8.3[b]	321.4 ± 7.4	269.8 ± 4.8	275.0 ± 4.3	286.6 ± 25.6	273.6 ± 50.2	178.5 ± 5.8	187.5 ± 7.0	24.8 ± 7.8[b]	67.7 ± 3.4

[a]Values are presented as means ± SEM. PS, phosphatidylserine; PI, phosphatidylinositol; SFA, saturated fatty acids; monoenes, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids. See Table 2 for other abbreviations.
[b]Statistically significant difference between treatment groups of the species in this column.
[c]Unsaturation index (unsat. ind.) = $\Sigma m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.

In mitochondrial CL, PUFA were the predominant fatty acids in both species, representing from 47.0–60.6% of the fatty acids in this phospholipid (Table 4). Cold acclimation of *M. mercenaria* increased the proportion of monoenes in CL (Table 4) due to increased levels of 18:1n-9 and 20:1 (Fig. 3). Additionally, there was a decrease in n-6 PUFA in this phospholipid (Table 4). Cold acclimation of *C. virginica* caused an increase in the content of n-6 PUFA as well as in 20:5n-3 while the level of the fatty acid 16:1 was found to decrease (Fig. 3).

PUFA were the most prevalent fatty acids in mitochondrial membrane PS in both species, ranging from 35.7–53.8% of total fatty acid content. This phospholipid was not affected by cold acclimation in *M. mercenaria*; however, there was an increase in the content of 20:4n-6 in this mitochondrial membrane phospholipid in *C. virginica* increasing from 0.57 ± 0.16 to 2.48 ± 0.96%.

In mitochondrial membrane PI, SFA were the predominant fatty acids in both species equaling 70–90%. This result is due to the 16:0 content in this phospholipid (Fig. 4). Cold acclimation of *M. mercenaria* did not affect the fatty acid content of this phospholipid. In *C. virginica*, cold acclimation caused a decrease in SFA content due to a decrease in 16:0. Total PUFA content of PI as well as n-3 PUFA content, n-6 PUFA content, and the unsaturation index were found to increase in

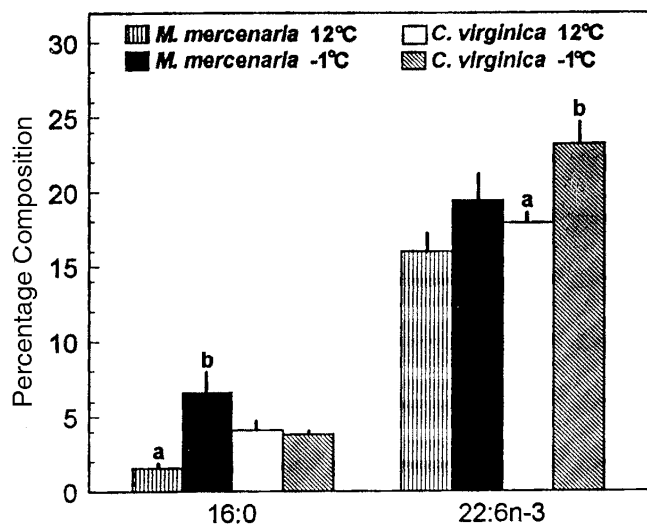

FIG. 1. Percentages of the fatty acids 16:0 and 22:6n-3 in the mitochondrial membrane phospholipid phosphatidylethanolamine of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or –1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

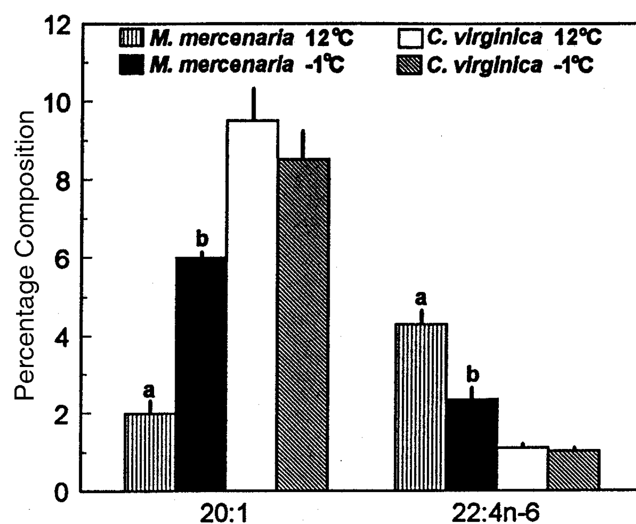

FIG. 2. Percentages of the fatty acids 20:1 and 22:4n-6 in the mitochondrial membrane phospholipid phosphatidylcholine of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or –1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

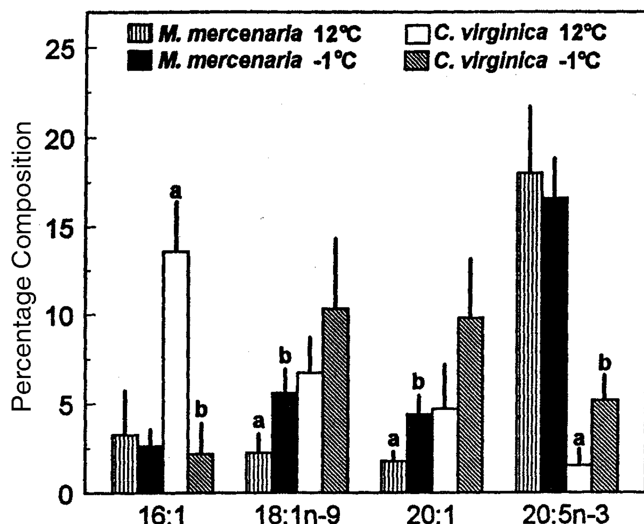

FIG. 3. Percentages of the fatty acids 16:1, 18:1n-9, 20:1, and 20:5n-3 in the mitochondrial membrane phospholipid cardiolipin of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

C. virginica with cold acclimation (Table 4). These results were due to increases in the fatty acids 20:2n-6 and 20:5n-3 (Fig. 4). The monoene 20:1 was also found to increase with cold acclimation in this phospholipid with cold acclimation (Fig. 4).

Fatty acid composition of krill. SFA were the most abundant fatty acids in the total lipid equaling 52%, PUFA composed primarily of 20:5n-3 and 22:6n-3 represented 32% of total fatty acid content (Table 5).

Mitochondrial protein. No change occurred in the concentration of mitochondrial protein with cold acclimation in either of the two species. In *M. mercenaria* mitochondrial protein was 6.43 ± 0.81 mg/g gill in animals acclimated to 12°C and 7.02 ± 0.92 mg/g gill in animals acclimated to −1°C. In *C. virginica*, mitochondrial protein was 10.14 ± 1.37 mg/g gill and 11.03 ± 1.52 mg/g gill in animals acclimated to 12 and −1°C, respectively. All protein values presented are from an *n* of 4.

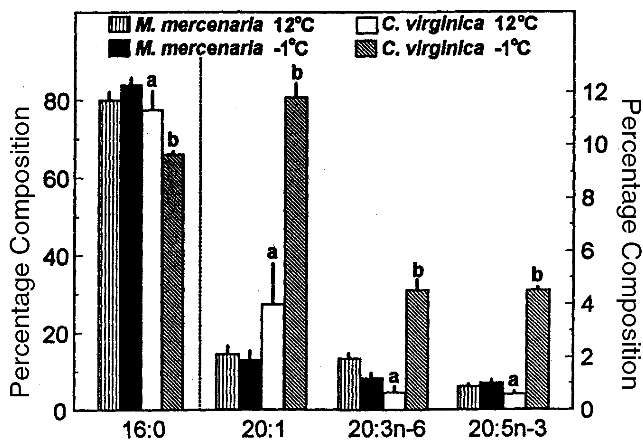

FIG. 4. Percentages of the fatty acids 16:0, 20:1, 20:3n-6, and 20:5n-3 in the mitochondrial membrane phospholipid phosphatidylinositol of the bivalve mollusks, quahog clam, *Mercenaria mercenaria,* and American oyster, *Crassostrea virginica,* acclimated to either 12 or −1°C. Values, within species, are statistically different if they are marked with the letters a and b. Values presented as means ± SEM.

TABLE 5
Percentages of Individual Fatty Acids in the Total Lipids Extracted from the Frozen Krill Used to Feed Both Species of Mollusk During the Thermal Acclimation Study

Fatty acid	mol%
14:0	13.70
14:1	0.97
16:0	22.08
16:1	8.86
18:0	16.00
18:1n-9	4.66
18:3n-3	1.27
18:4n-3	3.24
20:0	0.02
20:1	1.33
20:3n-3	0.07
20:4n-3	0.21
20:5n-3	17.68
22:0	0.068
22:1	0.77
22:2n-6	0.51
Saturated fatty acids	52.00
Monounsaturated fatty acids	16.84
Polyunsaturated fatty acids	31.38
n-3 Polyunsaturated fatty acids	30.7
n-6 Polyunsaturated fatty acids	0.67
n-3/n-6	45.57
Unsaturation index[a]	173.05

[a]Unsaturation index = $\sum m_i \cdot n_i$, where m_i is the mole percentage and n_i is the number of C–C double bonds of the fatty acid *i*.

DISCUSSION

Many of the observed changes in the composition of the mitochondrial membranes in the marine mollusks in the present study differ from what was previously reported in other ectothermic species during thermal acclimation. Previous laboratory studies demonstrated that the ratio of bilayer-stabilizing to bilayer-destabilizing lipids decreases in the cellular membranes of ectothermic animals with cold acclimation (16–18). As in other cellular membranes, PC stabilizes the bilayer in mitochondrial membranes as it favors the formation of a laminar bilayer, while PE destabilizes the bilayer by keeping the membrane close to the phase transition between laminar and hexagonal (H_{II}) phase conformations (16,19). Mitochondria contain a second bilayer-destabilizing phospholipid, CL. This phospholipid adopts the H_{II} phase conformation in the presence of Ca^{2+} (20,21) and the membrane protein cytochrome C oxidase (22), both of which are associated with the inner mitochondrial membrane. Cold acclimation of either species of mollusk in the present study did not alter the ratio of PC/(PE + CL), indicating that alterations of membrane-stabilizing to

-destabilizing lipids are not used by either of these animals to maintain membrane function at low temperatures.

The increase in the proportion of CL in the mitochondrial membranes of *M. mercenaria* with cold acclimation represents an acclimatory response to lowered environmental temperature. No change occurred in the ratio of PC/(PE + CL), therefore the likelihood that this alteration would affect bilayer stability is slight. Since CL is a required component for the function of several mitochondrial proteins (15), the observed changes may relate to shifts in metabolic organization. Previous studies demonstrated that the proportion of PUFA increases at the expense of SFA in the tissue lipids upon the acclimation of ectothermic animals to low temperatures (1,16,18,23–25). Compared to SFA, PUFA have lower melting points and, when included in a membrane, disrupt the monolayer due to their permanently "kinked" acyl chain (16,23,26). These two characteristics of PUFA increase the fluidity of a biological membrane and, it is suggested, enable the membranes of cold-acclimated animals to remain fluid and functioning (1,16,23,). In the present study no increase of PUFA took place in the mitochondrial membranes of *M. mercenaria* with cold acclimation, while the fatty acid 22:4n-6 actually decreased, suggesting that this species of marine mollusk is not utilizing PUFA to maintain membrane function at low temperatures and therefore may be utilizing a different strategy. Only in *C. virginica* were PUFA found to increase with cold acclimation, with n-3 PUFA increasing in PE, n-6 PUFA increasing in CL, and total PUFA increasing in PI. This response indicates that these animals are actively manipulating membrane composition and that PUFA may be important to membrane function at low temperatures at least in this mollusk.

While PUFA levels in the membranes of *M. mercenaria* did not increase with cold acclimation, monoene levels did. The fatty acid 18:1n-9 increased in CL while 20:1 was found to increase in PC and CL. Monoenes also were demonstrated to increase in tissue phospholipids and cellular membranes of other ectothermic animals with cold acclimation (18,25,27, 28). However, in these previous studies, PUFA increased in parallel with monoenes. Only in PI from the gills of *C. virginica* in the present study was such a result noted. An increase in monoenes in animals acclimated to cold temperatures may be related to the maintenance of membrane fluidity. The presence of the monoene 18:1 in the *sn*-1 position of PE of cold-acclimated or adapted fish was suggested to increase the influence of that phospholipid on the membrane (27). This would act to increase the fluidity of the membrane and therefore help maintain membrane function at low temperatures. The results of the present study suggest that *M. mercenaria* is increasing monoene content, not PUFA, to help maintain membrane function at low temperatures.

In both marine bivalves, the SFA 16:0 constitutes 66–84% of the fatty acids in PI. High levels of 16:0 were previously demonstrated to occur in gill mitochondrial membranes of *C. virginica* (8); therefore, the levels reported in the current study are not unexpected. The increase in 16:0 found in PE from the membranes of cold-acclimated *M. mercenaria* would act to stabilize the membrane at low temperature. The significance of this particular finding is not known at this time.

Limited study has been made of the metabolism of dietary fatty acids in bivalve mollusks. It was demonstrated that the oyster, *C. virginica*, and the yellow clam, *Mesodesma matroides*, are capable of elongation and desaturation of fatty acids (29–31). The fatty acid composition of the diet (krill) fed to these animals throughout the experiment has a higher proportion of SFA and lower proportion of PUFA and monoenes than the fatty acid composition of the membrane phospholipids of both species used in this study. Both mollusks therefore accumulated these fatty acids in their membranes in a higher proportion to what is found in the diet. The long-chain PUFA 22:6n-3 is lower in the krill-based diet than in the mitochondrial membranes. Long-chain n-3 fatty acids such as 22:6n-3 can only be made from other shorter n-3 fatty acids which are all synthesized from the precursor 18:3n-3. This fatty acid must be obtained from the diet. The diet used contained sufficient concentrations of all precursors for the synthesis of longer-chained PUFA.

The ratio of n-3 to n-6 fatty acids (n-3/n-6) of the mitochondrial membrane phospholipids of the marine mollusks in the present study is lower compared to those of previously published values for marine teleost fish. This ratio is 1.0 in *M. mercenaria* and 2.0 in *C. virginica* at 12°C, while it is 16 in the winter flounder *Pseudopleuronectes americanus*, acclimated to 10°C (32), and 76 in Arctic char, *Salvelinus alpinus*, caught from 4°C seawater (33). This is due to the lower levels of n-6 fatty acids in the phospholipids in the marine teleosts compared to that of the marine bivalves. Within wild populations of marine organisms, low levels of n-6 fatty acids are common when compared to similar freshwater species. This difference was attributed to fatty acid precursors available in the diet (34). However, the diet fed the mollusks throughout the current study had an n-3/n-6 ratio of 45. This is much higher than that of the marine bivalves due to the low content of n-6 fatty acids This indicates that n-6 fatty acids are being concentrated in the mitochondrial membrane, suggesting a functional significance of these fatty acids in the membrane.

The seasonal temperatures experienced by most temperate zone marine organisms in northern latitudes range from −1.5°C in winter to 12°C in summer. The large alterations of membrane phospholipid composition seen in previous thermal acclimation studies were in response to correspondingly large changes in environmental temperature for example: 5 vs. 30°C (35), 7 vs. 27°C (24), and 10 vs. 32°C (25). The results of the present study indicate that during thermal shifts such as those occurring during seasonal changes in environmental temperature changes in membrane phospholipids do occur in mollusks.

Unlike thermal acclimation in fishes and some other organisms, the ratio of membrane-stabilizing phospholipids to membrane-destabilizing phospholipids was not altered. Most of the acclimation response is due to changes in phospholipid fatty acids such as alterations in PUFA content (*C. virginica*) or monoene levels (*M. mercenaria*).

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Biosynthesis and Localization of Phosphatidyl-*scyllo*-Inositol in Barley Aleurone Cells

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ABSTRACT: A novel isomer of phosphatidylinositol (PI), phosphatidyl-*scyllo*-inositol, was characterized in the aleurone cells of barley seeds. In this investigation, the subcellular localization of *scyllo*-PI and the relative rates of biosynthesis and accumulation of [^{32}P]phosphoric acid ([^{32}Pi])-labeled *scyllo*- and *myo*-phosphoinositides in the plasma membrane and intracellular membrane pools were investigated. About 25% of the [^{32}Pi]-labeled phospholipids were present in plasma membrane and 75% in intracellular membranes. Incorporation of [^{32}Pi] into *scyllo*-PI was greater than into *myo*-PI in both the plasma membranes and intracellular membranes at all time points investigated, thus suggesting a higher rate of biosynthesis; however, the data do not preclude reduced breakdown of labeled *scyllo*-PI as a contributing factor. *In vitro* studies were conducted to investigate the presence of cytidinediphosphate diacylglycerol (CDP-DG):*scyllo*-inositol 3-phosphatidyltransferase (*scyllo*-PI synthase) and to optimize enzymatic activity. The inclusion of nonionic detergents (Brij 58 and Triton X-100) effected significant enhancement in the biosynthesis of *scyllo*-PI, whereas anionic, cationic, and zwitterionic detergents had little or no effect. This is the first evidence for CDP-DG:*scyllo*-inositol 3-phosphatidyltransferase activity.

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Phosphoinositides, a group of negatively-charged phospholipids, are important constituents of cell membranes. The critical role that *myo*-inositol-containing phosphoinositides play in signal transduction is well established. Plasma membrane-bound *myo*-phosphatidylinositolbisphosphate undergoes hormone-triggered hydrolysis to generate two second messengers, diacylglycerol and *myo*-inositol(1,4,5)trisphosphate (1). Numerous investigations aimed at understanding the role of phosphoinositides in plant cells have been published (2–4). Spatially and metabolically distinct pools of phosphoinositides occur in cells (1,2); therefore, a complete and accurate understanding of the role of phosphoinositides requires information on the biosynthesis, transport, and accumulation of phospholipids in cell membranes.

During seed development, the outer endosperm cells differentiate into functionally and morphologically distinct cells called the aleurone cells (5). Mature aleurone cells of common cereals, including barley, are packed with protein bodies, phytin grains, and lipid bodies. During germination, the aleurone layer plays a major role in the mobilization of reserves to the developing seedling by its involvement in the hormone-controlled synthesis and secretion of hydrolytic enzymes including α-amylase, β-galactosidase, phytase, and proteases. Rapid proliferation of the protein-synthesizing apparatus and intracellular membranes also occurs soon after imbibition (5,6). The ready availability of viable aleurone layers has made this a good model system for the study of plant hormone action (5–7).

We have described our efforts to characterize phosphoinositides in the aleurone tissue of barley (8,9), including the structural characterization of a novel phosphoinositide, *scyllo*-phosphatidylinositol (PI), in barley seeds (10,11). We are interested in the biosynthesis and accumulation of *myo*- and *scyllo*-phosphoinositides in the plasma membrane and intracellular membrane pools and the incorporation of [^{32}P]phosphoric acid ([^{32}Pi]) into these phosphoinositides. Toward this end, our first step (12) was to investigate the fractionation of membrane vesicles from barley aleurone cells, starting with the isolation and assessment of purity of plasma membrane and intracellular membrane vesicles using the aqueous polymer two-phase method (13,14). Whereas [^{32}Pi]-labeled monophosphorylated PI (PIP) and bisphosphorylated PI were clearly evident only in the plasma membrane (if present in intracellular membranes, they occur in undetectably low amounts), *myo*-PI and *scyllo*-PI are present in the intracellular membranes as well as in the plasma membrane. In this paper, results on the biosynthesis of *scyllo*- and *myo*-phosphoinositides by *in vivo* and *in vitro* studies are presented. Data on *in vivo* [^{32}Pi] labeling of the spatially distinct pools of phosphoinositides in plasma membrane and intracellular membranes are presented. Finally, the consequence of these results on signal transduction studies are discussed.

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Abbreviations: CDP-DG, cytidinediphosphate diacylglycerol; HPTLC, high-performance thin-layer chromatography; PI, phosphatidylinositol; [^{32}Pi], ^{32}P-labeled phosphoric acid; PIP, monophosphorylated PI.

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EXPERIMENTAL PROCEDURE

***Plant material*.** Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1991 harvest) were purchased from Seed Technology, Department of Agronomy and Soil, University of Washington, Pullman, WA.

Labeling with radioactive precursors. Aleurone layers were isolated from barley seeds (15). Aleurone layers were incubated at 25°C in a solution (1 mL/10 layers) containing succinate buffer (20 mM, pH 5), $CaCl_2$ (20 mM), chloramphenicol (30 μM), and [^{32}Pi], as indicated in figure legends; the [^{32}Pi], dissolved in 0.02 M HCl (DuPont/NEN, Boston, MA), was neutralized just before use with 0.02 M NaOH (9). To double-label with [^{32}Pi] and ^{14}C, the layers were incubated with [^{32}Pi] (20 μCi per five layers) and *myo*-[^{14}C(U)]inositol (from American Radiolabeled Chemicals Inc., St. Louis, MO; 2.5 μCi per five layers). After 20 to 26 h the layers were freed of excess isotope by washing three times with media without isotope (8). To separate the plasma membrane and intracellular membrane vesicles, the aleurone layers were homogenized in a cold mortar and pestle with 25 mL of homogenization buffer containing sucrose (0.5 M), Hepes-KOH (50 mM, pH 7.5), ascorbic acid (5 mM), dithiothreitol (1 mM), and polyvinylpyrrolidone (0.6%, wt/vol) and the separation was carried out as described previously (13,14). The concentration of both polymers, Dextran T500 and polyethyleneglycol 3350, in the aqueous polymer two-phase method was 6.2%.

Extraction and separation of phospholipids. Phospholipids were extracted from aleurone layers or from membrane pellets by a modified acidic Bligh-Dyer method (10,11). The washed aleurone layers were homogenized in a mortar and pestle with a small amount of sand and water (1 mL), and the slurry was transferred to a test tube. With membrane pellets, water (1 mL) was added and mixed well with a vortex mixer. To the suspension was added 3 mL of CH_3OH, 2.5 mL of $CHCl_3/CH_3OH$ (2:1, vol/vol), 0.75 mL of 2.4 N HCl, and 2.1 mL of $CHCl_3$. The solution was vortex-mixed and centrifuged, and the lower organic phase was removed. The aqueous phase was reextracted with 1.7 mL of $CHCl_3$. The combined organic extract was washed twice with 0.75 mL of 2.4 M HCl and 3.0 mL of CH_3OH/H_2O (1:1, vol/vol). Unlabeled phospholipids (0.075 mg) from soybean were added to the organic extract and separated on a high-performance thin-layer chromatography (HPTLC) plate [using a modified Folch solvent system, $CHCl_3/CH_3OH$/aqueous NH_4OH, 28–30% of NH_3 in H_2O, w/w (90:90:20, by vol)] as described previously (10). In some experiments the phospholipids were converted to glycerophospholipids by deacylation using methylamine and then separated by high-voltage paper electrophoresis or high-performance liquid chromatography (10).

Isolation of PI synthase. Barley seeds (300–400) were imbibed for 60–63 h at 30°C (15). The seeds were homogenized in a cold mortar and pestle in Tris/MES [2-(*N*-morpholino)ethanesulfonic acid] buffer (pH 7.5) containing Tris (25 mM), sucrose (0.30 mM), KCl (10 mM), $MgCl_2$ (1.0 mM), dithiothreitol (10 mM), and polyvinylpyr-rolidone (6.0%). The homogenate was centrifuged for 10 min at 750 × *g*. The supernatant was removed and centrifuged at 50,000 × *g* for 30 min. (Initial studies were conducted with 18,000 × *g* and 100,000 × *g* pellets, and the 50,000 × *g* pellet was used for all subsequent experiments.) The microsomal pellet was suspended in Tris/HCl buffer (pH 8.0) containing Tris (40 mM), sucrose (0.46 mM), and KCl (15 mM). A homogeneous suspension was obtained with difficulty, after repeated efforts to break up the pellet with a flattened glass rod.

PI synthase assay. PI synthase activity was assayed at 30°C in a solution containing 20 mM Tris/HCl (pH 8.0), 0.23 M sucrose, 7.5 mM KCl, 1 mM cytidinediphosphate diacylglycerol (CDP-DG) made from egg phosphatidylcholine (Doosan Serdary Research Laboratory, Englewood Cliffs, NJ), 5mM $MnCl_2$, 1 mM *myo*-[2-^{3}H(N)]inositol (40,000 to 200,000 cpm per assay) or *scyllo*-[^{3}H(N)]inositol (100,000 to 200,000 cpm per assay (American Radiolabeled Chemicals Inc.), and 0.03% Brij 58 (polyoxyethylene 20 cetyl ether) or 0.2% Triton X-100 in a total volume of 0.4 mL. CDP-DG was added to the solution containing Tris/HCl, sucrose, and KCl and sonicated for 30–60 s. After the solution appeared homogeneous, inositol and $MnCl_2$ were added. The reaction was initiated by addition of the PI synthase enzyme (150–300 μg protein) and was terminated after 3 h by the addition of 3.3 mL $CHCl_3/CH_3OH/H_2O$ (1:2:0.3, by vol). The phospholipids were extracted by the acidic Bligh-Dyer method (10).

RESULTS AND DISCUSSION

In vivo *[^{32}Pi] labeling of plasma membrane and intracellular membrane phosphoinositides.* To monitor the relative rates of incorporation of phosphoinositides in the plasma membrane and intracellular membrane pools, we followed the incorporation of [^{32}Pi] into phospholipids as a function of

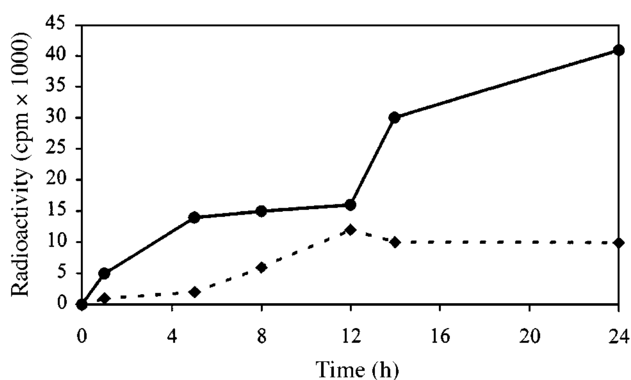


FIG. 1. Incorporation of [^{32}P]phosphoric acid ([^{32}Pi]) into the phospholipids of plasma membranes (◆)and intracellular membranes (●) in barley aleurone layers. Aleurone layers were incubated in media containing 75 μCi [^{32}Pi]/10 layers for the indicated time period. Plasma membranes and intracellular membranes were isolated and phospholipids were extracted from the membrane vesicles. The content of radioactivity in the phospholipid extracts was determined by liquid scintillation counting. This graph represents two independent experiments.

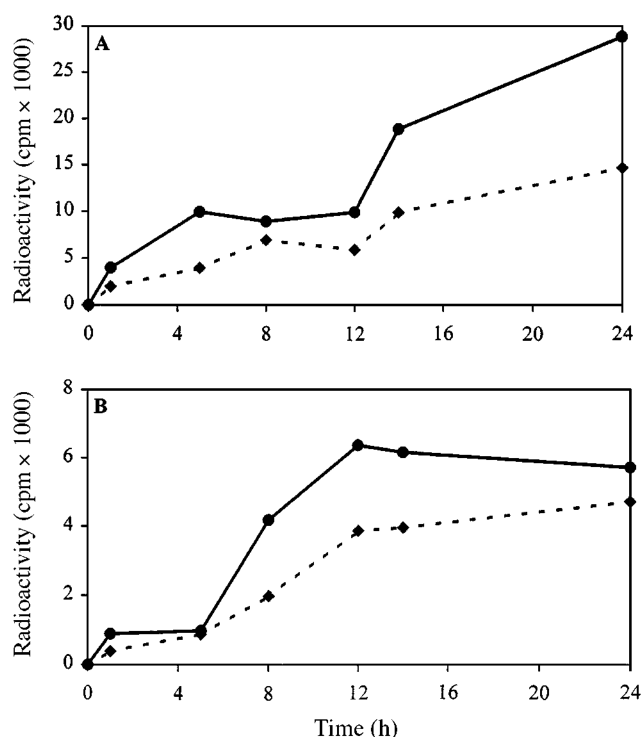


FIG. 2. Incorporation of [^{32}Pi] into *myo*-phosphatidylinositol (PI) and scyllo-PI of (A) intracellular membranes and (B) plasma membranes in barley aleurone layers. Aleurone layers were incubated in media containing 75 µCi [^{32}Pi]/10 layers for the indicated time period. Plasma membranes and intracellular membranes were isolated by aqueous polymer two-phase separation. The phospholipids from membrane vesicles were extracted, deacylated to glycerophospholipids, and separated by high-performance liquid chromatography. The content of radioactivity in the fractions corresponding to *myo*-glycerophosphoinositol (GPI: ◆) and *scyllo*-GPI (●) (7) was determined by liquid scintillation counting.

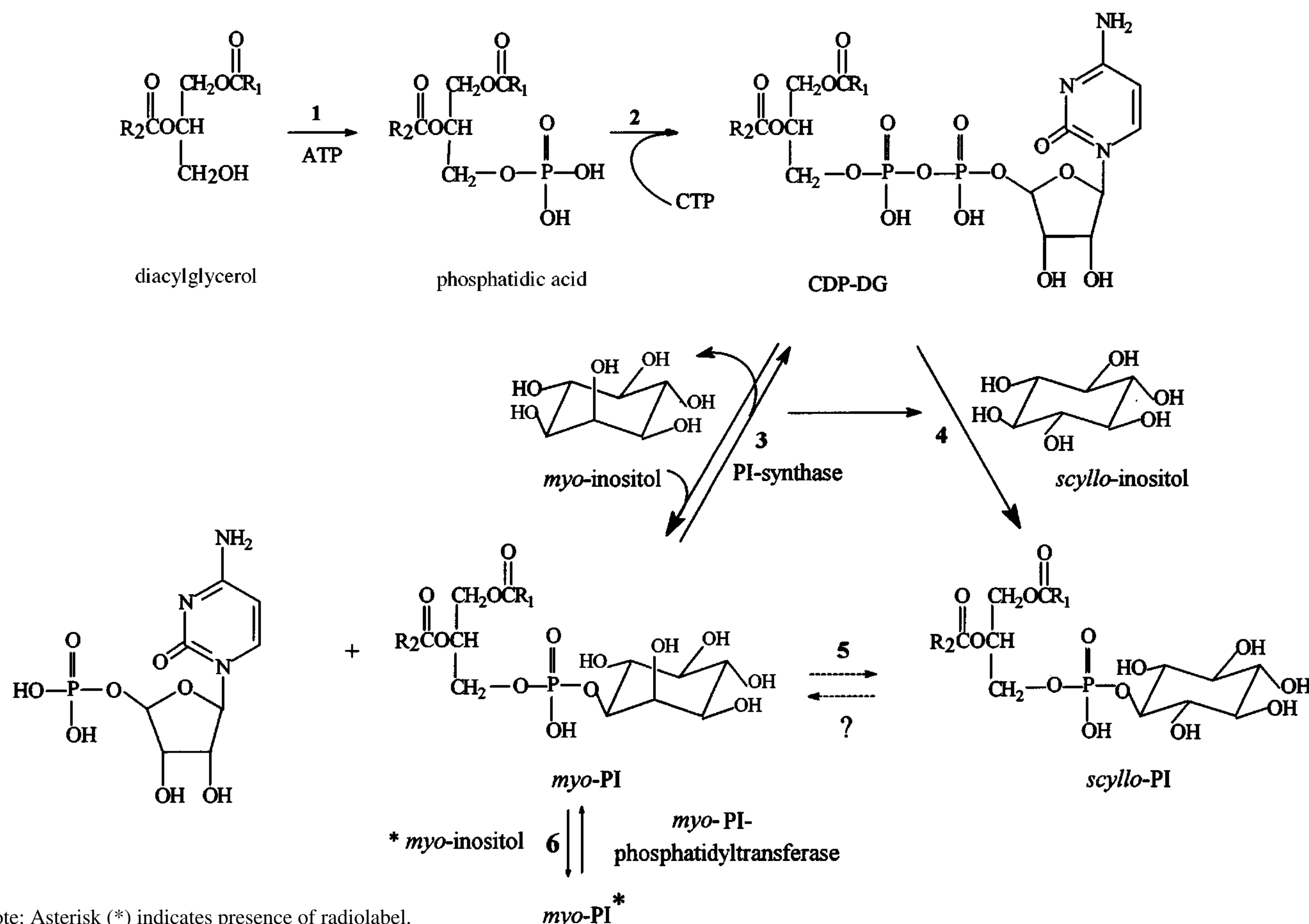
time. Incorporation of [^{32}Pi] into lipid extract increased for the first 24 h (Fig. 1). Figure 1 illustrates that phospholipids in intracellular membranes incorporate radioactivity faster than phospholipids in plasma membranes possibly due to the fact that phospholipids are biosynthesized in the endoplasmic reticulum and subsequently transported to the plasma membrane. After 14 h, about 75% of the radiolabeled cellular phospholipids was present in intracellular membranes and 25% in plasma membranes. [^{32}Pi] incorporation into the phospholipids of the plasma membranes had reached isotopic equilibrium in about 10–12 h, whereas incorporation into intracellular membranes continued to increase. To determine the relative rate of biosyntheses of *scyllo*-PI and *myo*-PI, incorporation of [^{32}Pi] into *scyllo*-PI and *myo*-PI was monitored by extracting phospholipids from plasma membranes and intracellular membranes, converting to glycerolipids, and separating by HPLC. The preferential incorporation of [^{32}Pi] into *scyllo*-PI compared to *myo*-PI in intracellular membranes as well as in plasma membranes was evident as early as 1.5 h and at all subsequent time points studied (Fig. 2A,B). In addition, whereas the rate of biosynthesis of *myo*-PI and *scyllo*-PI in the intracellular membranes continued to increase for 24 h (Fig. 2A), incorporation into both isomers had reached isotopic equilibrium in the plasma membranes in about 14 h (Fig. 2B).

These results indicate that in barley aleurone cells only 25% of the total [^{32}Pi]-labeled phospholipids is present in the plasma membrane. In addition, these data indicate that the times required to label the plasma membrane and intracellular membrane pools to isotopic equilibrium are different. The rapid rate of incorporation of [^{32}Pi] into *scyllo*-PI compared to *myo*-PI, in both plasma membranes and intracellular membranes, at all times points studied could reflect relative rates of turnover. A high turnover rate is characteristic of lipids that play a role in signal transduction, such as *myo*-PIP and *myo*-bisphorylated PI (1,2).

Biosyntheses of myo- *and* scyllo-*PI:* in vivo *studies*. The biosynthesis of *myo*-PI occurs *via* CDP-DG (Scheme 1). The reaction of CDP-DG with *myo*-inositol to yield *myo*-PI and cytidine monophosphate is catalyzed by CDP-DG:inositol 3-phosphatidyltransferase (EC 2.7.8.11), also called *myo*-PI synthase. This reaction is localized in the endoplasmic reticulum (16).

An enzyme that catalyzes the exchange of the head group, inositol, or *myo*-PI:*myo*-inositol phosphatidyltransferase (Scheme 1, Step **6**), has been observed in a number of cells including plant cells (16,17). The exchange enzyme is believed to be localized in the endoplasmic reticulum and the Golgi apparatus (16). Two forms of exchange activity, CMP-dependent and CMP-independent, have been characterized (17). It has been suggested that the CMP-dependent exchange activity may be due to the reversal of PI synthase activity (Step **3**) and incorporation of free inositol, whereas the CMP-independent exchange activity is due to the presence of a second enzyme (Step **6**). Recent cloning studies in mammalian cells indicated that a single polypeptide exhibits both the synthase and exchange activities (17), thereby providing evidence that, in mammalian cells, the CMP-dependent exchange activity is due to PI synthase catalyzing the reverse and forward reactions sequentially. The physiological significance of the exchange reaction is unknown. Analogous to the biosynthesis of *myo*-PI, *scyllo*-PI could be produced by the reaction of CDP-DG with *scyllo*-inositol (Step **4**). Alternatively, the head group of *myo*-PI, *myo*-inositol, could be exchanged with *scyllo*-inositol to form *scyllo*-PI (Step **5**).

Our [^{32}Pi] incorporation experiments indicate that preferential incorporation of ^{32}P into *scyllo*-PI compared to *myo*-PI occurred at all time points studied. Increased ^{32}P radioactivity in *scyllo*-PI could be due to the higher rate of biosynthesis of *scyllo*- PI compared to *myo*-PI, slower rate of subsequent metabolic degradation of *scyllo*-PI compared to *myo*-PI, or both. Although subsequent metabolism of *myo*-PI occurs rapidly, we currently have no information on subsequent metabolism of *scyllo*-PI. However, the increased rate of incorporation of [^{32}Pi] at all time points investigated strongly points to a higher rate of biosynthesis of *scyllo*-PI rather than the possibility that *scyllo*-PI was produced from *myo*-PI by the



Note: Asterisk (*) indicates presence of radiolabel.

SCHEME 1

exchange reaction (Step **5**). Evidence in favor of the latter possibility would have suggested a possible cellular role for the CMP-independent exchange enzyme for which there is currently no known biological role. A double-labeling experiment using [^{32}Pi] and *myo*-[^{14}C(U)]inositol was conducted to monitor the incorporation of both the phosphate and the head group inositol moieties. We chose to use *myo*-[^{14}C(U)]inositol rather than *myo*-[2-^{3}H]inositol for the double-labeling experiments to avoid the possibility of losing the ^{3}H at the C-2 position of *myo*-inositol during the isomerization of *myo*-inositol to *scyllo*-inositol, as this involves the epimerization of the C-2 carbon (10,18). Aleurone layers were incubated with [^{32}Pi] and *myo*-[^{14}C(U)]inositol for 24 h at 25°C, and the radiolabeled phospholipids were extracted and separated by HPTLC. The data in Table 1 indicate that, in contrast to the [^{32}Pi] studies, the amount of *myo*-[^{14}C(U)]inositol incorporated into *myo*-PI was much greater (eightfold) than that incorporated into *scyllo*-PI. (The region between *scyllo*-PI and *myo*-PI on the HPTLC plate contained very little radioactivity, about 60 dpm [^{14}C], which is the background value, thus indicating that the radioactivity in *scyllo*-PI is not due to tailing of *myo*-PI.) The low incorpora-

TABLE 1
Incorporation of Radiolabeled Precursors [^{32}Pi] and *myo*-[^{14}C(U)]Inositol into *myo*-PI and *scyllo*-PI[a,b]

Phospholipid	[^{14}C] Content (dpm)	[^{32}P] Content (dpm)	Ratio of [^{14}C]/[^{32}P]
myo-PI	1,059	1,899	0.56
scyllo-PI	132	1,196	0.11

[a]Abbreviations: [^{32}Pi], [^{32}P]phosphoric acid; PI, phosphatidylinositol; dpm, disintegrations per minute.
[b]Barley aleurone layers were incubated with radiolabeled precursors for 24 h.

tion of ^{14}C from *myo*-[^{14}C(U)]inositol into *scyllo*-PI compared to *myo*-PI is probably due to the low rate of conversion of *myo*-inositol to *scyllo*-inositol (18).

Biosynthesis of myo- *and* scyllo-*PI:* in vitro *studies.* To investigate the presence of *scyllo*-PI synthase in aleurone cells, *in vitro* experiments were conducted with microsomal membrane vesicles. Previous research has shown that *myo*-PI synthase enzyme is located in the microsomal pellet, which contains membrane vesicles from endoplasmic reticula, Golgi membranes, and plasma membranes (15). In initial experiments, the presence of *myo*-PI synthase and the formation of *myo*-PI were monitored by using *myo*-[2-^3H(N)]inositol as the precursor and measuring the ^3H content in the organic layer following enzymatic reaction and phospholipid extraction. Unreacted *myo*-[2-^3H(N)]inositol partitions into the aqueous phase and the *myo*-[2-^3H(N)]inositol that is incorporated into phospholipids partitions into the organic phase; consequently, the content of radioactivity in the organic phase is indicative of the extent of conversion. The microsomal pellet isolated after centrifugation of the cellular suspension at 18,000 × *g* did not catalyze the production of *myo*-PI in the presence of detergent whereas the 50,000 × *g* pellet did, thus suggesting that the membrane vesicles containing *myo*-PI synthase were not pelleted at 18,000 × *g*. The 100,000 × *g* microsomal pellet did not affect increased biosynthesis of *myo*-PI compared to the 50,000 × *g* pellet, therefore the 50,000 × *g* pellet was used as the source of enzyme in subsequent experiments. To confirm the formation of *myo*-PI, comigration with standard phospholipids was used to characterize the structure of the labeled product. The ^3H-labeled products were spotted alongside [^{32}Pi]-labeled phospholipids (obtained from an *in vivo* experiment) on an HPTLC plate. The results (Fig. 3, left lane) indicate that the radiolabeled product had the same migratory properties as *myo*-PI thereby providing evidence that *myo*-PI was produced in the *in vitro* experiment *via* the PI-synthase pathway. The presence of radiolabeled product in the PIP region suggests that the microsomal pellet also contains PI kinase that converts PI to PIP.

To detect the presence of *scyllo*-PI synthase (CDP-DG:*scyllo*-inositol transferase) we conducted the experiment with *scyllo*-[2-^3H(N)]inositol. When the *scyllo*-[2-^3H(N)]inositol was checked for purity by paper chromatography, the radiolabeled precursor used contained both *myo*-[^3H]inositol and *scyllo*-[^3H]inositol in tritium ratio of 1:2, respectively). Radioactivity was detected in the organic layer when Brij 58 or Triton X-100 was included in the reaction medium, suggesting that enzymatic conversion of *scyllo*-inositol to phospholipids had occurred. To confirm the formation of *scyllo*-PI, comigration with standard phospholipids was used to obtain structural information of the radiolabeled product. The ^3H-labeled products were spotted alongside [^{32}Pi]-labeled phospholipids (obtained from an *in vivo* experiment) on an HPTLC plate. As illustrated in Figure 3, right lane, radioactivity was detected in regions corresponding to both *scyllo*-PI and *myo*-PI, thereby indicating the presence of *scyllo*-PI synthase in the microsomal pellet.

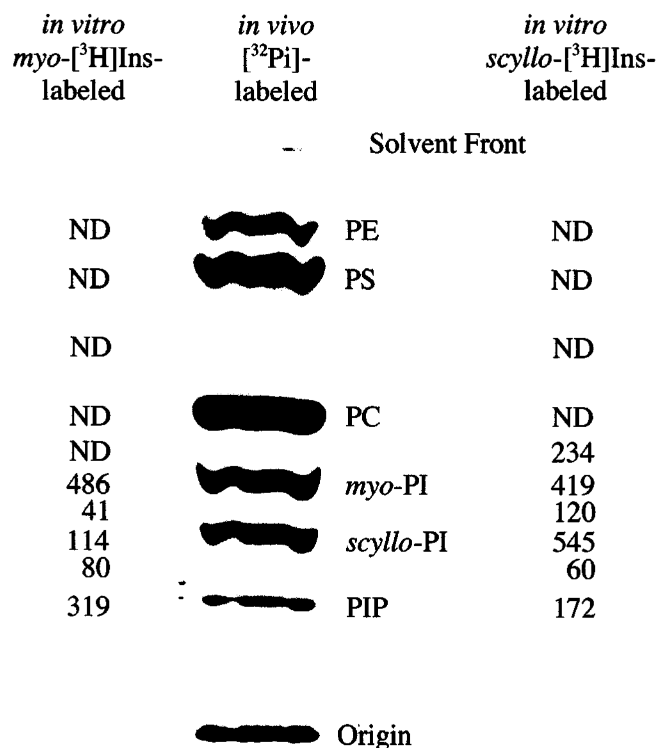


FIG. 3. Separation of *in vivo* [^{32}Pi]-labeled phospholipids (middle lane) alongside *in vitro myo*-[2-^3H(N)]inositol-labeled (left lane) and *scyllo*-[^3H(N)]inositol-labeled (right lane) phospholipids from PI-synthase assay. The *in vitro* assays were incubated with *myo*-[2-^3H(N)]inositol (200,000 cpm) or *scyllo*-[^3H(N)]inositol (400,000 cpm), and the labeled phospholipids were extracted. Aliquots of the [^{32}Pi]- and [^3H]-labeled phospholipids were mixed with carrier phospholipids and separated on a high-performance thin-layer chromatography plate. The *in vivo* [^{32}Pi]-labeled phospholipids (in the middle lane) were localized by autoradiography and silica gel (left and right lanes) corresponding to *myo*- and *scyllo*-PI, PC, and PIP; other regions of the plate were scraped and the [^3H] contents determined by liquid scintillation counting. The numbers in the left and right lanes indicate [^3H] values above background. ND, areas in which radioactivity above background (10–15 cpm) was not detected. These data are representative of two experiments. Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PIP, phosphatidylinositol-monophosphate; PS, phosphtidylserine; Ins, inositol; for other abbreviations see Figures 1 and 2.

Effect of detergent on PI synthase activity. Investigations of membrane-bound enzymes are conducted in the presence of detergents to facilitate solubilization of either substrate or enzymes or both (19). Detergents can be divided into four categories on the basis of charge: cationic, anionic, nonionic, and zwitterionic. The best detergent and the optimal concentrations required for maximal enzymatic activities vary with enzyme and cell type. A number of different types of detergents were included in order to determine the best detergent for the reaction. Deoxycholate, an anionic detergent, cetylpyridinium bromide, a cationic detergent, cholamidopropyldimethylammonio-1-propanesulfonate, a zwitterionic detergent, and two nonionic detergents, Brij 58 and Triton X-100, were included in our experiments. The data (Fig. 4) indicate that, consistent

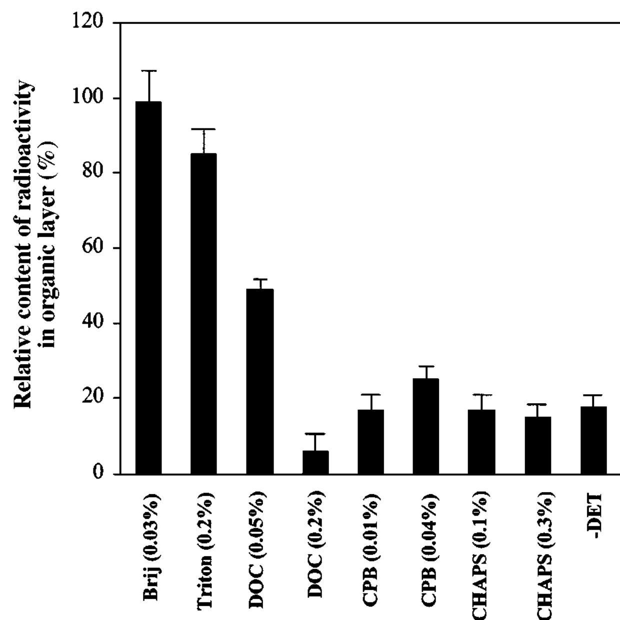


FIG. 4. Effect of various detergents on the biosynthesis of *myo*-PI. Barley seeds (350–400) were imbibed (60 h) and homogenized, and aliquots from the microsomal pellet were added to buffer containing *myo*-[2-^{3}H(N)]inositol (about 100,000 cpm) and various detergents, as indicated, to initiate the PI-synthase reaction. Reactions were terminated after 3 h by the addition of $CHCl_3/CH_3OH/H_2O$ (1:2:0.3, by vol), and the phospholipids were extracted, concentrated, and the content of radioactivity determined by liquid scintillation counting. All experiments was performed in duplicate; error bars represent the standard deviation. The amount of radioactivity at 100% corresponds to about 3,200 cpm. This graph is representative of three independent experiments. Abbreviations: DOC, deoxycholate; CPB, cetylpyridinium bromide; CHAPS, cholamidopropyldimethylammonio-1-propanesulfonate; –DET, no detergent. For other abbreviation see Figure 2.

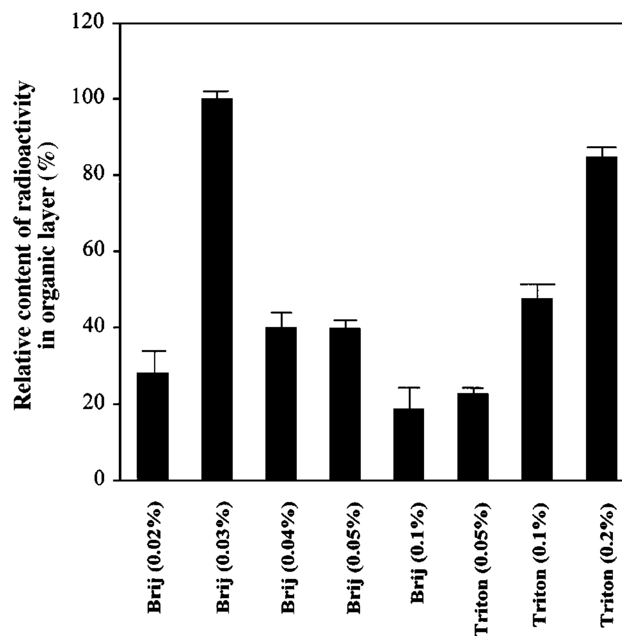


FIG. 5. Effect of Triton X-100 and Brij 58 on the biosynthesis of *myo*-PI. Barley seeds (350–400) were imbibed (60 h) and then homogenized, and aliquots from the microsomal pellet were added to buffer containing *myo*-[2-^{3}H(N)]inositol (about 100,000 cpm) and detergents, as indicated. Reactions were terminated after 3 h by the addition of $CHCl_3/CH_3OH/H_2O$ (1:2:0.3, by vol), and the phospholipids were extracted, concentrated, and the content of radioactivity in the organic phase determined by liquid scintillation counting. All experiments were performed in duplicate; error bars represent the standard deviation. The amount of radioactivity at 100% corresponds to about 4,200 cpm. This graph is representative of three independent experiments.

with previous experiments in soybeans and castor beans (20,21), Brij 58 and Triton X-100 gave the best yield of *myo*-PI. The biosynthesis of *myo*-PI in the presence of Brij 58 increased fivefold compared to the conversion in the absence of detergent. The inclusion of Triton X-100 in the reaction mixture stimulated biosynthesis fourfold compared to the absence of detergent. The inclusion of deoxycholate (0.05%) showed an increase in biosynthesis (2.5-fold) as compared to the absence of detergent, but was only about half as effective as Brij 58. Cetylpyridinium bromide and cholamidopropyldimethylammonio-1-propanesulfonate had no significant effect on the biosynthesis of *myo*-PI. The dependence of the formation of *myo*-PI and *scyllo*-PI on the concentration of Brij 58 and Triton X-100 was investigated. The formation of *myo*-PI in the presence of various concentrations of Brij 58 (0.02 to 0.1%) and Triton X-100 (0.05 to 0.2%) is illustrated in Figure 5. The optimal concentration of Brij 58 was clearly 0.03% while Triton X-100 proved to be 90% as effective at 0.2%. In contrast to *myo*-PI biosynthesis, the presence of Triton X-100 effected increased formation (150%) of *scyllo*-PI as compared to Brij 58. [The concentrations of Triton X-100 (0.2%, 3.3 mM) and Brij 58 (0.03%, 0.27 mM) in the assay were above the critical micelle concentration of both detergents: Triton X-100 (0.30 mM) and Brij 58 (0.19 mM)]. The differences in activity could be due to the unique response (or responses) of one (or two) enzyme(s) located on the same or different membranes.

In summary, *in vivo* and *in vitro* studies indicate that *scyllo*-PI is biosynthesized by CDP-DG:*scyllo*-inositol transferase. Like CDP-DG:*myo*-inositol transferase enzyme, CDP-DG:*scyllo*-inositol transferase activity is stimulated by the addition of the nonionic detergents Triton X-100 and Brij 58. This is the first report of CDP-DG:*scyllo*-inositol transferase activity in cells; however further work is required to demonstrate clearly that head group transfer activity is not involved.

Hormone-induced changes in phosphoinositides are often investigated by monitoring changes in the radioactive content of bulk, *in vivo*-labeled cellular phospholipids that are at isotopic equilibrium. The discovery of *scyllo*-PI and the difficulty in separating the many closely related *myo*- and *scyllo*-phosphoinositides and *myo*-inositol phosphates in plant cells complicate the analysis of phosphoinositides (10). In addition, spatially and metabolically distinct pools of phosphoinositide lipids are present in cells (1–3) and only phosphoinositides in plasma membranes are involved in the initial hormone-trig-

gered events. The hormone-sensitive pool in the plasma membrane is small and exhibits a high turnover rate compared to the hormone-insensitive pool, which includes the rest of the cellular phospholipids. This study shows that the hormone-sensitive plasma membrane pool in barley aleurone cells is less than 25% of the total cellular phospholipids, since all, or indeed most, of the phosphoinositides in the plasma membrane are not hormone sensitive, and 75% of the labeled phospholipids would not be expected to undergo any hormone-triggered change. Moreover, this investigation indicates that the incubation periods required for the plasma membrane and intracellular membranes to achieve isotopic equilibrium are different. Therefore, changes in plasma membrane phosphoinositides could be masked and/or lead to misleading conclusions when changes in ^{32}P content of total cellular phosphoinositides are monitored in signal transduction studies. Information on the subcellular localization of labeled phospholipids is essential for a more complete and accurate understanding of the role of phosphoinositides in signal transduction.

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The Isolation and Characterization of Right-Side-Out Plasma Membrane Vesicles from Barley Aleurone Cells

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ABSTRACT: Examination of organelle- and membrane-specific processes such as signal transduction necessitates the use of plasma membrane vesicles with cytoplasmic side-in orientation. We are interested in the structural identity and subcellular localization of *in vivo* [^{32}P]phosphoric acid ([^{32}Pi])-labeled phosphoinositides, including the recently discovered phosphatidyl-*scyllo*-inositol, for signal transduction studies. In the first part of this investigation, plasma membrane vesicles from barley aleurone cells were isolated employing the aqueous polymer (Dextran and polyethylene glycol) two-phase partition method. The membrane vesicles that partitioned into the upper and lower phases of the aqueous polymer two-phase system were characterized and the purity of the vesicles ascertained by assaying for two marker enzymes, K^+-stimulated, Mg^{2+}-dependent adenosine triphosphatase (EC 3.6.1.3, ATPase), localized in the plasma membranes, and cytochrome c oxidase, localized in the mitochondria. Inhibitors for ATPases such as azide, molybdate, and vanadate were used to distinguish between plasma membrane-associated and intracellular membrane-associated ATPases. These inhibitor studies suggest that the plasma membrane preparation contained about 7% of intracellular membrane vesicles and the intracellular membrane fraction contained about 6% of plasma membrane vesicles. Orientation of the plasma membrane vesicles was ascertained by measuring the latent ATPase activity. These latency studies suggest that about 95% of the plasma membrane vesicles were of cytoplasmic side-in orientation. In the second part of this investigation, intracellular distribution and *in vivo* [^{32}Pi] labeling of phosphoinositides in the plasma membranes and intracellular membranes were investigated. Preferential accumulation of [^{32}Pi]-labeled phosphatidyl-*myo*-inositol monophosphate (*myo*-PIP) and phosphatidyl-*myo*-inositol bisphosphate (*myo*-PIP_2) was observed in the plasma membrane. However, *scyllo*-phosphatidylinositol (*scyllo*-PI) was detected in both the plasma membrane and the intracellular membranes. The cellular concentration of *myo*-phosphoinositides was determined, and, after 24 h of labeling with [^{32}Pi], the ratio of radiolabel in *myo*-PI, PIP, and PIP_2 paralleled the relative concentrations in aleurone cells.

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Biochemical processes in living cells are compartmentalized. A number of fundamental processes such as signal perception, message transduction, ion uptake, and cell wall biosynthesis are localized in the plasma membrane. Plasma membrane vesicles of right-side-out orientation are essential for investigation of plasma membrane-specific processes such as signal transduction and ion transport. Many features of barley aleurone tissue make it particularly attractive for the study of plant hormone action, hence, gibberellin- and abscissic acid-regulated cellular events have been extensively studied in this model system (1,2). However, our understanding of the molecular mechanism of hormone perception, signal transduction, and biochemical regulation remains incomplete. In recent reports from this laboratory, we described our efforts to characterize phosphoinositides in the aleurone tissue of barley seeds (3,4, and references therein). We are interested in the structural identity of *in vivo*-labeled phosphoinositides including the subcellular localization of the recently discovered phosphatidyl-*scyllo*-inositol (3,4). To this end, our first step was to investigate the fractionation of membrane vesicles from barley aleurone cells.

Fractionation of intracellular organelles from aleurone tissue has presented problems. Previous attempts to isolate intracellular organelles, such as phytin bodies and endoplasmic reticulum, from aleurone tissue of wheat (5,6) and barley (7) indicated that the commonly used density centrifugation and differential centrifugation methods could not be easily applied to aleurone cells because of the tendency of intracellular organelles in aleurone cells to swell and aggregate. Aqueous polymer two-phase partitioning method has been successfully used to isolate plasma membrane vesicles from a number of plant tissues (8) including aleurone cells from wild oats (9). A solution containing Dextran, polyethylene glycol, water, and salts separates into two phases, each with unique ionic and hydrophobic properties, and these properties are dependent on salt composition, pH, and temperature (10–12). Plasma membrane and intracellular membrane vesicles fractionate by differential partitioning between the two phases based on their hydrophobicity and other surface properties

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Abbreviations: ATPase, ATP phosphohydrolase; GPI, glycerophosphoinositol; GPIP, glycerophosphoinositolmonophosphate; $GPIP_2$, glycerophosphoinositolbisphosphate; HVE, high-voltage paper electrophoresis; [^{32}Pi], ^{32}P-labeled phosphoric acid; PI, phosphatidylinositol; PIP, phosphatidylinositolmonophosphate; PIP_2, phosphatidylinositolbisphosphate.

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(10,11). In this paper, we will present results on the isolation, characterization, and assessment of purity of plasma membrane vesicles from barley aleurone cells using the aqueous polymer two-phase method (10,13). In addition, information regarding the orientation of the plasma membrane vesicles isolated by the aqueous polymer two-phase method will be presented. Then, characterization of *in vivo*-labeled phosphoinositides and the intracellular distribution of *scyllo*-phosphatidylinositol (*scyllo*-PI) will be discussed.

EXPERIMENTAL PROCEDURES

Plant material. Barley seeds (*Hordeum vulgare* L. cv. Himalaya, 1991 harvest) were purchased from Seed Technology, Department of Agronomy and Soil, University of Washington, Pullman, Washington. Fresh spinach leaves for preliminary studies on fractionation were purchased from a local grocery.

Preparation of microsomal fraction from aleurone layers for aqueous polymer two-phase partitioning (10). Plant material was maintained at 4°C throughout the experiment. Barley aleurone layers were isolated from barley half-seeds as described previously (3,4,14). Layers were homogenized in a cold mortar and pestle with 25 mL of homogenization buffer containing sucrose (0.5 M), Hepes-KOH (50 mM, pH 7.5), ascorbic acid (5 mM), dithiothreitol (1 mM), and polyvinylpyrrolidone (0.6%, wt/vol). The homogenate was filtered through cheesecloth and centrifuged at 10,000 × *g* for 10 min. The supernatant was centrifuged at 50,000 × *g* for 30 min to obtain the microsomal pellet.

Separation of intracellular membranes from plasma membranes by aqueous polymer two-phase partitioning. As suggested by Larsson *et al*. (10), preliminary experiments were conducted with spinach leaves, as partitioning of intracellular membranes, which include chloroplast membranes, can be ascertained visually. Successful separation was easily indicated by a green lower phase and a pale white upper phase. The following concentrations of both polymers, Dextran T500 and polyethylene glycol 3350, were tried: 5.8, 6.0, 6.2, 6.4, and 6.5% (wt/vol). In agreement with previous results, aqueous polymer solution containing 6.2% of both polymers gave the best separation. However, in our experience, the concentration of polymers necessary for partitioning into two phases and for providing the best separation varied with the manufacturer and batch.

The microsomal pellet from aleurone layers was suspended in buffer containing sucrose (0.33 M), KCl (3 mM), and potassium phosphate (5 mM, pH 7.8) to a total volume of 1.667 mL. Suspended membrane vesicles (1.5 g) were added to a test tube containing 4.5 g of aqueous polymer two-phase mixture containing the following: Dextran T500 (11.16 g of 20% w/w solution), polyethylene glycol 3350 (5.58 g of 40% w/w solution), sucrose (3.05 g), potassium phosphate buffer (0.675 mL of 0.2 M, pH 7.8 solution), KCl (0.041 mL of 2 M solution), and sufficient water to a final weight of 27.00 g. This aqueous polymer mixture was prepared the night before to allow adequate time for phase separation. The final concentration of polymer after the addition of membrane suspension was 6.2%. Final concentrations of the other reagents in the mixture were sucrose (0.33 mM), KCl (3 mM), and potassium phosphate (5 mM, pH 7.8). The test tube was inverted 20 to 30 times to ensure thorough mixing. Separation of the phases was facilitated by centrifugation at 1,100 × *g* for 5–10 min. The upper and lower phases were removed and washed, two times each, with equal volumes of fresh lower- and upper-phase solutions from the bulk solution B that was prepared as follows: Bulk phase solution B containing Dextran T500 (93.00 g of 20% w/w solution), polyethylene glycol 3350 (46.5 g of 40% w/w solution), sucrose (33.89 g), potassium phosphate buffer solution (7.50 mL of 0.2 M solution, pH 7.8), KCl (0.45 mL of 2 M solution), and sufficient water to a final weight of 300 g was prepared the night before, and the two phases were used for washing phase-partitioned membranes. The washed solutions were diluted with Tris-Mes buffer (300 mM, pH 6.5); the upper, plasma membrane-containing fraction was diluted twofold and the lower, intracellular membrane-containing fraction was diluted 10-fold, and the membrane pellets were obtained by centrifugation at 50,000 × *g* for 1 h. Protein was assayed by the BioRad protein assay (15) using bovine serum albumin as the standard.

Adenosine triphosphatase (ATPase) assay. ATPase activity was determined by monitoring the inorganic phosphate released during the reaction, which was assayed by a colorimetric method (16–18). The reaction mixture contained $MgSO_4$ (5 mM), KCl (50 mM), sucrose (125 mM), ATP (5 mM), Tris-Mes buffer (30 mM, pH 6.5 unless indicated otherwise), Triton X-100 (0.05% wt/vol), protein (10–50 µg), and activators or inhibitors as indicated, in a total volume of 500 µL. The reaction was initiated by the addition of ATP. The reaction mixture was incubated at 37°C for 20 min and the reaction stopped with the addition of 1.0 mL of a solution containing H_2SO_4 (0.5 M), sodium dodecylsulfate (5% wt/vol), and ammonium molybdate, $(NH_4)Mo_7O_{24}\cdot H_2O$ (0.5% wt/vol). Total inorganic phosphate released from ATP hydrolysis was determined spectrophotometrically by the addition of ascorbic acid (50 µL of 10% wt/vol). The reaction was allowed to proceed for 20 min and absorbance measured at 820 nm. Enzyme assays were performed in duplicate and mean values ± standard error of the mean are given. Specific activity of ATPase is µmol of total inorganic phosphate released/mg protein/min. A solution of sodium vanadate was prepared by the method of Gallagher and Leonard (19).

Cytochrome c oxidase assay. Cytochrome c oxidase activity was determined, at room temperature, by measuring the oxidation of reduced cytochrome c (17,20–22). The reduced form of cytochrome c shows characteristic absorption at 550 nm. Prior to the assay, cytochrome c from horse heart was reduced using sodium dithionite, $Na_2S_2O_4$. Excess dithionite was removed by air oxidation to SO_2. This oxidation can be achieved by passing air through the solution for a few minutes (17). Removal of excess reducing agent is necessary to prevent the reduction of oxidized product formed during enzymatic reaction.

The reaction mixture contained phosphate buffer (2.7 mL, 50 mM, pH 7.5), digitonin [100 μL, 0.3% (wt/vol)], membrane suspension (100 μL, 10–50 μg protein), and reduced cytochrome c from horse heart (100 μL, 0.45 mM in phosphate buffer). All reagents were added to a sample cuvette, and the reaction was initiated by the addition of reduced cytochrome c. Decrease in absorbance of the reaction mixture at 550 nm was monitored over time. Change in absorbance per minute was obtained from the initial slope of the curve using a millimolar extinction coefficient of 29.5 at 550 nm. Enzyme assays were performed in duplicate, and mean values ± standard error of the mean are given. Specific activity of cytochrome c oxidase is μmol of product produced/min/mg protein.

Labeling with radioactive precursors. Aleurone layers were isolated from barley seeds as previously described (3,4). Aleurone layers were incubated at 25°C in a solution (1 mL/10 layers) containing succinate buffer (20 mM, pH 5), $CaCl_2$ (20 mM), chloramphenicol (30 μM), and ^{32}P-labeled phosphoric acid ([^{32}Pi]) {the [^{32}Pi] in 0.02 M HCl (Dupont/NEN, Boston, MA) was neutralized just before use with 0.02 M NaOH} (3,4). After 24 h, the layers were freed of excess isotope by washing three times with media without isotope, and the plasma membrane and intracellular membranes were separated as described above.

Extraction and separation of phospholipids. Phospholipids were extracted from aleurone layers or from membrane pellets by a modified acidic Bligh-Dyer method (3,4). The washed aleurone layers were homogenized in a mortar and pestle with a small amount of sand and water (1 mL), and the slurry was transferred to a test tube. With membrane pellets, water (1 mL) was added and mixed well with a vortex mixer. To the suspension was added 3 mL of CH_3OH, 2.5 mL of $CHCl_3/CH_3OH$ (2:1 vol/vol), 0.75 mL of 2.4 N HCl, and 2.1 mL of $CHCl_3$. The solution was vortex-mixed and centrifuged, and the lower organic phase was removed. The aqueous phase was reextracted with 1.7 mL of $CHCl_3$. The combined organic extract was washed twice with 0.75 mL of 2.4 M HCl and 3.0 mL of CH_3OH/H_2O (1:1 vol/vol). Unlabeled phospholipids (0.075 mg) from soybean were added to the organic extract, converted to glycerophospholipids by deacylation using methylamine, and the products separated by high-voltage paper electrophoresis (HVE) (3,4).

Concentration of phosphoinositides in barley aleurone layers. The concentration of phosphoinositides was calculated by determining the phosphate content. Phosphoinositides from 225 aleurone layers were extracted, deacylated and the glycerophosphoinositides separated by high-voltage paper electrophoresis (HVE). The regions corresponding to *myo*-glycerophosphoinositol (GPI), -glycerophosphoinositolmonophospate (GPIP), and -glycerophosphoinositolbisphosphate ($GPIP_2$) were extracted from the paper with 1 mL of water, 5 times. The resulting solution was concentrated and the phosphate content determined according to Ames (16). The background phosphate content of an equivalent sheet of paper was determined at the same time (15 nmol) and subtracted from the phosphate content. The phosphate content in *myo*-phosphatidylinositol (PI), -phosphatidylinositolmonophosphate (PIP), and -phosphatidylinositolbisphosphate (PIP_2) was 330, 66, and 12.2 nmol per 200 layers, respectively. By assuming that there are 110,000 cells per aleurone layer and that the volume of each cell is 33 μL (23) the concentrations of *myo*-PI, -PIP, and -PIP_2 were found to be 0.8 ± 0.1, 0.08 ± 0.03, and 0.01 ± 0.005 mM, respectively.

RESULTS AND DISCUSSION

Fractionation of aleurone vesicles by aqueous polymer two-phase method: Assay of marker enzymes. Barley aleurone layers were homogenized and the microsomal pellet was separated by the aqueous polymer two-phase partition method of Larsson *et al.* (10). The membrane pellet from the lower phase contained approximately 80% of the recovered protein, and the pellet from the upper phase contained approximately 20%. About 70% of the protein in the microsomal pellet was recovered in the two phases. The protein recovery increased to 75% when the phases were centrifuged at 100,000 × *g*. We did not observe significant loss of membrane vesicles in the 10,000 × *g* pellet with barley aleurone cells, as was observed with wheat aleurone cells (9).

To ascertain the subcellular origin of the membrane vesicles that partition into the two phases, two marker enzymes, ATPase and cytochrome c oxidase, were assayed. We chose to assay ATPase activity because, in addition to providing information on the subcellular origin, the dependence of ATPase activity on factors such as detergent (24) and freeze/thaw treatment (25,26) provides information on the orientation (sidedness) of plasma membrane vesicles. ATPase activity is found in multiple locations in plant cells, such as mitochondria, plasma membrane, plastids, tonoplast, Golgi apparatus and endoplasmic reticulum. However, ATPase from different locations can be distinguished by differences in pH optima; susceptibility to inhibition by azide, molybdate and vanadate; and activation by K^+ ions (19). A marker widely used for plasma membrane is the K^+-stimulated, vanadate-inhibited, ATPase with optimal pH of 6.5 (10,11,19). ATPase activity of detergent-permeabilized membrane vesicles from the upper and lower phase was measured at pH 6.5, the optimal pH for plasma membrane-associated ATPase (11,17). ATPase activity in the upper phase was relatively insensitive to azide (1 mM), a mitochondrial ATPase inhibitor, and ammonium molybdate (0.1 mM), an acid phosphatase inhibitor (7% inhibition), whereas the addition of vanadate (0.1 mM), a plasma membrane ATPase inhibitor, either alone or in the presence of azide and molybdate, inhibited ATPase activity by 83 ± 1%, clearly indicating the sensitivity of ATPase in the upper phase to vanadate (Table 1). In contrast, ATPase in the lower phase was markedly sensitive to azide and molybdate (88% inhibition) and insensitive to vanadate (6% inhibition), as reported by Gallagher and Leonard (19) and confirmed by subsequent investigators (10,18,28).

Although vanadate is not a specific inhibitor of plasma

TABLE 1
Distribution of Marker Enzymes Before and After Aqueous Polymer Two-Phase Partitioning

Marker enzyme	Microsomal pellet Specific activity (μmol/mg protein/min)/(% control)	Upper phase Specific activity (μmol/mg protein/min)/(% control)	Lower phase Specific activity (μmol/mg protein/min)/(% control)
ATPase			
Control[a]	1.00 ± 0.03 (100)	0.55 ± 0.022 (100)	0.81 ± 0.03 (100)
Control + azide[b] + molybdate[c]	0.52 ± 0.02 (52)	0.51 ± 0.024 (93)	0.10 ± 0.02 (12)
Control + azide[b] + molybdate[c] + vanadate[d]	0.34 ± 0.04 (34)	0.10 ± 0.04 (18)	0.09 ± 0.04 (11)
Control + vanadate[d]	—	0.09 ± 0.04 (16)	0.76 ± 0.03 (94)
Cytochrome c oxidase	0.21 ± 0.013	0.080 ± 0.007	0.48 ± 0.024

[a]ATPase assay mixture contained $MgSO_4$ (5 mM), KCl (50 mM), sucrose (125 mM), ATP (5 mM), Tris-Mes buffer (30 mM, pH 6.5), Triton X-100 (0.05% wt/vol), protein (10–50 μg), and activators or inhibitors as indicated.
[b]Sodium azide (1 mM).
[c]Ammonium molybdate (0.1 mM).
[d]Sodium orthovanadate (0.1 mM). Abbreviation: ATPase, adenosine triphosphatase.

membrane ATPase and, at concentrations above 500 μM, does inhibit mitochondrial ATPase, previous investigations (19) showed that vanadate inhibits plasma membrane ATPases at concentrations (100 μM) which do not have a significant effect on mitochondrial ATPases. Our observations of ATPase inhibition at low vanadate concentrations (100 μM) were consistent with previous reports and suggest that the upper-phase fraction consists predominantly of plasma membrane vesicles.

The activity of a second marker enzyme, cytochrome c oxidase, located in the mitochondria, was determined. Specific activity in the lower-phase membrane fraction was six times that in the upper-phase membrane fraction, indicating that the lower phase was enriched in mitochondrial vesicles (Table 1). The specific activity of cytochrome c oxidase was similar to the value observed in wild oats (9).

ATPase activity in plasma membrane vesicles (upper phase fractions) is stimulated by K^+ ions, and the K^+-stimulated component is sensitive to vanadate inhibition (19). In the assay mixture described in the Experimental Procedures section detergent-permeabilized vesicles were assayed for vanadate-sensitive ATPase activity in the presence and absence of KCl. The addition of 50 mM KCl stimulated ATPase specific activity threefold, from 0.17 ± 0.01 to 0.49 ± 0.006 μmol (mg protein)$^{-1}$ min^{-1}. The addition of vanadate in the presence of KCl suppressed ATPase activity to 0.10 ± 0.005 μmol (mg protein)$^{-1}$ min^{-1}, whereas the addition of vanadate in the absence of KCl had no effect; the specific activity was 0.18 ± 0.007 μmol (mg protein)$^{-1}$ min^{-1}. The data illustrate that the inhibitory action of vanadate occurs only in the presence of K^+ ions and suggest that vanadate inhibits the K^+-stimulated component of plasma membrane ATPase, thus providing further proof that ATPase activity in the upper phase is due to the K^+-stimulated, vanadate-sensitive ATPase of the plasma membrane.

The pH dependence of ATPase activity in the vesicles in the upper phase was investigated. ATP-hydrolyzing activity at pH 6.5, the optimal pH of plant plasma membrane-associated H^+-pump, was 10 times the activity at pH 8.0, the optimal pH of mitochondrial and tonoplast ATPase (Table 2) (19). In fact, very little ATPase activity was observed at pH 8.0.

Taken together, the data (Table 3) demonstrate that the plasma membrane marker, the vanadate-sensitive component of ATPase, is enriched ninefold in the upper phase compared

TABLE 2
Effect of pH on ATPase Activity in the Upper-Phase Fraction

Treatment	Specific activity at pH 6.5 (μmol/mg protein/min)	Specific activity at pH 8.0 (μmol/mg protein/min)
Control[a]	0.55 ± 0.04	0.015 ± 0.01
Control + azide[b] + molybdate[c]	0.49 ± 0.03	0.02 ± 0.01
Control + azide[b] + molybdate[c] + vanadate[d]	0.14 ± 0.04	0.03 ± 0.01

[a]ATPase assay mixture contained $MgSO_4$ (5 mM), KCl (50 mM), sucrose (125 mM), ATP (5 mM), Tris-Mes buffer (30 mM, pH 6.5 or 8.0), Triton X-100 (0.05% wt/vol), protein (10–50 μg), and inhibitors as indicated.
[b]Sodium azide (1 mM).
[c]Ammonium molybdate (0.1 mM).
[d]Sodium orthovanadate (0.1 mM). See Table 1 for abbreviation.

TABLE 3
Relative Enrichment or Depletion of Enzymes in the Two Phases

Enzyme	Specific activity of enzyme (μmol/mg protein/min)		
	Microsomal fraction[a]	Upper phase	Lower phase
K^+,Mg^{2+}-ATPase, Vanadate-sensitive[b]	0.66	0.45	0.05
K^+,Mg^{2+}-ATPase, Azide and molybdate sensitive[c]	0.48	0.04	0.71

[a]Microsomal fraction, prior to separation by aqueous polymer two phase technique.
[b]The vanadate inhibition of the K^+,Mg^{2+}–ATPase.
[c]Azide and molybdate inhibition of the K^+,Mg^{2+}-ATPase. See Table 1 for abbreviation.

to the lower phase. In contrast, the intracellular markers, the azide- and molybdate-sensitive components of ATPase, are depleted 18-fold. The assay of cytochrome c oxidase, a mitochondrial marker, provided additional confirmation that mitochondrial membrane was enriched in the lower phase and intracellular membranes were depleted in the upper phase. The low inhibition of upper-phase membrane vesicles by azide and molybdate (7%, Table 1) and of lower-phase membrane vesicles by vanadate (6%, Table 1) suggests that the plasma membrane fraction contains about 7% contamination from intracellular membranes and that the intracellular membrane fraction contains about 6% contamination from the plasma membrane.

Orientation of plasma membrane vesicles. When membrane vesicles are formed during tissue homogenization, two orientations are possible; cytoplasmic side in, or right-side out, and cytoplasmic side out, or inside out. ATPase activity in cytoplasmic side-in vesicles is latent, as the active site and ATP-binding domain of ATPase in the plasma membrane are located on the inner, cytoplasmic side of the plasma membrane and therefore inaccessible to ATP (27). Latent ATPase activity can be unmasked in the presence of detergents, which render membranes more permeable to ATP. Thus, latent ATPase activity is an indication of the percentage of cytoplasmic side-in vesicles in plasma membrane preparations. An estimate of the latent ATPase activity can be determined by (i) increase in ATPase activity in the presence of a detergent that increases the permeability of membranes to ATP (18,24,25) and (ii) increase in ATPase activity when membrane vesicles are subjected to freeze/thaw treatment, whereupon cytoplasmic side-in membrane vesicles burst and reform (26,28). During revesiculation, the potential exists for the formation of a higher proportion of vesicles of cytoplasmic side-out orientation.

To unmask the latent ATPase sites two nonionic detergents were employed: Triton X-100 was selected because it is widely used in such studies and therefore allows for comparison, and Brij 58 [$C_{16}H_{33}(OCH_2CH_2)_{20}OH$] was chosen because it is able to permeabilize plasma membranes at low concentrations with minimal activating or inhibitory effects (24). The effect of varying concentrations of Triton X-100 and Brij 58 on ATPase activity at pH 6.5 was investigated (Fig. 1). ATPase activity was maximal when the concentration of Brij 58 was 0.005% (wt/vol), and further increases in the concentration of Brij 58 were inhibitory. When Triton X-100 was employed, 10 times as much detergent [0.05% (wt/vol)] was required to produce the same ATPase activity. However, the maximal ATPase activity observed in the presence of Triton X-100 (10 times that observed without detergent) was 150% that in the presence of Brij 58. Both Brij 58 and Triton X-100 were inhibitory at high concentrations. These data illustrate that a major portion of ATPase activity at pH 6.5 is latent in plasma membrane vesicles, suggesting a high proportion of cytoplasmic side-in vesicles. Brij 58 showed latency of about 84% [ratio of latent activity, (i.e., difference in activity measured ± detergent) to total activity, (i.e., activity measured with detergent)], whereas, with Triton X-100, a latency of 90% was observed.

Latent ATPase activity is dependent on the nature and concentration of the detergent. Use of detergents to determine ATPase latency is complicated by the fact that detergents, in addition to permeabilizing membrane vesicles, may have stimulatory or inhibitory effects (24–26) due to incorporation of detergents into the lipid bilayer of vesicles and consequent conformational changes in the ATPase molecule. The differ-

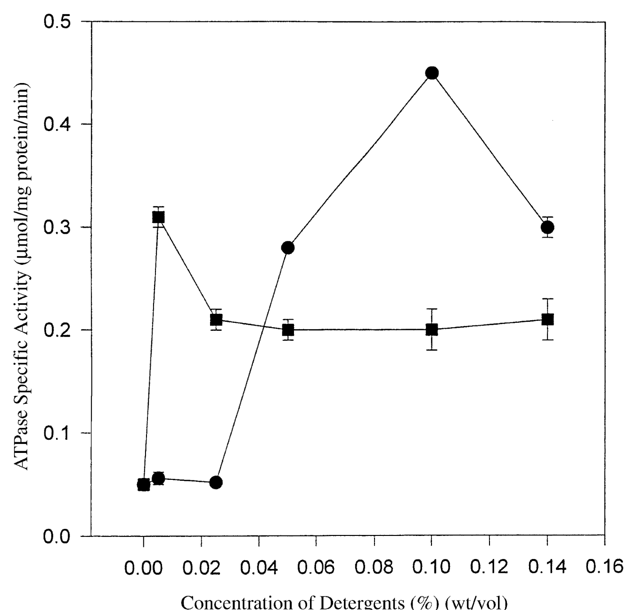

FIG. 1. Effect of varying concentrations of two nonionic detergents, Triton X-100 (●) and Brij 58 (■), on adenosine triphosphatase (ATPase) activity (pH 6.5) associated with the upper phase of the aqueous polymer two-phase partitioning method. All experiments were performed in duplicate. Error bars represent standard deviation about the mean. This graph is representative of three separate experiments.

ence in the maximal ATPase activity produced could be due to inhibition of ATPase at optimal concentration of Brij-58 or to activation of plasma membrane ATPase by Triton X-100. In contrast to our observations, others observed that Brij 58-permeabilized membranes exhibited higher ATPase activity than Triton X-100 and that Triton X-100 was inhibitory at higher concentrations (24,28).

The orientation of the isolated plasma membrane vesicles was investigated by freeze/thaw treatment. The vesicles were frozen in liquid nitrogen (for 2 min) and then thawed (in water at 25°C) to induce vesicle breakage and reformation, possibly with the opposite orientation. The freeze/thaw cycle was carried out four times, and ATPase activity assayed in the presence and absence of detergents (Table 4). ATPase activity increased dramatically (10-fold) after the freeze/thaw cycle. Interestingly, ATPase activity after freeze/thaw treatment was similar to that observed in the presence of Triton X-100. When Triton X-100 was added to vesicles that had been subjected to freeze/thaw treatment, a further increase (twofold) in ATPase activity was observed.

That freeze/thaw treatment significantly increased ATPase activity suggests that, when vesicles reform after cleavage during freezing, a higher proportion of inside-out vesicles are formed. A 10-fold increase after four freeze/thaw cycles is significantly greater than the fourfold increase reported previously (25). Further doubling of ATPase activity upon addition of Triton X-100 to plasma membrane vesicles that have been subjected to freeze/thaw treatment suggests that 50% of the reformed vesicles are cytoplasmic side in, and therefore sidedness during revesiculation of barley aleurone cells is a random event. The freeze/thaw data suggest that approximately 95% of ATPase activity is latent, and therefore 95% of the vesicles are cytoplasmic side in. This observation also suggests that Triton X-100 did not unmask all the latent ATPase activity. An alternative explanation could be that freeze/thaw treatment disturbs the integrity of the membranes, and the additional increase upon addition of Triton X-100 is due to activation of the enzyme and is not related to unmasking of latent activity.

Previous investigations have shown that upon homogenization, vesiculation with cytoplasmic side-in orientation is strongly favored (24,25,29). This preference could be due to (i) higher negative charge density of the outer surface compared to the inner surface, affecting membrane curvature, and (ii) the remaining inner cytoskeleton attached to the plasma membrane, affecting curvature (25). Since cytoplasmic side-in and cytoplasmic side-out vesicles can be expected to have different surface properties, the aqueous polymer two-phase partitioning method, in which separation is based on surface properties, can discriminate between these orientations. Indeed, plasma membrane vesicles isolated by the aqueous polymer two-phase partition method yield predominantly cytoplasmic side-in vesicles (25,26,30), and our investigations with barley aleurone cells are consistent with these observations.

In summary, we have demonstrated that the aqueous polymer two-phase partitioning method separates plasma membrane vesicles in barley aleurone cells from intracellular membrane vesicles. The assaying of K^+-stimulated, Mg^{2+}-dependent ATPase with activators and inhibitors and cytochrome c oxidase as marker enzymes suggests that the plasma membrane vesicles isolated by this method are over 90% pure and predominantly (95%) cytoplasmic side in.

Identification of phospholipids in the plasma membrane and intracellular membranes. To identify the phospholipids that are labeled in the plasma membrane and intracellular membranes pools, aleurone cells were incubated in [^{32}Pi] for 24 h, and the *in vivo*-labeled phospholipids were extracted from whole cells, plasma membranes, and intracellular membranes. Radioactivity in the lipid extract from intracellular membranes was three times that in plasma membranes. The phospholipids were converted to glycerophospholipids and separated by HVE. Comparison of the autoradiograms (Fig. 2) indicates that there are significant differences. In whole-cell extract, the ratio of radioactivity in *myo*-PI:-PIP:-PIP_2 (Bands 3, 5, and 7) after incubating the aleurone cells for 24 h was 100:11:3 (10,928:1236:340 cpm). In plasma membranes, the ratio of radioactivity in *myo*-GPI:-GPIP:-$GPIP_2$ was 100:30:15 (1000:295:153 cpm). The lack of radioactivity in the regions corresponding to *myo*-GPIP (Band 5) and *myo*-$GPIP_2$ (Band 7) in intracellular membranes is clearly evident and suggests the undetectably low concentration or lack of *myo*-PIP and *myo*-PIP_2 in intracellular membranes. The pres-

TABLE 4
Effect of Freeze/Thaw Treatment on ATPase Activity in the Upper Phase

Treatment	Specific activity (µmol/mg protein/min) (% of control)
Control[a] (without freeze/thaw) – Triton X-100	0.05 ± 0.002 (100)
Control subjected to freeze/thaw treatment[b] – Triton X-100[c]	0.5 ± 0.03 (1000)
Control + Triton X-100[c]	0.560 ± 0.03 (1120)
Control subjected to freeze/thaw treatment[b] + Triton X-100[c]	1.11 ± 0.013 (2220)

[a]ATPase assay mixture contained $MgSO_4$ (5 mM), KCl (50 mM), sucrose (125 mM), ATP (5 mM), Tris-Mes buffer (30 mM, pH 6.5), protein (10–50 g) and Triton X-100 as indicated.
[b]The vesicles in the upper phase were subjected to freeze/thaw cycle (four times) as described in Experimental Procedures section (26).
[c]Concentration of Triton X-100 was 0.10% (wt/vol). See Table 1 for abbreviation.

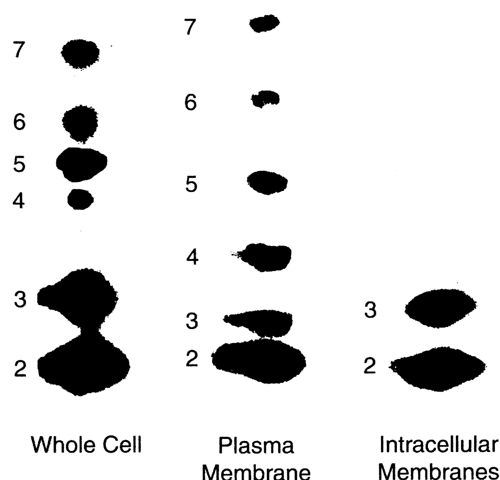

FIG. 2. Separation of anionic [^{32}P]phosphoric acid ([^{32}Pi])-labeled phospholipids from whole cells, plasma membranes and intracellular membranes. Aleurone layers from barley seeds of 1991 harvest were radiolabeled with [^{32}P]phosphoric acid (50 μCi/10 layers) for 24 h at 25°C. Total cellular phospholipid was extracted from 10 aleurone layers. Plasma membrane and intracellular membrane fractions were isolated from 40 aleurone layers using the aqueous polymer two-phase partitioning method, and phospholipids were extracted from the separated membranes. Phospholipids were deacylated, separated by high-voltage paper electrophoresis, and the radioactive compounds were detected by autoradiography. Data are representative of five independent experiments. Compounds 2, 3, 5, and 7 were identified as *scyllo*-glycerophosphoinositol (GPI), *myo*-GPI, *myo*-glycerophosphoinositolmonophosphate, and *myo*-glycerophosphoinositolbisphosphate. Compounds 4 and 6 are unidentified. See Narasimhan *et al.* (3) and references therein for more details.

ence of *scyllo*-PI (Band 2) in both intracellular and plasma membranes is also clear. In addition, radioactivity in *scyllo*-PI is greater than in *myo*-PI in whole-cell extract (2:1), plasma membrane (4:1), and intracellular membranes (2:1). The data suggest that preferential labeling of *scyllo*-PI, *myo*-PIP, and *myo*-PIP_2 occurs in the plasma membranes compared to intracellular membranes. The relative accumulation of labeled *myo*-PIP and *myo*-PIP_2 is consistent with that observed in other studies. Rincón and Boss (30) found that 90% of the labeled *myo*-PIP and *myo*-PIP_2 in prelabeled carrot cells was located in the plasma membranes and 10% in intracellular membranes. Sandelius and Sommarin (31,32) assayed *myo*-PI-kinase and *myo*-PIP-kinase in wheat seedlings and concluded that both of these enzymes are localized predominantly in the plasma membranes. The data presented above indicate that *myo*-PIP and -PIP_2 in plasma membranes incorporate more [^{32}Pi] than intracellular membranes. This could be due either to enhanced activity of PI-kinase and PIP-kinase in plasma membrane compared to intracellular membranes or to greater translocation of *myo*-PIP and *myo*-PIP_2 and *scyllo*-PI to plasma membrane.

Information on the chemical quantities of phosphoinositides in plant cells by direct determination of phosphoinositides is, to our knowledge, unavailable (33). Molar ratios of phosphoinositides is estimated from radiolabeling studies on the assumption that the relative ratio of radioactivity reflects chemical quantities after isotopic equilibrium has been reached. To obtain information on the molar amounts, we extracted phosphoinositides from barley aleurone layers, deacylated them to glycerophosphoinositols, separated by HVE, and determined the phosphorus content. After accounting for the differences in the number of phosphate groups (one, two, and three in *myo*-PI, *myo*-PIP and *myo*-PIP_2, respectively) the concentrations of *myo*-PI, -PIP, and PIP_2 were found to be 0.8 ± 0.1, 0.08 ± 0.03, and 0.01 ± 0.005 mM, respectively. The molar ratio of *myo*-PI:-PIP:-PIP_2 is therefore 100:10:1.25. Comparison of the ratios of chemical amount and [^{32}Pi]-labeling suggests that the ratio of radioactivity in phosphoinositides after 24 h of labeling is similar to chemical amounts. This is in contrast to animal cells where the quantitatively minor PIP and PIP_2 incorporate a disproportionate amount of the radiolabel, suggesting that these phospholipids are turning over rapidly (34). If one assumes that the [^{32}Pi] label in the whole cell reflects the chemical amounts and that the PI in plasma membranes is 25% of that found in the whole cell, whereas most or all of the *myo*-PIP and -PIP_2 are located in the plasma membrane, then the ratio of the chemical amounts of *myo*-PI:-PIP:-PIP_2 is 0.2:0.08:0.01 mM, or 100:40:5, which is comparable to the ratio of radiolabel (100:30:15).

In summary, the distribution of phosphoinositides in plasma membranes differed from that in intracellular membranes. Preferential accumulation of [^{32}Pi] label in *myo*-PIP and -PIP_2 was observed in the plasma membrane, and the amount found in the intracellular membranes was undetectably low. Both isomers of PI, *myo*- and *scyllo*-PI, were present in the plasma membranes as well as the intracellular membranes.

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Principal Component Analysis of Measured Quantities During Degradation of Hydroperoxides in Oxidized Vegetable Oils

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ABSTRACT: Decomposition of hydroperoxides in sunflower oil under strictly oxygen-free conditions was followed by measuring peroxide values against time, absorbance values at 232 and 268 nm, *para*-anisidine values, and by quantitative analyses of volatile products using various additives. The results were arranged in a matrix form and subjected to principal component analysis. Three principal components explained 89–97% of the total variance in the data. The measured quantities and the effect of additives were closely related. Characteristic plots showed similarities among the measured quantities (loading plots) and among the additives (score plots). Initial decomposition rate of hydroperoxides and the amount of volatile products formed were similar to each other. The outliers, the absorbance values, were similar to each other but carried independent information from the other quantities. *Para*-anisidine value (PAV) was a unique parameter. Since PAV behaved differently during the course of hydroperoxide degradation, it served as a kinetic indicator. Most additives were similar in their effects on the mentioned quantities, but two outliers were also observed. Rotation of the principal component axes did not change the dominant patterns observed. The investigations clearly showed which variables were worth measuring to evaluate different additives.

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Oxidation of edible oils causes serious flavor and taste problems in the food industry. During oxidation, hydroperoxides are formed which decompose into volatile organic compounds such as alcohols, aldehydes, ketones, and acids (1). These volatile end-products can lead to off-flavor of foods (2–4). Polyunsaturated vegetable oils heated or exposed to light in the presence of oxygen may undergo partial autoxidation resulting in hydroperoxides. During oxidation the decomposition of hydroperoxides is the most important process, which produces secondary initiation, leads to hydroxyl, alkoxyl and peroxyl radicals, propagating the chain, attacking the hydroperoxides and bulk triglycerides.

For characterization of oil oxidation and of hydroperoxide degradation, the following data are used generally: peroxide value (POV), absorbance values at 232 and 268 nm (E232 and E268, respectively), *para*-anisidine value (PAV), and concentrations of volatile products formed during the process. These data are termed "variables," "measured quantities," or "factors" later on. Different kinds of additives can accelerate and/or inhibit the decomposition of hydroperoxides. Herewith we limited our examinations to study solely the decomposition of hydroperoxides and to examine the effect of 10 additives or their combinations. Therefore oxygen-free conditions were applied. The hydroperoxide amount formed from radical-induced decomposition of hydroperoxides is far below the detection level under the circumstances applied.

Such a multicomponent–multivariable problem calls for a detailed multivariate analysis. Hence application of a multivariate technique to characterize the hydroperoxide decomposition seems to be appropriate. Principal component analysis (PCA), factor analysis, and related techniques became popular because they can offer information otherwise not accessible (5–7). This information includes, e.g., classification, searching similarities, finding relationships, outlier detection, modeling, data reduction, and finding physical significance to abstract factors.

PCA was successfully applied to various fields of chemistry especially in chromatography and spectroscopy (6). The usefulness of the technique was proved recently by its extension to quantitative structure–activity relationships (8,9), to reaction kinetics (10,11), and to chemical engineering (12). In food chemistry multivariate approaches are normally applied to correlate instrumental data and food components. The most commonly used multivariate techniques in food chemistry are PCA, discriminant analysis, cluster analysis, and their various combinations. These approaches are mainly used for classification and pattern recognition purposes (13–16). Classification of beer aroma volatile compounds is also reported (15). Multivariate statistical methods are routinely applied for authenticity control of edible oils (16,17),

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Abbreviations: BHA, butyl hydroxyanisole; BHT, butyl hydroxytoluol; CAR, β-carotene; E232, absorbance value at 232; E268, absorbance value at 268; HEP, 2-*trans*-heptenal; HEX, hexanal; LEC, lecithin; PAV, *para*-anisidine value; PCA, principal component analysis; POV, peroxide value; PRG, propylgallate; SAC, sum of all acid amounts; SALD, sum of all aldehyde amounts; TAC, tocopheryl acetate; TBHQ, *tert*-butyl hydroquinone; TCDEC, *cis,trans* 2,4-decadienal; TOC, α-tocopherol; TTDEC, *trans,trans* 2,4-decadienal; *w*, initial decomposition rate of hydroperoxides.

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or they can be applied to discover adulteration of vegetable oils (18). Cluster analysis and PCA were also carried out for feature selection and validation problems (19,20). It might be quite interesting to find out whether the application of PCA can be extended to other fields as well.

To our knowledge this is the first attempt to characterize measured quantities (factors) and additives by PCA for the deterioration of oils.

The aims of the present investigation were as follows: (i) to determine which measured quantities (factors) bear the same information and which are independent ones (what kinds of characteristics are worth measuring?); (ii) to classify the measured quantities and additives according to their similarities; (iii) to compare the results of hydroperoxide decomposition at 180 and 240 min; and (iv) to assess differences in additives. This paper was primarily concerned about the degradation products of vegetable oils and about the analysis of decomposition products. Secondarily, it was considered as a chemometrics paper having application of known principles and methods. The information extracted by PCA is used mainly useful for food chemists.

EXPERIMENTAL PROCEDURES

Standard refined (neutralized, bleached, deodorized) sunflower oil was mildly oxidized by oxygen under controlled conditions. The POV of the oxidized oil was measured to be 21. The oil was provided by Unilever Research Laboratory (URL), Vlaardingen, The Netherlands. All experiments of hydroperoxide decomposition were carried out under strictly oxygen-free conditions at 140°C using deoxygenated nitrogen atmosphere and sealed ampoules (21).

POV, PAV, and absorbances at 232 and 268 nm were determined by standard methods: POV {ISO 3960 or DGF C-VI 6a (84)}, PAV {DGF C-VI 6e (84)}, and ultraviolet absorptivity {AOCS Ch 5-91 (93)}. Volatile contents of sunflower oils were analyzed by headspace solid-phase microextraction (22–24) using a Finnigan MAT GCQ GC/MS apparatus (San Jose, CA) having a quadrupole ion-trap mass analyzer. Separation was carried out (21,25) on a CP WAX 52CB (ChromPack) column (Middleburg, The Netherlands) (30 m long with 0.25 mm i.d. and 0.25 µm film thickness). Electron impact mass spectra were taken by full-scan mode in 10–650 amu mass range. Detected components were identified by matching electron impact spectra against the NIST library containing about 100,000 compounds. Samples were inserted into the injection port by headspace solid-phase microextraction method using 100 µm poly(dimethylsiloxane) fiber (Supelco Inc., Bellefonte, PA). Optimal conditions were: 45 min adsorption time at 40°C from a 4-mL vol closed vial containing 1 mL of oil. A narrow (0.75 mm i.d.) inlet liner was applied in order to detect fairly sharp and well-defined peaks. Desorption time was 1 min at 275°C. Column temperature setting was programmed from 40 to 220°C (hold for 5 min) with 4°C/min increase rate. The carrier gas was helium at 35 cm/s constant velocity.

Variables PAV, absorbance, and amount of volatiles were measured at two time points: after 3 h and 1 h later. The time was determined by the average rate of hydroperoxide decomposition. The majority of hydroperoxides were decomposed at the end of the experiments. The initial rate of hydroperoxide decomposition (w) was calculated from the beginning of POV vs. time curves using 6–7 time points. The POV was measured by iodometric titration {ISO 3960 or DGF C-VI 6a (84)}.

All measurements concerning any quantities were repeated several times (at least twice, on average four, at maximum seven times), and only the averages were used later on. The following data pretreatment was applied: The data were arranged in a matrix form. The mean of each column was subtracted from the corresponding matrix elements. Then, every matrix element was divided by the variance of its own column. Thus, the data were centered to zero mean and scaled to unit variance. The approach presented here is called "finite population" approach in some scientific fields. On each entity the measurement can be considered as fairly accurate with little repetition error such that the observed variance related to PCA is entirely between additives, i.e., there is little within additive uncertainty. In other words, if the experiments are repeated, nearly the same results are expected.

THEORY

In the course of defining principal components, the original variables are transformed into new ones. The principal components are, in fact, linear combinations of the original variables. Their values are the component scores. The linear coefficients are called the component score coefficients. The linear coefficients of the inverse relation are the loadings, i.e., in our case the correlation coefficients between the original variables and the principal components. The algorithms for PCA can be found in standard chemometrics books and tutorials (5–7). The principal components are orthogonal (independent), in other words uncorrelated. Further on, they are ordered in such a way that the variance of the first principal component is the greatest, the variance of the second is second-greatest, and so on, whereas that of the last one is the smallest. The solution is obtained by an eigenvalue calculation.

The columns of data matrices are intercorrelated, i.e., the data are redundant. The method of PCA (5–7) makes use of the intercorrelations by starting from the correlation matrix of the variables, and it eliminates the redundancy from the data, i.e., it reduces their dimensionality by revealing several underlying components. The underlying components are represented by new variables called principal components.

A basic assumption in the use of PCA is that the score and loading vectors corresponding to the largest eigenvalues contain the most useful information relating to a specific problem and that the remaining ones constitute mainly noise, i.e., for a practical problem it is sufficient to retain only a few components accounting for a large percentage of the total variance (7).

TABLE 1
Arrangement of Matrices to be Analyzed (notations are in parentheses)[a]

Column designees		Row designees	
No.	Measured quantities	No.	Additives
1	Initial decomposition rate of hydroperoxides: (*w*)	1	Without any additive
2	Absorbance value at 232 nm (E232)	2	Butyl hydroxyanisol (BHA)
3	Absorbance value at 268 nm (E268)	3	Butyl hydroxytoluol (BHT)
4	*para*-Anisidine value (PAV)	4	*tert*-Butyl hydroquinone (TBHQ)
5	Amounts of hexanal (HEX)	5	Propylgallate (PRG)
6	Amounts of 2-*trans*-heptenal (HEP)	6	Lecithin (LEC)
7	Amounts of *cis,trans* 2,4-decadienal (TCDEC)	7	α-Tocopherol (TOC)
8	Amounts of *trans,trans* 2,4-decadienal (TTDEC)	8	Tocopheryl acetate (TAC)
9	Sum of all aldehyde amounts (SALD)	9	β-Carotene (CAR)
10	Sum of all acid amounts (SAC)	10	Additive 2 + 7: TOC + BHA
		11	Additive 4 + 7: TOC + TBHQ

[a]The measured quantities (reactivity values) are taken as variables (columns) and the various additives as mathematical–statistical cases (rows).

Each data set to be analyzed was essentially a data matrix containing the measured quantities. The arrangement of matrices is summarized in Table 1.

First, the correlation matrix of the original variables was computed (this matrix contained the correlation coefficients of each original variable with the others). Then, the component loadings were calculated from the eigenvectors and eigenvalues of the correlation matrix. Finally, the component scores were obtained from the component loadings and the original variables. The computer program used (26) furnishes the variances accounted for by the principal components, the loading patterns, the score coefficients for the standardized variables at mean zero and standard deviation one, and the component scores.

Three sets of data were analyzed: Data Set I was a matrix of 10×11; all the data referred to 180 min. The letter "A" and number "1" at the end of notations indicated the reaction time (180 min). The 10 measured quantities constituted the columns. The data points in 11 rows of the matrix represented measurements on oils with differing additives added. The first row corresponded to the results without any additives. All the other additives were denoted by numbers 2–11 (see Table 1). They were all conventional antioxidants. The last two entries showed an eventual synergistic effect since additives No. 10 and 11 were composites from additives No. 2 and 7 as well as 4 and 7, respectively.

Data Set II showed the results after 240 min. This was again a 10×11 matrix. The same measured quantities were used at 240 min (except *w* values which were identical to the ones in Data Set I). Only the notations differed a little: E232B and E268B instead of E232A and E268A as well as PAV2, HEX2, HEP2, etc. instead of PAV1, HEX1, HEP1, . . ., etc. indicated the time period of 240 min. The same additives were used as in Data Set I.

Data Set III consisted of a joined data set, i.e., Data Set I + Data Set II. It consisted of a 19×11 matrix using the same measured quantities and additives as above. (The initial decomposition rate was identical for the first two data sets, which is why only 19 measured variables were analyzed instead of 20.)

RESULTS AND DISCUSSION

Generally, it can be established for all sets of data that three principal components explain about 89–97% of the variance in the data. The selection of the number of principal components is somewhat arbitrary. Empirical rules suggest the involvement of components with eigenvalue larger than 1 or 0.5. Considering the above empirical rules, even two principal components to be retained are enough in the case of Data Set II. Analyzing the loading values of Data Sets I and II, a grouping can be seen immediately (Figs. 1 and 2).

Not only are the amounts of different aldehydes correlated with each other but also the initial decomposition rates of hydroperoxides: the decomposition reaction is kinetically controlled. The larger the initial decomposition rate is, the more volatile products are formed. The alternative would be the formation of some intermediates, and hence no direct link would be observed between decomposition rate and product formation. The absorbance values are outliers beyond doubt in both figures: the amount of conjugated dienes and trienes in the system carries independent information from the volatile compounds formed. Therefore, it is inappropriate to characterize the oil deterioration processes alone by measuring the volatile products and initial rates of hydroperoxides (as followed by POV).

PAV, characteristic of the conjugated oxo-diene content of the oil, is an outlier in Figure 1 (at 180 min, Data Set I), but its point is included in the cluster of product amounts (and *w*) in Figure 2 (at 240 min, Data Set II). From this observation it follows that α,β-unsaturated aldehydes "behave differently" within the time interval studied. Since after the degradation of hydroperoxides PAV is not an outlier anymore, this quantity can be used as a kinetic indicator.

The observations involve some far-reaching conclusions. For example, it is not worthwhile to quantify all the volatile products present in the system. Their values are related to each other at both time points. Naturally, it is expedient to measure the aldehyde present in the largest amount. The quantification of products can be avoided in case of quick

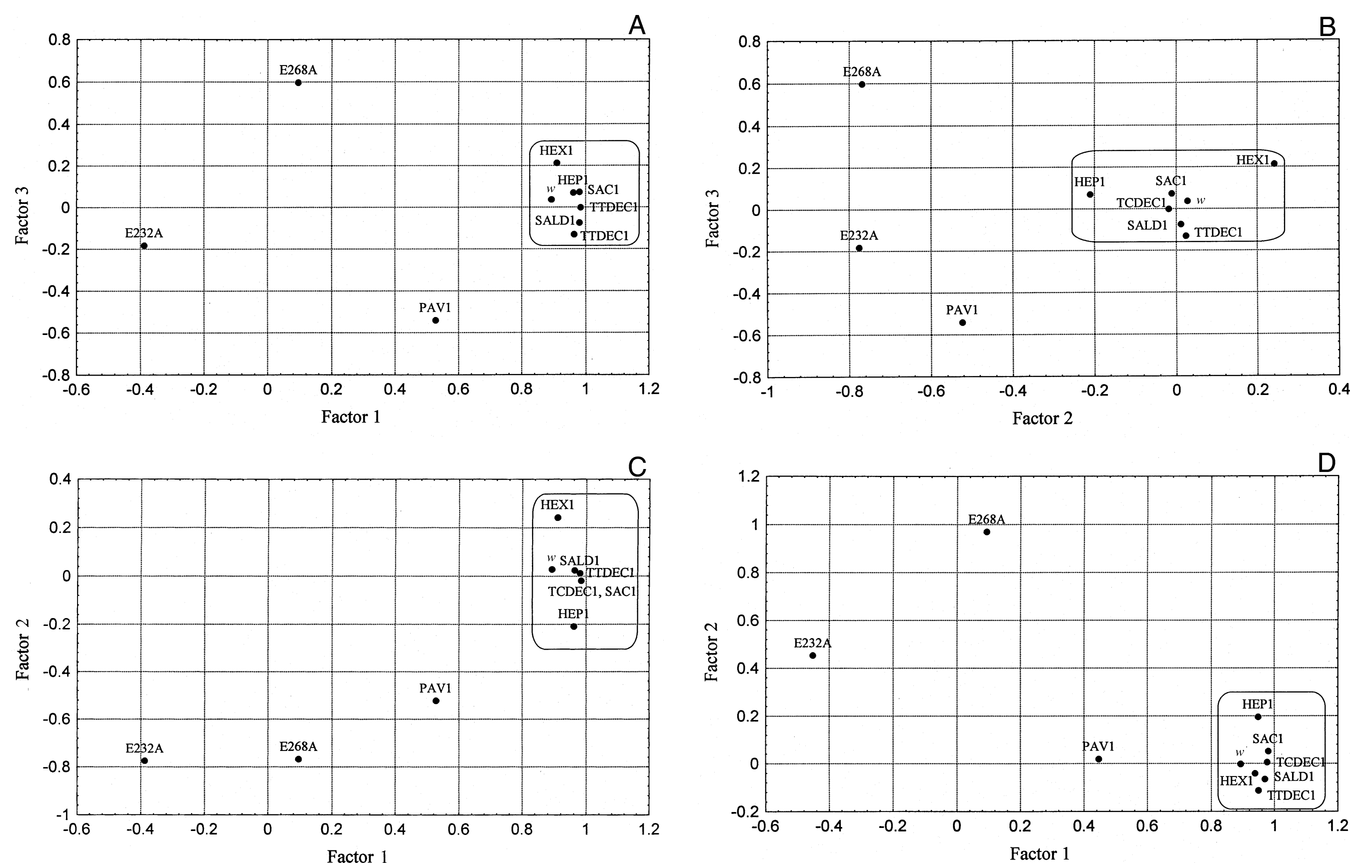

FIG. 1. Characterization of measured quantities. Factor loadings for Data Set I (at 180 min, 140°C). Parts A, B, and C are plots of unrotated loading values in three dimensions. Part D is the plot of rotated loading values. "A or 1" at the end of abbreviations means a reaction time of 180 min. HEX, hexanal; *w*, initial decomposition rate of hydroperoxides; SALD, sum of all aldehyde amounts; TTDEC, *trans,trans* 2,4-decadienal; TCDEC, *cis,trans* 2,4-decadienal; SAC, sum of all acid amounts; HEP, 2-*trans*-heptanal; PAV, *para*-anisidine value; E268A, absorbance value at 268 nm; E232A, absorbance value at 282 nm.

characterization of the decomposition process because *w* holds approximately the same information as the amounts of volatile products. Of course, gas chromatography/mass spectrometry measurements provide much useful information on the quality and quantity of the products formed. However, all amounts of aldehydes are similar to each other and to *w* from the point of view of additive effects on the decomposition of hydroperoxides. In other words, if one of the additives causes a slow decomposition rate of hydroperoxides, then the amounts of aldehydes will similarly be low and, on the contrary, large *w* involve large amounts of volatile aldehydes formed. This statement is relative. If we need the absolute values of products, the gas chromatography/mass spectrometry analysis (quantification) cannot be avoided.

The loading values of the first three principal components are provided in a table form for Data Set III (Table 2) instead of a loading plot.

The above tendencies are expressed even stronger if analyzing the joined data set. Correlation coefficients larger than 0.7 are indicated by bold in Table 2. It can be seen immediately that the first principal component is closely related to the "*w*" and to the amounts of products (both at 180 and 240 min). The initial decomposition rate of hydroperoxides carries the same information as the volatile products formed during degradation of hydroperoxides.

The second principal component closely resembles the absorbance values (E268A and E232B), whereas the third principal component correlates well with PAV at 180 min. Although the absorbance values show close similarity according to the second abstract factor, in a three-dimensional space of factors the points for absorbance values do not coincide. The absorbance values are proportional to the amounts of conjugated dienes and trienes. The amounts of dienes and trienes were correlated well [the corresponding absorbance values (E268A, E232B, and E268B) formed the second principal component almost exclusively]; nevertheless they also differ to some extent. This suggests that the absorbance values should be measured at both wavelengths and at different time points. Notably two absorbance values behave oppositely during the degradation process. At the earlier stage (180 min), absorbance at 268 nm correlates better with the second principal component, whereas at the later stage (240 min) absorbance at 232 nm does the same.

The third principal component is composed mainly of PAV1 and E232A. The correlation of the third principal component with other variables is almost always negligible, usu-

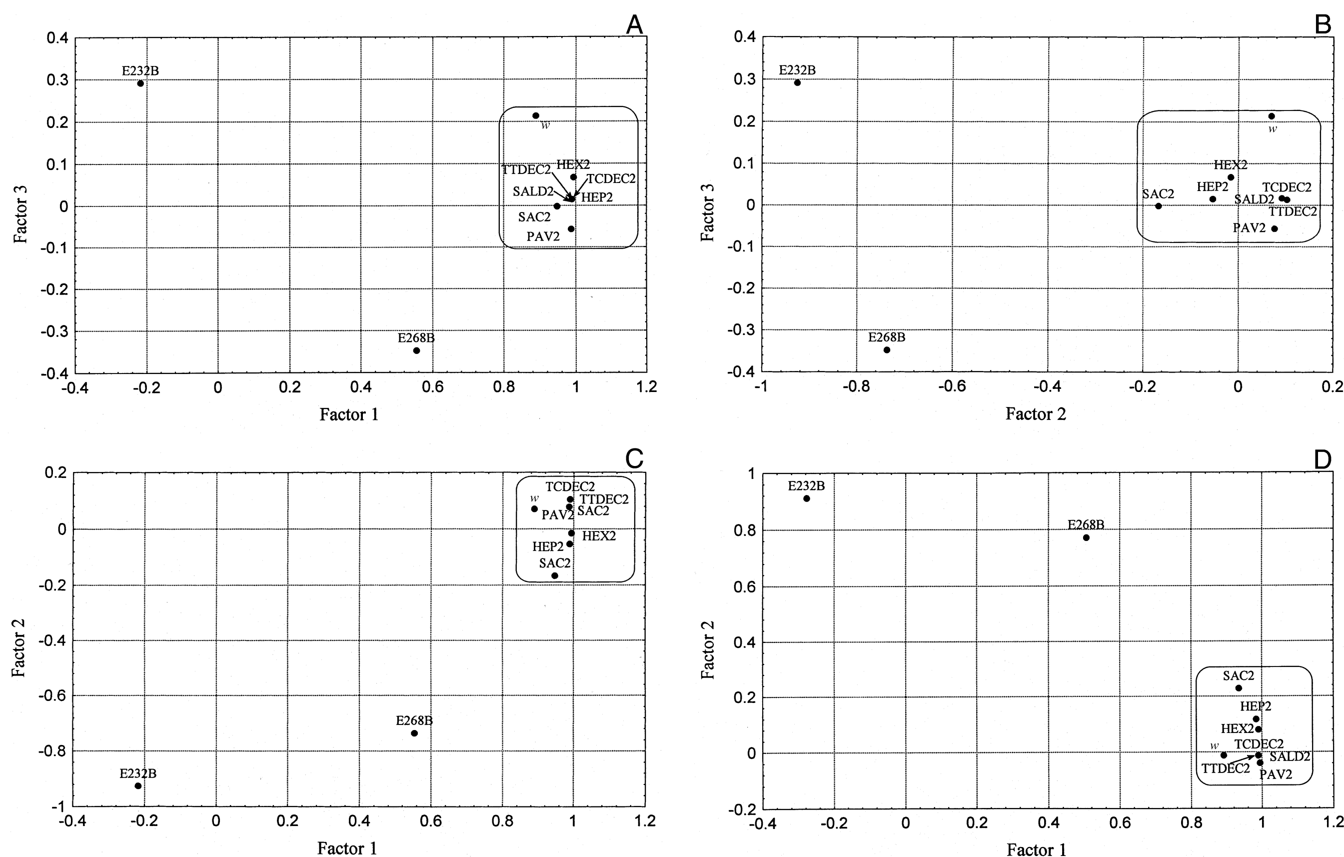

FIG. 2. Characterization of measured quantities. Factor loadings for Data Set II (at 240 min, 140°C). Parts A, B, and C are plots of unrotated loading values in three dimensions. Part D is the plot of rotated loading values. "B or 2" at the end of abbreviations means reaction time 240 min. See Figure 1 for abbreviations.

TABLE 2
Analysis of Data Set III[a]

	Loading 1	Loading 2	Loading 3
w	**0.860064**	0.03268	−0.04479
E232A	−0.34973	−0.37897	0.696142
E268A	0.12879	**−0.84462**	0.285092
PAV1	0.495745	0.097145	**0.723989**
HEX1	**0.884923**	0.001012	−0.33394
HEP1	**0.946533**	−0.15414	0.111498
TCDEC1	**0.965755**	0.064263	0.033119
TTDEC1	**0.937381**	0.211077	0.090639
SALD1	**0.956229**	0.149933	0.059774
SAC1	**0.967483**	−0.03936	−0.04778
E232B	−0.25789	**−0.8571**	−0.29604
E268B	0.499713	−0.69402	−0.21959
PAV2	**0.966594**	−0.01117	0.103332
HEX2	**0.970729**	−0.04536	−0.08824
HEP2	**0.965359**	−0.09802	0.012536
TCDEC2	**0.968713**	0.044089	0.009985
TTDEC2	**0.968707**	0.042321	0.007897
SALD2	**0.970213**	0.034787	0.004346
SAC2	**0.927096**	−0.17524	−0.07122
Explained variance	13.26752	2.227722	1.390905
Proportion of the total variance (%)	69.8291	11.7249	7.3206

[a]Loading values (unrotated), i.e., the correlation coefficients between the original and new variables. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("A or 1" and "B or 2" at the end of abbreviations means reaction times 180 and 240 min, respectively).

ally less than 0.3, and its absolute value reaches a maximum of 0.334 for HEX1. This behavior is quite common in the field of PCA. The most dominant pattern is expressed by the first two principal components, whereas PAV's "uniqueness" is in the third one.

This can help in the interpretation of the influence of additives. The score values are plotted in Figure 3.

Two outliers can be observed beside a grouping on the most informative Score 1 vs. Score 2 plots (Fig. 3C and D). Additive No. 6 (lecithin) is an outlier according to the first principal component, whereas additive No. 7 (α-tocopherol) is according to the second (the absorbance values). All the other additives are similar to each other. Although the third abstract factor can differentiate among them, it must be kept in mind that this factor explains only about 7% of variability in the data. The outlying observations suggest that, in all probability, lecithin and α-tocopherol react with different mechanisms than the other antioxidants. Lecithin causes a high initial rate of decomposition and a large amount of volatile products. α-Tocopherol differs from the other additives in the amounts of conjugated dienes and trienes.

Rotation of the principal component axes can help in the identification of abstract factors. Moreover, if the dominant pattern remains after rotation, the reliability of the pattern found is enhanced. There are many ways to rotate the axes.

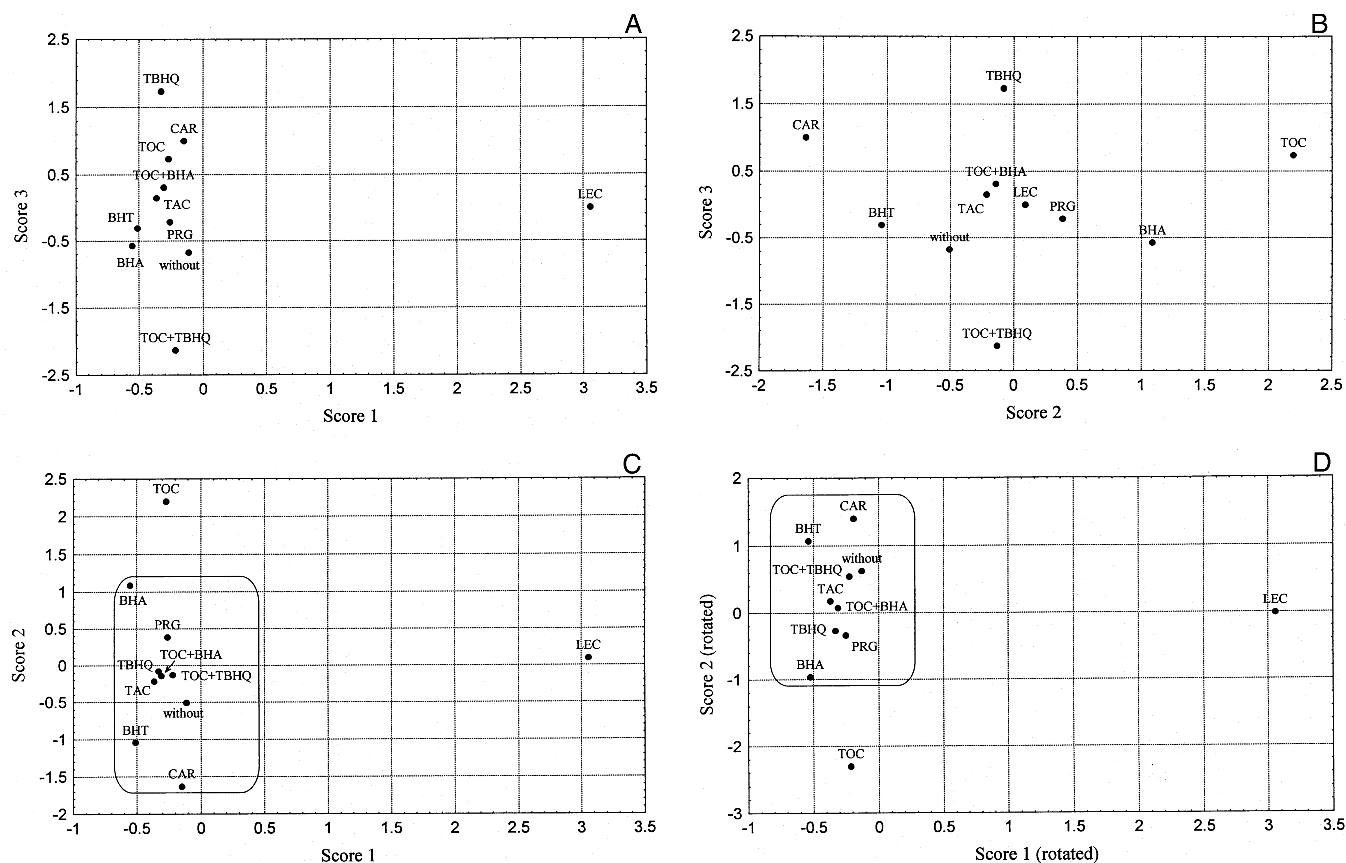

FIG. 3. Characterization of additives. Score plots for Data Set III (joined data for 180 and 240 min, at 140°C). Parts A, B, and C are plots of unrotated scores in three dimensions. Part D is the plot of rotated scores. TOC, α-tocopherol; BHA, butylated hydroxyanisole; PRG, propylgallate; TBHQ, *tert*-butyl hydroquinone; TAC, tocopheryl acetate; BHT, butyl hydroxytoluol; CAR, β-carotene; LEC, lecithin.

We have chosen the varimax rotation (normalized) which preserves the orthogonality of the axes. Varimax rotation of the factor axes enhances the role of the third factor, slightly decreasing the role of the first two abstract factors. The role of PAV1 becomes more expressed if analyzing Data Set I. Although E232A and PAV1 replace each other in their loading values (Data Set III), PAV2 will not correlate with the third abstract factor, influencing rotation and suggesting that the different behavior of PAV1 and PAV2 is not an artifact. Lack of influence of rotation on the dominant patterns strengthens our conclusion, suggesting that the effects are real and not the result of random effects or calculation artifacts.

It should be mentioned that during the reviewing process some additional measurements were made. Although the numbers in the input matrix changed a little, all the conclusions drawn remained the same. This again supports the reliability of the findings.

Naturally, all these statements are valid within the scope of the investigation, i.e., within the measured quantities and additives applied. However, in the case of new additives or additive combinations, the calculations can easily be extended.

The experimental error, as usual, can affect the data and hence the statements. However, PCA has a favorable characteristic: it is not sensitive on the error level or small changes of experimental errors. The underlying philosophy of PCA is that the "noise" comprises the last principal components not even considered and discussed here.

The input matrices (Data Sets I–III; Supplementary Table 1), all results of calculations [tables of loading and score values (Supplementary Tables 2–7 and Supplementary Tables 8–13, respectively) and their plots] are available from the authors upon request.

In conclusion, PCA of measured quantities (and additives) unambiguously shows a close similarity among measured quantities (and additives). Although the findings are applicable exclusively to the additives studied, no doubt similar analysis can be carried out on other, even different type of, additives. Three abstract factors explain 89–97% of variances present in the data. A definite cluster is observed on the loading plots, i.e., initial decomposition rate of hydroperoxides and amounts of volatiles measured by gas chromatography/mass spectrometry are closely related. The outliers are the absorbance values. That is, they are different from the variables in the cluster and carry independent information. PAV is unique at 180 min. It is different from all other quantities, and from the value at 240 min. This latter value fits into the cluster of other quantities. The analysis makes differences in ad-

SUPPLEMENTARY TABLE 1
Transpose of the Input Matrix[a]

	Without	BHA	BHT	TBHQ	PRG	LEC	TOC	TAC	CAR	TOC + BHA	TOC + TBHQ
w	0.189	0.18	0.167	0.227	0.28	0.667	0.202	0.297	0.19	0.401	0.289
E232A	6.71	6.24	7.72	7.82	5.24	5.23	6.23	7.63	7.73	7.26	3.96
E268A	3.73	3.02	4.47	4.55	3.79	4.12	3.09	3.86	4.85	3.91	4.05
PAV1	14.2	12.7	10.2	18.9	16.9	19.9	17.1	14.4	18.5	15.8	10.8
HEX1	11.3	2.3	1.4	1	3.6	26.2	4.6	5.6	3.5	3.8	11.8
HEP1	6.1	1.3	1.2	5.6	2.1	23.7	2.3	3.8	7.9	3.5	3.7
TCDEC1	6.3	10.3	9.2	19	7.6	221	18	5	8.8	5.2	9.4
TTDEC1	16.4	25.3	24.6	52.8	16.1	601	170	12.8	21.5	12.9	19.7
SALD1	46.6	39.2	36.4	79	29.4	906	198	33.6	52.1	32	54.1
SAC1	5.3	0	0	0.5	0	27.6	0	1.1	1.6	0.42	1.4
E232B	7.18	6.46	7.28	6.33	6.09	6.12	5.35	6.99	7.33	6.9	7.05
E268B	4.18	3.27	4.13	3.42	4.5	4.84	2.64	3.67	4.64	3.75	4.05
PAV2	15.3	10.8	15.8	16.4	17.1	62.3	18	13.5	20	15.8	13.1
HEX2	13.6	1.7	5.4	3.1	5	92.5	3.9	8.6	9.3	8.1	14.6
HEP2	11.9	0.54	2.5	2.1	2.8	56.1	2.2	5.2	10.3	4.2	2.6
TCDEC2	17	1.3	20.7	12.2	19.6	610	26.5	5.3	4.7	6.9	1.9
TTDEC2	37.6	3.7	54.1	32.5	51.9	1730	66	14.3	12	17.3	9
SALD2	87	7.7	82.7	51.3	80.4	2340	100	40.2	44.7	43	36.6
SAC2	5.8	0	0	0	0	16.7	0	0.71	4.4	0.42	2

[a]Data Set I: columns were *w*, E232A, E268A, PAV1, HEX1, HEP1, TCDEC1, TTDEC1, SALD1, SAC1, rows were without additive and BHA, BHT, TBHQ, PRG, LEC, TOC, TAC, CAR, TOC + BHA, TOC + TBHQ. Data Set II: columns were *w*, E232B, E268B, PAV2, HEX2, HEP2, TCDEC2, TTDEC2, SALD2, SAC2; rows were without additive and BHA, BHT, TBHQ, PRG, LEC, TOC, TAC, CAR, TOC + BHA, TOC + TBHQ. Data Set III: Data Set I + Data Set II. For abbreviations see Table 1 in the paper. ("A or 1" at the end of abbreviations means reaction time 180 min and "B or 2" at the end of abbreviations means reaction time 240 min.)

SUPPLEMENTARY TABLE 2
Analysis of Data Set I[a]

	Loading 1	Loading 2	Loading 3
w	**0.893434**	0.028384	0.036163
E232A	–0.38788	**–0.77532**	–0.18348
E268A	0.09577	**–0.76834**	0.596667
PAV1	0.527503	–0.52296	–0.54145
HEX1	**0.910042**	0.241871	0.212199
HEP1	**0.962098**	–0.20993	0.06894
TCDEC1	**0.984261**	–0.01872	–0.00316
TTDEC1	**0.963815**	0.024092	–0.13234
SALD1	**0.980582**	0.011718	–0.07646
SAC1	**0.980958**	–0.01128	0.072582
Explained variance	6.811444	1.569528	0.762573
Proportion of the total variance (%)	68.1144	15.6953	7.6257

[a]Loading values (unrotated), i.e., the correlation coefficients between the original and new variables. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("A or 1" at the end of abbreviations means reaction time 180 min).

SUPPLEMENTARY TABLE 3
Analysis of Data Set II[a]

	Loading 1	Loading 2	Loading 3
w	**0.888758**	0.069859	0.213401
E232B	–0.21725	**–0.92681**	0.291227
E268B	0.554371	**–0.73626**	–0.34826
PAV2	**0.987504**	0.077275	–0.05757
HEX2	**0.99411**	–0.01561	0.067134
HEP2	**0.9897**	–0.05335	0.014663
TCDEC2	**0.990031**	0.103633	0.013027
TTDEC2	**0.99014**	0.10169	0.015161
SALD2	**0.991785**	0.092617	0.016869
SAC2	**0.948105**	–0.16708	–0.00249
Explained variance	7.930421	1.47257	0.260367
Proportion of the total variance (%)	79.3042	14.7257	2.6037

[a]Loading values (unrotated), i.e., the correlation coefficients between the original and new variables. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("B or 2" at the end of abbreviations means reaction time 240 min).

ditives clear. Rotation of the axes does not change the dominant patterns found supporting our conclusions. The investigations provide us with the factors having independent information content, simplifying the necessary measurements in analogous systems.

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SUPPLEMENTARY TABLE 4
Analysis of Data Set III[a]

	Loading 1	Loading 2	Loading 3
w	**0.860064**	0.03268	–0.04479
E232A	–0.34973	–0.37897	0.696142
E268A	0.12879	**–0.84462**	0.285092
PAV1	0.495745	0.097145	**0.723989**
HEX1	**0.884923**	0.001012	–0.33394
HEP1	**0.946533**	–0.15414	0.111498
TCDEC1	**0.965755**	0.064263	0.033119
TTDEC1	**0.937381**	0.211077	0.090639
SALD1	**0.956229**	0.149933	0.059774
SAC1	**0.967483**	–0.03936	–0.04778
E232B	–0.25789	**–0.8571**	–0.29604
E268B	0.499713	–0.69402	–0.21959
PAV2	**0.966594**	–0.01117	0.103332
HEX2	**0.970729**	–0.04536	–0.08824
HEP2	**0.965359**	–0.09802	0.012536
TCDEC2	**0.968713**	0.044089	0.009985
TTDEC2	**0.968707**	0.042321	0.007897
SALD2	**0.970213**	0.034787	0.004346
SAC2	**0.927096**	–0.17524	–0.07122
Explained variance	13.26752	2.227722	1.390905
Proportion of the total variance (%)	69.8291	11.7249	7.3206

[a]Loading values (unrotated), i.e., the correlation coefficients between the original and new variables. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("A or 1" and "B or 2" at the end of abbreviations means reaction time 180 and 240 min, respectively).

SUPPLEMENTARY TABLE 5
Analysis of Data Set I[a]

	Loading 1	Loading 2	Loading 3
w	**0.893345**	–0.002656	0.047604
E232A	–0.451344	0.452379	0.613899
E268A	0.093802	**0.969165**	0.086327
PAV1	0.446268	0.018624	**0.803377**
HEX1	**0.938171**	–0.042115	–0.223089
HEP1	**0.948751**	0.195163	0.190367
TCDEC1	**0.977389**	0.005092	0.117540
TTDEC1	**0.949303**	–0.113396	0.181662
SALD1	**0.969732**	–0.066737	0.150631
SAC1	**0.980773**	0.050599	0.056544
Explained variance	6.748785	1.204043	1.190718
Proportion of the total variance (%)	67.4878	12.0404	11.9072

[a]Rotated loading values, i.e., the correlation coefficients between the original and new variables after varimax rotation. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("A or 1" at the end of abbreviations means reaction time 180 min).

SUPPLEMENTARY TABLE 6
Analysis of Data Set II[a]

	Loading 1	Loading 2
w	**0.891418**	–0.012046
E232B	–0.276927	**0.910758**
E268B	0.505432	**0.770681**
PAV2	**0.990437**	–0.013040
HEX2	**0.991002**	0.080080
HEP2	**0.984153**	0.117449
TCDEC2	**0.994669**	–0.039178
TTDEC2	**0.994651**	–0.037232
SALD2	**0.995704**	–0.028071
SAC2	**0.935266**	0.228246
Explained variance	7.903234	1.499757
Proportion of the total variance (%)	79.0323	14.9976

[a]Rotated loading values, i.e., the correlation coefficients between the original and new variables after varimax rotation. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("B or 2" at the end of abbreviations means reaction time 240 min).

SUPPLEMENTARY TABLE 7
Analysis of Data Set III[a]

	Loading 1	Loading 2	Loading 3
w	**0.860606**	–0.00134	–0.04626
E232A	–0.35916	0.225047	**0.755574**
E268A	0.107019	**0.774699**	0.446815
PAV1	0.498336	–0.22556	0.692925
HEX1	**0.88454**	0.087418	–0.32334
HEP1	**0.942272**	0.153079	0.144243
TCDEC1	**0.967104**	–0.04499	0.024388
TTDEC1	**0.942553**	–0.20094	0.05166
SALD1	**0.959804**	–0.13445	0.033561
SAC1	**0.966127**	0.072581	–0.03445
E232B	–0.28006	**0.891849**	–0.12225
E268B	0.481539	**0.736182**	–0.07589
PAV2	**0.966018**	0.01509	0.108111
HEX2	**0.969203**	0.086526	–0.07292
HEP2	**0.962508**	0.118098	0.036241
TCDEC2	**0.969532**	–0.02058	0.005705
TTDEC2	**0.96948**	–0.01843	0.004007
SALD2	**0.970789**	–0.01031	0.002021
SAC2	**0.922234**	0.209343	–0.03081
Explained variance	13.26015	2.202218	1.42378
Proportion of the total variance (%)	69.7903	11.5906	7.4936

[a]Rotated loading values, i.e., the correlation coefficients between the original and new variables after varimax rotation. Values larger than 0.7 are indicated in bold. For abbreviations see Table 1 in the paper ("A or 1" and "B or 2" at the end of abbreviations means reaction time 180 and 240 min, respectively).

SUPPLEMENTARY TABLE 8
Analysis of Data Set I[a]

	Score 1	Score 2	Score 3
Without	–0.15866	0.307082	0.199146
BHA	–0.55083	1.175433	–0.85113
BHT	–0.67487	–0.41785	1.395982
TBHQ	–0.24811	–1.51108	–0.38706
PRG	–0.27462	0.482151	–0.40567
LEC	**2.970218**	–0.02929	0.079001
TOC	–0.13806	0.712349	–1.81068
TAC	–0.33617	–0.23555	–0.09011
CAR	–0.22675	–1.69937	0.304374
TOC + BHA	–0.24327	–0.29816	–0.30339
TOC + TBHQ	–0.11889	1.514294	1.869534

[a]Score values (unrotated) i.e., the principal components. For abbreviations see Table 1 in the paper. The outlier is indicated by bold.

SUPPLEMENTARY TABLE 9
Analysis of Data Set II[a]

	Score 1	Score 2
Without	–0.11987	–0.9187
BHA	–0.54917	0.629923
BHT	–0.38675	–0.82801
TBHQ	–0.40111	0.689464
PRG	–0.19797	0.126853
LEC	**2.988513**	0.257176
TOC	–0.4157	**2.288214**
TAC	–0.31833	–0.18703
CAR	–0.12414	–1.38107
TOC + BHA	–0.22061	–0.10529
TOC + TBHQ	–0.25487	–0.57154

[a]Score values (unrotated) i.e., the principal components. For abbreviations see Table 1 in the paper. The outliers are indicated by bold.

SUPPLEMENTARY TABLE 10
Analysis of Data Set III[a]

	Score 1	Score 2	Score 3
Without	–0.11221	–0.50827	–0.67716
BHA	–0.55108	1.086275	–0.57568
BHT	–0.51061	–1.04377	–0.3092
TBHQ	–0.32661	–0.0808	1.732329
PRG	–0.25682	0.382741	–0.21752
LEC	**3.057406**	0.091768	–0.00474
TOC	–0.26887	**2.197432**	0.733193
TAC	–0.361	–0.21614	0.146776
CAR	–0.14711	–1.635	0.999472
TOC + BHA	–0.3056	–0.14275	0.306666
TOC + TBHQ	–0.2175	–0.13148	–2.13414

[a]Score values (unrotated) i.e., the principal components. For abbreviations see Table 1 in the paper. The outliers are indicated by bold.

SUPPLEMENTARY TABLE 11
Analysis of Data Set I[a]

	Score 1	Score 2	Score 3
Without	–0.12148	–0.09193	–0.36868
BHA	–0.54056	–1.43842	–0.21956
BHT	–0.58447	1.253299	–0.81636
TBHQ	–0.377	0.858419	1.271113
PRG	–0.27476	–0.62776	–0.05359
LEC	**2.958483**	0.054852	0.271412
TOC	–0.23863	–1.74477	0.838995
TAC	–0.35706	0.115773	0.188994
CAR	–0.3116	1.462982	0.891353
TOC + BHA	–0.28618	0.017858	0.397383
TOC + TBHQ	0.133252	0.139694	–2.40106

[a]Score values applying varimax rotation, i.e., the rotated principal components. For abbreviations see Table 1 in the paper. The outlier is indicated by bold.

SUPPLEMENTARY TABLE 12
Analysis of Data Set II[a]

	Score 1	Score 2
Without	–0.17922	0.908986
BHA	–0.50714	–0.66423
BHT	–0.43966	0.801172
TBHQ	–0.35553	–0.71404
PRG	–0.18932	–0.13943
LEC	**2.998902**	–0.06273
TOC	–0.26635	**–2.31036**
TAC	–0.32979	0.165978
CAR	–0.21349	1.370103
TOC + BHA	–0.22698	0.090749
TOC+TBHQ	–0.29141	0.553799

[a]Score values applying varimax rotation, i.e., the rotated principal components. For abbreviations see Table 1 in the paper. The outliers are indicated by bold.

SUPPLEMENTARY TABLE 13
Analysis of Data Set III[a]

	Score 1	Score 2	Score 3
Without	–0.12554	0.628953	–0.56404
BHA	–0.52302	–0.96486	–0.7814
BHT	–0.53751	1.070964	–0.09952
TBHQ	–0.328	–0.27116	1.712617
PRG	–0.24692	–0.33865	–0.29001
LEC	**3.058755**	–0.01135	–0.00818
TOC	–0.21175	**–2.30504**	0.283723
TAC	–0.36642	0.173661	0.184828
CAR	–0.18897	1.401197	1.30183
TOC + BHA	–0.30908	0.071583	0.327349
TOC + TBHQ	–0.22155	0.544704	–2.06721

[a]Score values aplying varimax rotation, i.e., the rotated principal components. For abbreviations see Table 1 in the paper. The outliers are indicated by bold.

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ARTICLE

Lymphatic Fatty Acid Absorption Profile During 24 Hours After Administration of Triglycerides to Rats

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ABSTRACT: In this study we determined in rats the complete 24-h lymphatic fatty acid profile after administration of either rapeseed oil (RO) or rapeseed oil interesterified with 10:0 (RO/C_{10}) with special emphasis on the transition from absorptive to postabsorptive phase. Rats were subjected to cannulation of the main mesenteric lymph duct and the next day oils were administered through a gastric feeding tube. Lymph was collected in 1-h fractions for the following 24 h. The time for maximum lymphatic transport of fatty acids was at 4 h with fast changes in fatty acid composition from the fatty acids of endogenous origin to those of the administered oils. Seven to eight hours after administration the transport was significantly lower than maximum, indicating the change from absorptive to postabsorptive phase. At 24 h after administration of either oil the transport of total fatty acids, palmitic acid (16:0), and linoleic acid (18:2n-6) together with oleic acid (18:1n-9) after RO had not returned to the transport at baseline. In contrast, the transport of decanoic acid (10:0) and α-linolenic acid (18:3n-3) returned to baseline values between 12 and 15 h. This indicated that the absorption of purely exogenous fatty acids (illustrated by 10:0 and 18:3n-3) was complete at 15 h and that the fatty acids transported between 15 and 24 h were derived mostly from endogenous stores.

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Dietary triacylglycerols are hydrolyzed by preduodenal lipases and pancreatic lipases (1–3). By the action of these lipases the triacylglycerols are hydrolyzed to 2-monoacylglycerols and free fatty acids prior to absorption (3–5). Triacylglycerols are resynthesized from monoacylglycerols and long-chain fatty acids in the enterocyte (6) and are assembled together with phospholipids, cholesterol, cholesterol esters, and apolipoproteins to form chylomicrons secreted to the lymph (7–9). After reaching the circulation the triacylglycerols are degraded in the extrahepatic tissues by lipoprotein lipase and the resulting fatty acids are absorbed by most tissues for triacylglycerol synthesis or for energy provision (10,11). Short- and medium-chain fatty acids are absorbed to a higher degree through the portal vein and transported in the blood bound to albumin (12).

Bollman *et al.* (13) developed a rat model, used by many other research groups as well, to investigate the lymphatic absorption of fatty acids. Researchers often observe the absorption for 24 h with frequent collections of lymph samples during the absorptive phase and a pooled sample in the phase supposed to be postabsorptive (14–18), while some concentrate on investigating the absorptive phase (19–21). Others collect samples less frequently during the 24-h experiment (22) or only one sample from 0 to 24 h (23). To our knowledge, nobody has examined in detail the changes in fatty acid profile during the postabsorptive phase.

In the present study we determined the complete fatty acid absorption profile after administration of rapeseed oil (RO) or rapeseed oil interesterified with 10:0 (RO/C_{10}) to lymph-cannulated rats by collecting 1-h lymph samples throughout a period of 24 h. The purpose was to investigate in more detail the phase from maximal absorption to baseline in order to decide the optimal time to finish a lymph absorption study. This was performed partly to report the most correct results and partly from an ethical point of view, i.e., to reduce the collection time for the animals involved.

EXPERIMENTAL PROCEDURES

Animals and surgery. The following experiment was approved by the Danish Committee for Animal Experiments. Male albino Wistar rats were obtained from Møllegaard Breeding and Research Centre, Ll. Skensved, Denmark. They were fed a standard rat chow diet (Altromin No. 1324, Chr. Petersen A/S, Ringsted, Denmark) and weighed 250–280 g at the time of surgery. Nonfasted rats (6 in each group) were anesthetized intraperitoneally with pentobarbital (approximately 0.05 mg/g body weight) and were subjected to cannulation of the main mesenteric lymph duct (13) with a clear vinyl tube (o.d. 0.8 mm, i.d. 0.5 mm; Critchley Electrical Products Pty. Ltd., NSW, Australia). A feeding silicone tube (o.d. 3.0 mm, i.d. 1.0 mm; Polystan, Værløse, Denmark) was inserted into the fundus region of the stomach and fixed with a purse-string suture. Following surgery the rats were placed in individual restraining cages (24) with tap water freely

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Abbreviations: RO, rapeseed oil; RO/C_{10}, rapeseed oil interesterified with 10:0.

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available, no food, but a steady infusion of physiological saline (0.9% NaCl) at 3 mL/h through the feeding tube.

Administration of oil and collection of lymph. The next day, the experiment was started by collection of a baseline fraction of lymph from −1 to 0 h. At time zero 0.3 mL of oil was injected through the feeding tube followed by 0.5 mL saline, and the infusion of saline was continued at 3 mL/h. For the following 24 h, lymph was collected in 1-h fractions in tubes containing 100 μL of a 10% (wt/vol) $Na_2EDTA \cdot 2H_2O$. They were frozen immediately after collection and kept at −20°C until analysis.

Oils and lipid analysis. RO (Aarhus Olie, Aarhus, Denmark) and RO/C_{10} (manufactured at the Department of Biotechnology, DTU, Lyngby, by random interesterification of rapeseed oil with tri-10:0 using sodium methoxide as catalyst) were used for the experiment. The fatty acid profile of triacylglycerols was determined by gas–liquid chromatography after methylation with KOH in methanol (25). The resulting fatty acid methyl esters were analyzed using a Hewlett-Packard 5890 chromatograph with a fused-silica capillary column (SP-2380, 60 m, i.d. 0.25 mm), flame-ionization detection (FID), and helium as a carrier gas. Initial oven temperature was 70°C followed by temperature programming: 15°C/min until 160°C, followed by 1.5°C/min until 200°C which was maintained for 15 min, and finally the temperature was raised to 225°C and maintained for 5 min. Peak areas were calculated using a Hewlett-Packard computing integrator and were used to calculate the mol% of fatty acids following correction for response factors based on calibrated standards (Nu-Chek-Prep, Elysian, MN).

Analysis of lymph lipids. Total lipid was extracted from lymph fractions according to the method by Folch *et al.* (26) after addition of internal standards (13:0 and 17:0 methyl esters). After methylation with KOH in methanol the fatty acids were analyzed by gas–liquid chromatography as described above. The internal standards were used to calculate the amounts of fatty acids transported in lymph, 13:0 for chain lengths up to C_{14} and 17:0 for chain lengths of C_{16} and above.

Statistics. Results were expressed as mean ± standard error of the mean (SEM). Differences between lymphatic transport at different time points were determined by paired *t*-test using the Jandel SigmaStat statistical package (Jandel Corporation, Erkrath, Germany). The level of statistical significance was taken as $P < 0.05$.

RESULTS

Fatty acid composition of oils (Table 1). The major fatty acids (in mol%) in RO were oleic acid (18:1n-9, 55.3%), linoleic acid (18:2n-6, 21.8%), and α-linolenic acid (18:3n-3, 9.7%), while those in RO/C_{10} were decanoic acid (10:0, 40.8%), 18:1n-9 (33.0%), 18:2n-6 (12.7%), and 18:3n-3 (5.8%).

Lymphatic transport. The peak of total fatty acid lymphatic transport was at 4 h, reaching values of 15.7 ± 1.9 mg/h after RO and 20.1 ± 4.2 mg/h after RO/C_{10}, and thereafter declining toward baseline values (Fig. 1). The average lymph flow after administration of RO was 3.0 ± 0.8 mL/h and 3.0 ± 0.7 mL/h after RO/C_{10}. The fatty acids of lymph lipids [mainly palmitic (16:0), stearic (18:0), linoleic (18:2n-6), and arachidonic acid (20:4n-6) (not shown)] changed quickly after administration of oils to reflect the administered oils. A particularly steep rise in the content of 18:1n-9 was observed between 1 and 4 h (Fig. 2A and 2B). The lymphatic transport at baseline differed between fatty acids with transport values close to 0 for 10:0, 18:1n-9, and 18:3n-3, indicating that these fatty acids were not present in the fasting animal in appreciable amounts.

Considering the lymphatic transport of total fatty acids after administration of RO and RO/C_{10} (Fig. 1) significantly higher values ($P < 0.05$) were observed at 24 h compared with 0 h, indicating that the transport had not returned to baseline at the end of the experiment. After administration of RO significant differences were observed between the transport at 24 h and the values from 2 to 10 h. After administration of

TABLE 1
Fatty Acid Composition (mol%) of Oils[a]

Fatty acid	Rapeseed oil (RO)	Rapeseed oil/10:0 (RO/C_{10})
10:0	0.0	40.8
16:0	5.4	2.9
18:0	1.7	1.1
18:1n-9	55.3	33.0
18:1n-7	3.5	1.9
18:2n-6	21.8	12.7
18:3n-3	9.7	5.8
20:0	0.6	0.3
20:1n-9	1.1	0.7
Others	0.9	0.8

[a]Values represent the mean of three determinations.

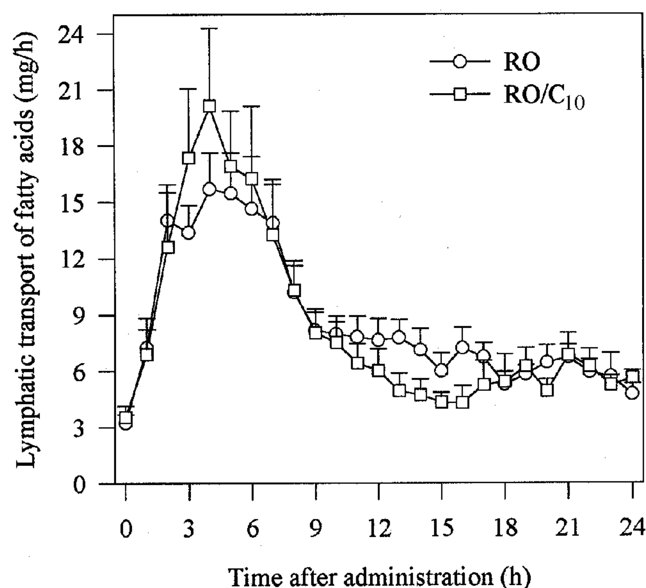

FIG. 1. Lymphatic transport of total fatty acids following intragastric administration of rapeseed oil (RO) and rapeseed oil interesterified with decanoic acid (RO/C_{10}). Values are means ± SEM (n = 6).

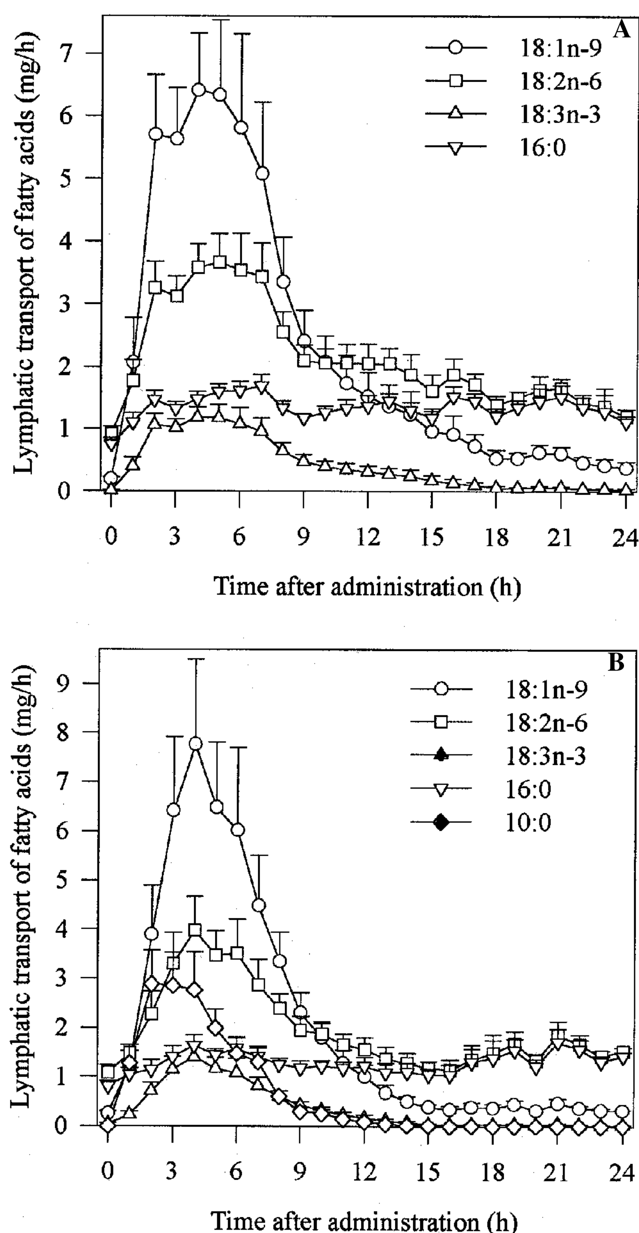

FIG. 2. Lymphatic transport of fatty acids (10:0, 16:0, 18:1n-9, 18:2n-6 and 18:3n-3) following intragastric administration of (A) RO and (B) RO/C_{10}. Values are means ± SEM ($n = 6$). See Figure 1 for abbreviations.

RO/C_{10}, differences were observed between values at 24 h and the values at 3 to 7 h, indicating a more rapid decline from maximal transport toward baseline after this oil compared with RO, although not reaching baseline.

The lymphatic transport of 16:0 and 18:2n-6 after administration of both oil, and of 18:1n-9 after administration of RO (Fig. 2) had not returned to baseline at 24 h (significantly higher than the value at 0 h; $P < 0.05$). There was no difference ($P > 0.05$) between 0 h and 15 to 24 h values for lymphatic transport of 18:3n-3 after RO. After RO/C_{10} no differences were observed between baseline and 15- to 24-h values for 10:0, 14- to 24-h values for 18:1n-9, and 12- to 24-h values for 18:3n-3, indicating that the transport of these fatty acids had returned to baseline before the end of the experiment.

The value at the time for maximal transport of fatty acids after RO administration was significantly higher than the transport values from 8 to 24 h ($P < 0.05$) for total fatty acids and for C_{18}-fatty acids. After RO/C_{10} administration the maximal transport was significantly higher ($P < 0.05$) than the values from 7 to 24 h for 18:1n-9, 18:3n-3, and total fatty acids, and higher than the values from 8 to 24 h for 10:0 and 18:2n-6. There was no difference between the transport at 8 h and the values at 9 to 14 h for total fatty acids and for 18:2n-6, and between the values at 8 h and 9 to 11 h for 18:1n-9 and 18:3n-3 after RO. Compared with this, no differences were observed after RO/C_{10} administration between the return from maximal transport and the values at 8 h for 18:1n-9 and 18:3n-3, 9 to 10 h for 10:0, 9 to 11 h for 18:2n-6, and 8 to 11 h for total fatty acids.

DISCUSSION

The profiles of fatty acid transport were similar for total fatty acids after administration of RO and RO/C_{10} with maximal lymphatic transport at 4 h and thereafter decreasing toward the transport at baseline. The return toward baseline was slower for total fatty acids, 16:0 and 18:2n-6, after both oils together with 18:1n-9 after RO (significant differences between 0 and 24 h), than for 18:3n-3 after RO and for 10:0, 18:1n-9, and 18:3n-3 after RO/C_{10} (no significant differences). This indicates that the fatty acid composition of lymph at 24 h does not necessarily parallel the fatty acid composition at baseline, and if a complete return to baseline is considered important for the interpretation of an experiment, it must exceed 24 h. Furthermore, the absorption profiles for fatty acids like 10:0 and 18:3n-3 show that these fatty acids are exclusively exogenous fatty acids with transport values close to 0 in the fasting state and a return to baseline between 12 and 15 h after administration. Thus, 10:0 and 18:3n-3 could act as markers for total absorption of exogenously administered fat, indicating that the transport of fatty acids after 15 h is almost purely endogenously derived.

For practical reasons, a collection period of 15 h is not very suitable and the performed experiments will be a compromise between acceptable recoveries and reasonable collection periods. From our data it is possible to calculate the underestimate if the experiment is brought to an end before 24 h. Ending the experiment after RO/C_{10} administration at 8 or 10 h would give a 10:0 recovery compared with 24 h values of 95 and 98%, respectively. The same values for 18:3n-3 are 81 and 90%, respectively. Compared with this, RO administration leads to 18:3n-3 recovery of 73 and 81% at 8 and 10 h, respectively. From these calculations it is obvious that RO/C_{10} is absorbed more rapidly than RO and that 18:3n-3 is absorbed at a slower rate compared with 10:0. Furthermore, ending the experiment 8 h after oil administration leads especially to a considerable underestimate of 18:3n-3 absorption.

There are marked differences in the way different researchers perform lymph absorption experiments concerning the frequency with which samples are collected and the duration of the total period of collection. From our experiment it is evident that remarkable changes can happen during 1-h collections, especially during the absorptive phase. Collection of lymph with frequency less than 1 h is not advisable if the purpose is a detailed analysis of the changes in fatty acid composition during absorption or concerns individual fatty acids. Depending on the aim of the experiment, less frequent sampling could suffice, like Nilsson *et al.* (15), Ikeda *et al.* (18), and Reicks *et al.* (22), primarily investigating the recovery of fatty acids.

Because of differences in fatty acid compositions, administered fats give rise to different absorption profiles and thereby return toward baseline values at different times, as seen in this experiment when comparing the absorption of RO and RO/C_{10}, and as seen by Degrace *et al.* (17), Rayo *et al.* (20), and Bergstedt *et al.* (27). It is therefore important to cover the entire absorption process in time when absorption of different dietary fats is compared. In our experiment, transport values significantly lower than maximal values were not reached until 7 to 8 h after administration, indicating the transition from absorptive to postabsorptive phase. This means that it is necessary to collect frequent samples for 8 h after the administration in order to identify and pass the absorption maximum. Investigators like Degrace *et al.* (17) and Chernenko *et al.* (21) stop the frequent collection of lymph samples 6 h after administration of fat. Although absorption of different fats results in different absorption profiles, 6 h after administration could not be considered the end of the absorptive phase. On the other hand, nothing is gained in continuing the frequent collection of samples beyond this point because in this experiment no differences were observed between the transport at 7 and 8 h (values significantly lower than maximum) and the transport until 11 to 14 h for most fatty acids, the only exceptions being 18:1n-9 and 18:3n-3 after RO/C_{10} (values at 9 h significantly lower than values at 7 h).

The results from this experiment led to the conclusions that collection of lymph samples for 8 h after administration of fat is sufficient to cover the major part of the absorptive phase. From an ethical point of view, the animals could be killed after this period because a fatty acid composition of lymph at 24 h, similar to that of the fasting animal, will not be obtained anyway. On the other hand, collection of lymph for more than 8 h is necessary to estimate the recovery of administered fat, because the absorption of exogenous fat has not been completed at 8 h after administration, as illustrated by the 10:0 and 18:3n-3 absorption profiles. Continuing the collection for 24 h leads to a higher degree of mixing exogenous fatty acids with endogenously derived fatty acids.

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Dietary Effects of Conjugated Octadecatrienoic Fatty Acid (9 *cis*, 11 *trans*, 13 *trans*) Levels on Blood Lipids and Nonenzymatic *in vitro* Lipid Peroxidation in Rats

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ABSTRACT: The present study examined the antioxidant activity of conjugated octadecatrienoic fatty acid (9 *cis*,11 *trans*,13 *trans*-18:3), α-eleostearic acid, of karela seed *(Momordica charantia)*, fed to rats for 4 wk. The growth pattern of rats and the effect on plasma cholesterol and high density lipoprotein (HDL) cholesterol and peroxidation of plasma lipid, lipoprotein, eryhrocyte membrane, and liver lipid were measured. Rats were raised on diets containing sunflower oil mixed with three different levels of conjugated trienoic fatty acid (9*c*,11*t*,13*t*-18: 3) 0.5, 2, and 10% by weight; the control group was raised with sunflower oil as dietary oil as the source of linoleic acid (9*c*,12*c*-18:2). The growth pattern of the three experimental groups of rats showed no significant difference compared to the control group of rats, but the group with 10% 9*c*,11*t*,13*t*-18:3 had slightly higher body weight than the control group of rats. Concentrations of total cholesterol, HDL-cholesterol, and non-HDL-cholesterol in plasma were similar in all four groups. Plasma lipid peroxidation was significantly lower in the case of 0.5% 9*c*,11*t*,13*t*-18:3 group than the control group and the 2 and 10% 9*c*,11*t*,13*t*-18:3 dietary groups as well. Lipoprotein oxidation susceptibility test with 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups was significantly less susceptible to lipoprotein peroxidation when compared with sunflower oil dietary group, and the dietary group with 0.5% 9*c*,11*t*,13*t*-18:3 showed least susceptibility. There was significant lowering in erythrocyte ghost membrane lipid peroxidation in the 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups compared to the sunflower oil groups. Nonenzymatic liver tissue lipid peroxidation was significantly lower in the group of rats raised on 0.5% 9*c*,11*t*,13*t*-18:3, but the groups on 2 and 10% 9*c*,11*t*,13*t*-18:3 acid did not show any significant difference compared with the control group of rats.

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Polyunsaturated fatty acids (PUFA) are susceptible to autoxidation giving rise to peroxyl free radical. Peroxidation takes place both in *in vivo* (1) and *in vitro* (2) conditions. *In vivo* lipid peroxidation takes place either by free radicals or enzymatically (2). Growing evidence exists that the oxidation of lipids plays a significant role in the development of atherosclerosis (3). Free radicals are constantly being generated, and antioxidant defense mechanism neutralizes them, making them ineffective (4). When the free-radical generation exceeds the scavenging capacity of the antioxidant defenses, the result is oxidative stress; there is a preponderance of free radicals that initiate *in vivo* peroxidation of PUFA of membrane lipids (5). There are currently four nutrients—ascorbic acid (5); conjugated linoleic acid (CLA) (6); one food preservative (butylated hydroxytoluene); and one drug (probucol) (7)—that function as *in vivo* antioxidants. The latter two also are antiatherosclerotic in animal models (7,8).

Growing interest exists in dietary conjugated fatty acids, especially CLA, which was shown by several workers to be an effective agent in reducing the incidence of chemically induced cancers and to act as chemoprotective agent (9). Anticarcinogenic effects of CLA might be attributed to their antioxidant activity (10) that serves as an *in-situ* defense mechanism against membrane attack by free radicals. However, recent reports from Van den Berg *et al*. (11) and Chen *et al.* (12) raised some doubts regarding the antioxidant property of CLA.

Conjugated linolenic acid (α-eleostearic acid: 9*c*,11*t*,13*t*-18:3) commonly found in karela seed (*Momordica charantia*) was nutritionally evaluated in our laboratory (13). However, its role as an antioxidant was not established.

In the present study, an attempt was made to determine the antioxidant effects of α-eleostearic acid 9*c*,11*t*,13*t*-18:3 supplemented at three different percentages (0.5, 2, 10%) in the diet by measuring plasma lipid, lipoprotein, erythrocyte membrane (EM), and liver lipid peroxidations. We also studied the growth rate and lipid profile of plasma using the same rat model.

EXPERIMENTAL PROCEDURES

Dietary fat sources. Authentic karela seeds, obtained from the local market of Calcutta, India, were crushed into fine particles,

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Abbreviations: CLA, conjugated linoleic acid; EM, erythrocyte membrane; HDL, high density lipoprotein; I.P., Indian Pharmacopia; LOS, lipoprotein oxidation susceptibility; PUFA, polyunsaturated fatty acid; TBARS, thiobarbituric acid-reactive substance.

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and the oil was extracted in a Soxhlet apparatus with *n*-hexane (40–60°C boiling point range). The free fatty acids present in the oil were then removed by miscella refining process (14). The extracted oil containing hexane, known as miscella (hexane/oil, 2:1) was mixed with 10% NaOH solution (20% excess of the theoretical amount required) at 40°C for 30 min to neutralize the free fatty acids. The soap formed was removed by centrifugation and the organic phase was washed with water. Deacidified oil was recovered after removing the solvent under vacuum distillation and drying under vacuum.

The refined oil was then bleached with tonsil earth optimum (1% w/w) (15) obtained from P.T. Sud-Chemic (Jakarta, Indonesia) and activated carbon (0.2% w/w), supplied by E. Merck India Pvt. Ltd. (Bombay, India) at 60°C under vacuum for 20 min. After the bleaching operation, the oil was recovered by vacuum filtration and stored at −20°C under nitrogen.

Dietary fat blends. Sunflower oil (trade name Sundrop) was obtained from I.T.C., Ltd. (Hyderabad, India). Sunflower oil was mixed with 1, 4, and 20% karela oil to give a final oil mixture containing 0.5, 2, and 10% by weight eleostearic acid, respectively; see Table 1 for fatty acid composition of the dietary oil mixtures.

Analysis of fat products. Gas chromatography was used as described previously (16). Fatty acid methyl esters were prepared following published procedures (17). Briefly, approximately 50 mg of oil was dissolved in 0.5 mL of diethyl ether, and 1.0 mL of 0.5 N methanolic KOH solution was added. The reaction mixture was shaken for 10 min at room temperature, and shaken vigorously after the addition of 1.0 mL of 1 N HCl. Methyl esters were extracted with 3 × 1 mL portions of petroleum ether. The extracts were combined and the solvent removed by a flow of nitrogen in a screw-capped test tube.

Conjugation of α-eleostearic acid present in karela seed oil was determined by ultraviolet spectrophotometric analysis at 262, 268, and 274 nm (18).

Feeding experiment. Animal experiments were designed and carried out as reported previously (19). Male albino rats of Charles Foster strain were housed in individual cages and were fed the dietary oils and fresh water *ad libitum*. Daily food consumption and weekly body weight gain were recorded. The feeding experiment was conducted to evaluate the antioxidant effect of α-eleostearic acid supplementation at three different levels.

Thirty-two rats (70–80 g body weight) were divided into four groups each consisting of eight rats having equal average body weight. The rats were fed experimental diets composed of fat-free casein, 18%; fat, 20%; starch, 55%; salt mixture, 4% [composition of salt mixture No. 12 (in g): NaCl, 292.5; KH_2PO_4, 816.6; $MgSO_4$, 120.3; $CaCO_3$, 800.8; $FeSO_4 \cdot 7H_2O$, 56.6; KCl, 1.66; $MnSO_4 \cdot 2H_2O$, 9.35; $ZnCl_2$, 0.5452; $CuSO_4 \cdot 5H_2O$, 0.9988; $CoCl_2 \cdot 6H_2O$, 0.0476] (20); cellulose, 3%; one multivitamin capsule [vitamin A I.P. (Indian Pharmacopia) 10,000 units; thiamine mononitrate I.P. 5 mg; vitamin B I.P. 5 mg; calcium pantothenate USP 5 mg; niacinamide I.P. 50 mg; ascorbic acid I.P. 400 units; cholecalciferol USP 15 units; menadione I.P. 9.1 mg; folic acid I.P. 1 mg, vitamin E USP 0.1 mg] per kg of diet. The diets were adequate in all nutrients.

Rats were maintained on the above diets *ad libitum* for 4 wk. For each rat the amount of daily diet consumed and weekly body weight gain were noted. Rats were killed while under mild anesthesia, blood was collected, and liver was immediately excised, blotted, and stored at −40°C before analysis.

Lipid analysis. According to the standard methods, the lipid components such as total cholesterol (21) and high density lipoprotein (HDL)-cholesterol (22) of plasma were analyzed using enzymatic kits supplied by Ranbaxy Diagnostics Ltd. (New Delhi, India).

Plasma lipid peroxide was measured by the assay of thiobarbituric acid-reactive substances (TBARS) according to the standard method (23). The amount of malondialdehyde formed was calculated by taking the extinction coefficient of malondialdehyde to be 1.56×10^5 M^{-1} cm^{-1}.

Lipoprotein oxidation susceptibility (LOS) test. Non-HDL-cholesterol oxidation was carried out by precipitating apoB-containing lipoprotein (low density lipoprotein and very low density lipoprotein) according to Bachorik and Albers (24). The LOS test was carried out according to the method described by Phelps and Harris (25).

Preparation and oxidative sensitivity of EM ghost. After plasma separation, the erythrocytes were washed three times with 3 vol 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 EM ghosts were prepared by using hypotonic solution of NaCl according to the method of Rose and Oklander (26).

A modification of the 2-thiobarbituric acid test (27) was used to measure lipid peroxides. A 0.5-mL aliquot of the red blood corpuscle membrane suspension was mixed with 1.0 mL of 10% trichloroacetic acid and 2.0 mL of 0.67% 2-thiobarbituric acid. The mixture was heated at 95°C for 15 min, cooled, and centrifuged. The absorbance of the supernatant was measured at 534 nm in a Shimadzu spectrophotometer (Tokyo, Japan), and the relative amounts of lipid peroxides were expressed in absorbance units, A_{534} nm (28).

Liver tissue lipid peroxidation. For lipid peroxide measurement, *ca.* 1 g of liver tissue was homogenized and extracted using the method of Bligh and Dyer (29).

2-Thiobarbituric acid test for liver lipid peroxidation. The present test was performed according to the method described by Schmedes and Hølmer (30).

RESULTS

Antioxidant property of conjugated octadecatrienoic fatty acid, α-eleostearic (9*c*,11*t*,13*t*-18:3) acid was investigated.

The fatty acid composition of the dietary oils and oil blends are shown in Table 1. The oleic (18:1) and linoleic (18:2) contents were generally similar in all the dietary fats.

The mean body weight gain of rats fed the 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups for 4 wk showed no statistically significant difference when compared with the sun-

TABLE 1
Fatty Acid Composition of the Dietary Oils and Oil Mixtures

	Fatty acid composition (area%)[a]				
Dietary fats	16:0	18:0	18:1	18:2	18:3(9*c*,11*t*,13*t*-18:3)
Sunflower oil	6.5	3.5	31.9	58.1	—
Karela seed oil	2.0	31.7	8.0	7.1	51.1
Sunflower oil + karela seed oil (99:1, w/w)	6.4	3.8	31.7	57.6	0.5
Sunflower oil + karela seed oil (96:4, w/w)	6.3	4.6	30.9	56.1	2.1
Sunflower oil + karela seed oil (80:20, w/w)	5.6	9.1	27.2	47.9	10.2

[a]As determined by gas chromatography. 9*c*,11*t*,13*t*-18:3, α-eleostearic acid. $n = 8$.

TABLE 2
Plasma Cholesterol Profile of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing 0.5, 2, and 10% α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3)

Diets	Total cholesterol (mg/dL)	HDL-cholesterol (mg/dL)	Non-HDL-cholesterol (mg/dL)
Sunflower oil	46.3 ± 7.3[a]	23.3 ± 2.4	23.01 ± 6.7
0.5% 9*c*,11*t*,13*t*-18:3	35.8 ± 2.5	18.1 ± 1.7	17.8 ± 3.2
2% 9*c*,11*t*,13*t*-18:3	40.1 ± 3.7	20.1 ± 2.2	20.0 ± 5.2
10% 9*c*,11*t*,13*t*-18:3	44.3 ± 4.8	19.9 ± 4.6	24.3 ± 2.4

[a]All values are means of eight rats/diet. HDL, high density lipoprotein.

flower oil control group (Fig. 1). Although statistically insignificant, the dietary group with 10% 9*c*,11*t*,13*t*-18:3 showed maximal gain in weight at the end of week 4. In a previous nutritional experiment (13) the mean body weight gain was shown to be significantly more in rats raised on 51.1% 9*c*,11*t*,13*t*-18:3 compared to rats raised on 9*c*,12*c*,15*c*-18:3 dietary fat, which would support our present observation.

The amounts of plasma total cholesterol, HDL-cholesterol, and non-HDL-cholesterol of the rats raised on 0% 9*c*,11*t*,13*t*-18:3 (sunflower oil) and 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diet are included in Table 2. A comparison of the plasma total cholesterol of rats fed 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 and 0% 9*c*,11*t*,13*t*-18:3 (sunflower oil) showed that there was a trend of greater amount of plasma total cholesterol in the 0% 9*c*,11*t*,13*t*-18:3 diet compared to the other three 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diets. The total cholesterol was minimal in the group fed 0.5% 9*c*,11*t*,13*t*-18:3 and increased gradually as the level of 9*c*,11*t*,13*t*-18:3 was raised. There was no significant difference between the 0% 9*c*,11*t*,13*t*-18:3 and three 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups. HDL-cholesterol content in the plasma (Table 2) was maximal in the 0% 9*c*,11*t*,13*t*-18:3 dietary group, but there was no significant difference between the four dietary groups.

Table 3 includes the *in vitro* plasma lipid peroxidation and LOS of the 0, 0.5, 2, and 10% 9*c*,11*t*,13*t*-18:3 diet. It is evident that 9*c*,11*t*,13*t*-18:3 supplementation effectively reduced the susceptibility of the plasma lipid to peroxidation; the minimal susceptibility was achieved with 0.5% 9*c*,11*t*,13*t*-18:3 along with the gradual increase of 9*c*,11*t*,13*t*-18:3 supplementation (2%, 10%). The 0% 9*c*,11*t*,13*t*-18:3 group (sunflower group) showed maximal peroxidation of plasma lipid. Increasing the level of 9*c*,11*t*,13*t*-18:3 in the diet from 0.5 to 2% resulted in 25% increase in sensitivity to *in vitro* plasma lipid peroxidation. When the dietary level of 9*c*,11*t*,13*t*-18:3 was further increased to 10%, the sensitivity to *in vitro* plasma peroxidation increased by an additional 29%, but this change was not significant.

The oxidative sensitivity of the EM ghost and liver lipid

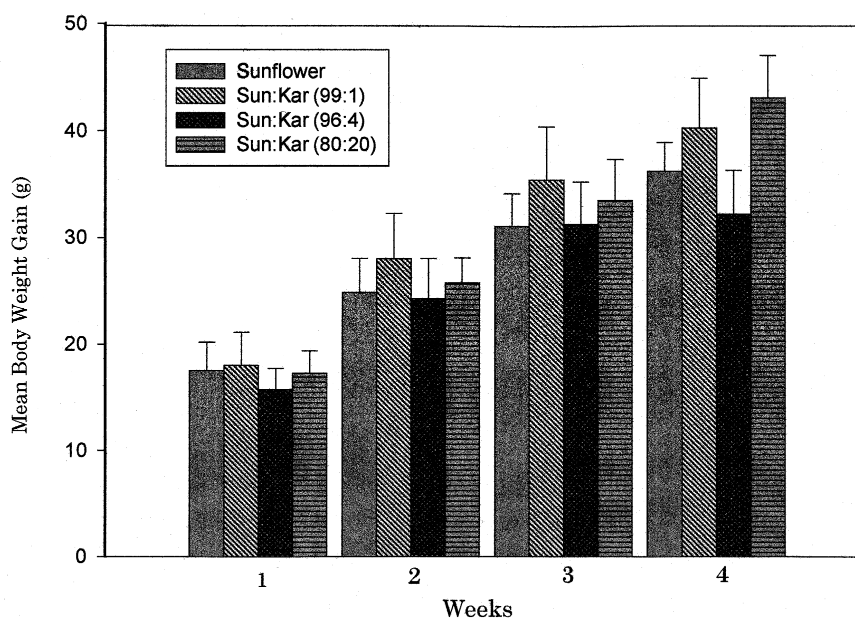

FIG. 1. Mean body weight gain of rats fed sunflower oil and sunflower (Sun):karela seed oil (Kar) blends at different weeks. $n = 8$ rats.

TABLE 3
Plasma Lipid Peroxidation and Lipoprotein Peroxidation of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing Different Levels of α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3) at 20% Fat in Diet

Diets	Plasma lipid peroxidation (nmole of MDA/mL of plasma)	Lipoprotein oxidation susceptibility (LOS) (nmole of MDA/mg of non-HDL-cholesterol)
Sunflower oil	9.3 ± 1.6[a]	51.1 ± 9.8
0.5% 9*c*,11*t*,13*t*-18:3	4.1 ± 0.7[b,c,d]	12.8 ± 2.7[b]
2% 9*c*,11*t*,13*t*-18:3	6.8 ± 1.1	14.7 ± 2.7[b]
10% 9*c*,11*t*,13*t*-18:3	9.0 ± 1.1	16.1 ± 6.6[e]

[a]All values are means of 8 rats/diet. MDA, malondialdehyde. See Table 2 for other abbreviation.
[b]Experimental group vs. control group $P < 0.01$.
[c]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group $P < 0.01$.
[d]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 2% 9*c*,11*t*,13*t*-18:3 group $P < 0.05$.
[e]Experimental group vs. control group $P < 0.02$.

TABLE 4
Lipid Peroxidation of Erythrocyte Membrane (EM) Ghost and Liver Tissue of Rats Fed Sunflower Oil and Sunflower:Karela Seed Oil Blended Products Containing Different Levels of α-Eleostearic Acid (9*c*,11*t*,13*t*-18:3) at 20% Fat in Diet

Diets	EM lipid peroxidation (nmole of MDA/mg of protein)	Liver tissue lipid peroxidation (nmole of MDA/mg of tissue lipid)
Sunflower oil	3.1 ± 0.4[a]	1.8 ± 0.3
0.5% 9*c*,11*t*,13*t*-18:3	1.8 ± 0.2[b]	0.6 ± 0.1[c,d,e]
2% 9*c*,11*t*,13*t*-18:3	2.1 ± 0.3[f]	1.5 ± 0.5
10% 9*c*,11*t*,13*t*-18:3	1.7 ± 0.4[g]	1.5 ± 0.3

[a]All values are means of eight rats/diet. $n = 8$. See Table 3 for other abbreviation.
[b]0.5% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.05$.
[c]0.5% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.01$.
[d]0.5% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group.
[e]2% 9*c*,11*t*,13*t*-18:3 group vs. 10% 9*c*,11*t*,13*t*-18:3 group $P < 0.01$.
[f]2% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.01$; $P < 0.05$.
[g]10% 9*c*,11*t*,13*t*-18:3 group vs. sunflower group $P < 0.05$.

peroxidation of the four dietary fat groups of rats are presented in Table 4. TBARS production in EM ghosts from the 0% 9*c*,11*t*,13*t*-18:3 group was significantly higher when compared with TBARS production in the EM ghosts of the 0.5, 2, 10% 9*c*,11*t*,13*t*-18:3 dietary groups. But there was no significant difference between the 9*c*,11*t*,13*t*-18:3 supplemented groups.

Liver lipid peroxidation results showed that *in vitro* production of TBARS in liver tissue of dietary group supplemented with 0.5% 9*c*,11*t*,13*t*-18:3 was significantly lower than the 0, 2, and 10% 9*c*,11*t*,13*t*-18:3 dietary groups. Thus the TBARS formation in the *in vitro* condition was lowest in the dietary group that was supplemented with least amount of 9*c*,11*t*,13*t*-18:3.

DISCUSSION

The study showed that 0.5 and 2% 9*c*,11*t*,13*t*-18:3 fatty acid in dietary oil had no effect on the body growth of rats, but there was an improved weight gain with 10% 9*c*,11*t*,13*t*-18:3, though not significant. The previous nutritional experiment (13) showed that 20% 9*c*,11*t*,13*t*-18:3 in the diet improved the body weight gain of rats in comparison with the rats raised on 9*c*,12*c*,15*c*-18:3 dietary fat which supports the present observation. Chin *et al*. (31) studied the effect of CLA on rat growth and development. Pups that continued to receive the CLA-supplemented diet after weaning had significantly greater body weight gain compared to control animals.

For assessment of the effect of 9*c*,11*t*,13*t*-18:3 on atherosclerosis in rats, total cholesterol, HDL-cholesterol, and non-HDL-cholesterol were estimated in plasma. There was no evidence of significant difference in the total cholesterol and various forms of cholesterol between the experimental and the control groups of rats. It can be concluded that the four dietary groups were nutritionally equivalent since the PUFA content of the four dietary fats was more than adequate, generally equal in content, and the levels of 9*c*,11*t*,13*t*-18:3 were low. A diet supplemented with 0.5 g CLA for 12 wk reduced low density lipoprotein-cholesterol and triglycerides markedly (32).

Plasma lipid peroxidation and LOS were maximally reduced at 0.5% of dietary 9*c*,11*t*,13*t*-18:3, and peroxidation increased gradually with the increased levels of 9*c*,11*t*,13*t*-18:3 in the diet group. Therefore, the antioxidant efficacy of 9*c*,11*t*,13*t*-18:3 is maximal at 0.5% in the diet. Ip *et al*. (33) showed that 0.25% CLA in the diet had maximal antioxidant effect, and CLA lowered mammary tissue malonaldehyde formation.

EM lipid peroxidation data indicate that 9*c*,11*t*,13*t*-18:3 had reduced lipid peroxidation possibly after being incorporated into the lipids of red blood cell membrane. Sunflower oil containing high levels of linoleic acid and no 9*c*,11*t*,13*t*-18:3 showed increased membrane lipid peroxidation. Cunningham *et al.* (34) observed that supplementation of linoleic acid increased intracellular lipid peroxide concentrations in normal human mammary epithelial cells. Ha *et al.* (10) from his investigations concluded that CLA might serve as an *in situ* defense mechanism against membrane attack by free radicals which may explain the anticarcinogemic properties of CLA.

Liver tissue lipid peroxidation also reduced at 0.5% 9*c*,11*t*,13*t*-18:3 which may have been due to incorporation of this conjugated linolenic acid. Belury and Kempa-Steczko (35) showed that dietary CLA was incorporated in neutral and phospholipids at the expense of linoleic acid in the diet.

Normally conjugated fatty acids are more rapidly oxidized than the nonconjugated PUFA (11). Various investigators showed that the linolenates oxidize approximately twice as fast as the linoleates (36). According to Swern (37) conjugated triene esters oxidize more rapidly than nonconjugated triene esters. In compounds with more than two conjugated double bonds, conjugation increased the rate of oxidation (38). Thus, in the *in vivo* study conjugated trienoic fatty acids are also likely to be more rapidly oxidized than linoleates by picking up more free radicals, thereby eliminating or reducing the formation of hydroperoxides. CLA in higher concentrations

acts as prooxidant rather than antioxidant (9). At 0.5%, 9*c*,11*t*,13*t*-18:3 showed antioxidant property that decreased with increase in its level. At higher concentrations it could have possibly acted as a prooxidant similar to CLA, also reported to act as a prooxidant at higher concentration (9).

Another possible mechanism could be that oxidation of conjugated trienoic fatty acid resulted in the formation of conjugated dienoic fatty acids (CLA) that acted as antioxidants (9). Brauer and Steadman (38) measured the oxidation of β-eleostearic acid by means of ultraviolet spectrophotometry and observed that the conjugated triene decreased as oxidation proceeded, while the absorption due to conjugated diene increased. This also occurred during oxidation of α-eleostearic acid (39). Allen and Kummerow (40) found that the amount of triene conjugation lost and the amount of diene conjugation formed were proportional to the amount of oxygen absorbed. Thus it may be possible that in the *in vivo* conditions these conjugated linolenic fatty acids may have reduced the formation of hydroperoxides by lowering the generation of free radicals and peroxidation of PUFA occurring in EM and other lipids.

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Dietary Menhaden, Seal, and Corn Oils Differentially Affect Lipid and *Ex vivo* Eicosanoid and Thiobarbituric Acid-Reactive Substances Generation in the Guinea Pig

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ABSTRACT: This investigation was carried out to characterize the effects of specific dietary marine oils on tissue and plasma fatty acids and their capacity to generate metabolites (prostanoids, lipid peroxides). Young male guinea pigs were fed nonpurified diet (NP), or NP supplemented (10%, w/w) with menhaden fish oil (MO), harp seal oil (SLO), or corn oil (CO, control diet) for 23 to 28 d. Only the plasma showed significant n-3 polyunsaturated fatty acid (PUFA)-induced reductions in triacylglycerol (TAG) or total cholesterol concentration. Proportions of total n-3 PUFA in organs and plasma were elevated significantly in both MO and SLO dietary groups (relative to CO), and in all TAG fractions levels were significantly higher in MO- than SLO-fed animals. The two marine oil groups differed in their patterns of incorporation of eicosapentaenoic acid (EPA). In guinea pigs fed MO, the highest levels of EPA were in the plasma TAG, whereas in SLO-fed animals, maximal incorporation of EPA was in the heart polar lipids (PL). In both marine oil groups, the greatest increases in both docosahexaenoic acid (22:6n-3, DHA) and docosapentaenoic acid (22:5n-3, DPA), relative to the CO group, were in plasma TAG, although the highest proportions of DHA and DPA were in liver PL and heart TAG, respectively. In comparing the MO and SLO groups, the greatest difference in levels of DHA was in heart TAG (MO > SLO, $P < 0.005$), and in levels of DPA was in heart PL (SLO > MO, $P < 0.0001$). The only significant reduction in proportions of the major n-6 PUFA, arachidonic acid (AA), was in the heart PL of the SLO group (SLO > MO = CO, $P < 0.005$). Marine oil feeding altered *ex vivo* generation of several prostanoid metabolites of AA, significantly decreasing thromboxane A_2 synthesis in homogenates of hearts and livers of guinea pigs fed MO and SLO, respectively ($P < 0.04$ for both, relative to CO). Lipid peroxides were elevated to similar levels in MO- and SLO-fed animals in plasma, liver, and adipose tissue, but not in heart preparations. This study has shown that guinea pigs respond to dietary marine oils with increased organ and plasma n-3 PUFA, and changes in potential synthesis of metabolites. They also appear to respond to n-3 PUFA-enriched diets in a manner that is different from that of rats.

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It has been suggested that diets containing moderate levels of oily fish, which are enriched in n-3 polyunsaturated fatty acids (PUFA), have a variety of health benefits, including prevention of fatal cardiac arrhythmias (1,2 and references therein) and support of normal brain and retinal development (3). The molecular mechanisms underlying these effects of n-3 PUFA are not fully understood, but before we can understand them, we need to know the extent to which individual n-3 polyenes are incorporated into different body organs. It will be important also to know whether incorporation varies with the source and PUFA composition of the dietary oil, an issue that has been studied to only a limited extent (4,5).

Dietary studies, which have been done primarily with n-6 PUFA, have demonstrated that guinea pigs are closer to humans than are the more commonly used laboratory animal species, including rats, in terms of their lipid and fatty acid metabolism. Examples include their lipid profiles in some organs, particularly heart (6,7), their plasma lipoprotein profile (8) and its response to dietary fat saturation (9), and their distribution of hepatic cholesterol pools (10). Also, a small number of studies have suggested that guinea pigs fed a standard laboratory diet are relatively n-3 PUFA deficient (5,11). This condition appears to be be readily reversed by diets containing n-3 PUFA, although the effects produced in major organs and blood have not yet been fully characterized.

The low levels of n-3 PUFA in the guinea pig also make this species a good model for determining whether the dietary source of n-3 PUFA (e.g., oils from fish *vs.* oils from marine mammals) influences any change that may occur with respect to organ/blood lipid and fatty acid profiles. A little-appreciated fact regarding marine oils is that their chemical composition and structure can vary significantly. One difference is in the relative proportions of their major n-3 PUFA, eicosapentaenoic (EPA, 20:5n-3) and docosahexaenoic (DHA, 22:6n-3) acids (12). Also, oils from marine mammals, but not fish, contain substantial amounts of the less well-known n-3 long-chain PUFA, docosa-

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Abbreviations: AA, arachidonic acid (20:4n-6); CO, corn oil; DHA, docosahexaenoic acid (22:6n-3); DPA, docosapentaenoic acid (22:5n-3); EPA, eicosapentaenoic acid (20:5n-3); LPO, lipid peroxide; MO, menhaden fish oil; MUFA, monounsaturated fatty acid(s); NP, nonpurified; PG, prostaglandin(s); PGI_2, prostacyclin; PL, polar lipid; PUFA, polyunsaturated fatty acid(s) (polyenes); SLO, harp seal oil; TAG, triacylglycerol; TBARS, thiobarbituric acid-reactive substances; TC, total cholesterol; TX, thromboxane. Fatty acid nomenclature: 20:5n-3 refers to the number of carbons:number of double bonds, and n-*x* refers to the position of the first double bond relative to the methyl terminus of the fatty acid.

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pentaenoic acid (DPA, 22:5n-3). It constitutes approximately 5% of the total fatty acids in seal oil, which was the dominant fat in the diet of Greenland Eskimos (13), the population in which an association was first made between a low incidence of cardiovascular disease and high dietary intake of n-3 PUFA. Another potentially important difference between oils from marine mammals and fish is the positional distribution of these PUFA in triacylglycerols (TAG). In fish oils, n-3 PUFA are esterified primarily at the *sn*-2 position, whereas in seal oil they are found mainly at *sn*-1 and *sn*-3 (14–16). This structural difference could result in differences in absorption, distribution, and systemic metabolism of the n-3 PUFA, although its significance regarding organ/cell structure and function remains unknown.

For the representative fish oil in this study, we used menhaden oil (MO). It contains ≈30% (by wt) n-3 PUFA, of which EPA is 13.4% and DHA is 9.9%. The U.S. Food and Drug Administration recently approved its use as a human food supplement (17), and as such, it is quantitatively the most important fish oil in North America. Harp seal oil (SLO) contains ≈21% n-3 PUFA, has DHA (8%) ≥ EPA (7%), and has a relatively high proportion of DPA (4–6%). SLO is also of interest since there is little information regarding its effects on organ structure and metabolite production, and particularly since it is being investigated for use in the food and pharmaceutical industries (16).

This investigation was undertaken to determine the effects of marine oil-enriched diets on organ-specific uptake, metabolism, and incorporation of n-3 PUFA into polar lipids (PL) and/or TAG in guinea pig organs (heart, liver, brain, adipose tissue) and plasma. Effects of the diets on the *ex vivo* generation of select prostanoids and lipid peroxide (LPO) markers were also determined.

MATERIALS AND METHODS

Materials. The marine oils used were refined menhaden fish (*Brevoortia tyrannis*) oil (MO), a gift of the United States Department of Commerce, National Marine Fisheries Services, Southeast Fisheries Science Center (Charleston, SC), and harp seal (*Phoca groenlandicus*) oil (SLO), a gift of Gateway Maritime Inc. (Brigus, Newfoundland, Canada). Corn oil (CO) was obtained commercially (Best Foods Canada Inc., Etobicoke, Ontario). Reagents for fatty acid analyses and colorimetric assays and α-tocopherol were purchased from Sigma Chemical Co. (St. Louis, MO) or Diagnostic Chemicals (Charlottetown, Prince Edward Island, Canada).

Animals and diets. The diets were prepared with a standard nonpurified guinea pig diet (NP; PROLAB Guinea Pig Formula, Agway Inc., Syracuse, NY). It was ground, mixed with the marine oils or CO (10 g/100 g diet), and reconstituted into pellets in a California pelletizer at the Laboratory of Fisheries and Oceans Canada (Halifax, Nova Scotia, Canada). Antioxidant (100 mg α-tocopherol/kg diet) was added to each of the oil-enriched diets. The diets were made up at the beginning of the study and were stored at –20°C under N_2 in 100-g portions. The fatty acid compositions of the diets were reported in detail previously (5). Ten- to 14-d-old male albino guinea pigs (Harlan, Indianapolis, IN) weighing 120–240 g were fed the diets (4–9/dietary group) for 23–28 d. The animals were given free access to food and to water supplemented with ascorbic acid (200 mg/L, wt/vol) and kept in 12-h light:dark cycles in the Animal Care Quarters of the Sir Charles Tupper Medical School, Dalhousie University (Halifax, Nova Scotia, Canada). They were fed at the same time each day, at which time uneaten food was weighed and discarded, and the cups were washed thoroughly. The guinea pigs were weighed every 2 to 3 d throughout the feeding period. They were killed with an overdose of euthanol, and blood was taken by cardiac puncture into 10 mL syringes that contained EDTA (19 mg) and indomethacin (7.2 mg). Plasma and red blood cells were separated by centrifugation. Organs were quickly removed, weighed, flash frozen and stored at –86°C. These studies were carried out in full compliance with the regulations of the Canadian Council on Animal Care.

Lipid extraction and fatty acid analysis. Lipids were extracted from hearts, livers, brains, and adipocytes using chloroform/methanol (2:1, vol/vol) under an N_2 atmosphere, according to the method of Bligh and Dyer (18). On the day of extraction, tissues were brought to room temperature, weighed, and homogenized in a Sorvall Omni-mixer (Mandel Scientific Co., Guelph, Ontario, Canada) using double volumes of solvents relative to tissue weights to ensure complete homogenization. The chloroform phase was allowed to settle overnight in a glass separatory funnel. The solvent was removed under water aspirator vacuum in a rotary evaporator and finally under mechanical vacuum. For extraction of plasma lipids, 10 vol of chloroform/methanol (2:1, vol/vol) were added to the samples, the solutions were vortexed vigorously for 2 min, and 1 vol of 0.9% saline was added. The mixture was centrifuged, and the bottom chloroform layer was removed by Pasteur pipette. The upper phase was reextracted with chloroform/methanol (2:1, vol/vol), and the chloroform layers were pooled and dried over sodium sulfate under N_2. Where indicated, lipid classes (PL, TAG) were isolated by thin-layer chromatography on Soft Layer Adsorbosil Plus-1 plates (Mandel Scientific) using the solvent system hexane/anhydrous ethyl ether/glacial acetic acid (85:15:1, by vol). Lipids were recovered from the appropriate bands by extraction with chloroform/methanol (2:1, vol/vol). The methyl esters of fatty acids of the individual lipid classes were prepared using the method described previously (19). Methyl esters were separated by gas–liquid chromatography (GLC) with a 30 m × 0.25 mm Omegawax 320 column (Supelco Sigma-Aldrich Canada Ltd., Oakville, Ontario, Canada) in a Perkin-Elmer AutoSystem gas chromatograph (Perkin-Elmer, Norwalk, CT) equipped with a flame-ionization detector. Fatty acids were identified by comparison with authentic standards, and peak areas were converted to weight percentages with flame-ionization detector response correction.

Prostanoid analyses. Frozen tissue samples were analyzed for prostanoids essentially as described by Abeywardena *et al.* (20). Briefly, the samples (approximately 100 mg) were minced, washed several times in Tris-EDTA with the washes removed by rapid centrifugation, and the washed tissues were reconstituted in a modified Tyrode's solution. This preparation was in-

cubated at 37°C for 45 min on a shaking water bath, after which the tubes were cooled on ice for 5 min and centrifuged. The supernatants were acidified with 1 N HCl, extracted with ethylacetate, and analyzed for prostanoids as described below. Plasma samples (stored at −86°C in the presence of EDTA and indomethacin) were also acidified and extracted with ethylacetate. The samples were first purified and concentrated by passage through either C2 or C18 Amprep™ Minicolumns (Amersham Canada Ltd., Oakville, Ontario, Canada). Levels of prostaglandin (PG) E_2, thromboxane (TX) A_2 (measured as TXB_2), and prostacyclin (PGI_2, measured as 6-keto-$PGF_{1\alpha}$) were determined in sample aliquots by enzyme immunoassay; $PGF_{2\alpha}$ was measured using radioimmunoassay. All assays were done with kits from Amersham, and color intensities in wells of the enzyme immunoassay plates were read using a Bio-Rad Model 3550 Microplate Reader (Bio-Rad, Mississauga, Ontario, Canada). It was appreciated that there could be some cross reactivity of the antibodies with the trienoic prostanoids.

Quantitation of lipid peroxide products. Tissue levels of LPO were quantitated using a combination of colorimetric and high-performance liquid chromatography (HPLC) methods, essentially as described by Wong *et al.* (21). Organ homogenates or plasma were incubated under acidic conditions with thiobarbituric acid (TBA) to form TBA-reactive substances (TBARS). LPO-specific TBARS were isolated and quantitated using a Varian high-performance liquid chromatograph (Model 9010) equipped with a 25 cm C_{18} column that was attached to a 2 cm LC-18 guard column, frit filter and 50 µL sampling loop. The eluting solvent was methanol/50 mM phosphate buffer (pH 6.8; 2:3, vol/vol), which was filtered and degassed prior to use. Flow rate was 1.5 mL/min. After the columns had been equilibrated for 30 min with methanol/buffer, samples were injected and absorbance was monitored at 532 nm for 10 min. Mean elution time for the LPO-TBARS was 3.6 min. Products were quantitated by comparison with peak heights of TBARS products of known amounts of the standard, 1,1,3,3-tetramethoxypropane.

Colorimetric analyses. Protein was analyzed according to Lowry *et al.* (22) using bovine serum albumin as a standard. Total cholesterol (TC) and TAG were quantitated using kits from Sigma Chemical Co. and Diagnostic Chemicals, respectively.

Statistical analysis. Data are expressed as mean ± SEM. They were analyzed for statistical significance by a one-way analysis of variance using a Bonferroni correction factor for multiple testing. Differences were considered to be significant when $P < 0.05$.

RESULTS

Growth and diet tolerance. Weight gains and growth rates were similar for all dietary groups, as we had found in an earlier study (5), although guinea pigs fed the nonsupplemented NP diets ate approximately 30% more food each day than did those in the oil-supplement feed groups. As the NP and oil-supplemented diets differed in caloric densities, and since animals fed the latter ingested lower amounts of other nutrients (e.g., vitamins, protein,

TABLE 1
Major Polyunsaturated Fatty Acids and Fatty Acid Class Profiles in Total Lipids of Nonpurified (NP) Guinea Pig Diets and NP Supplemented with Corn Oil, Menhaden, or Seal Oils[a,b]

	Diet			
	NP	NP+CO	NP + MO	NP + SLO
	(g/100 g fatty acids)			
Fatty acid				
18:2n-6	29.9	49.3	10.0	10.3
18:3n-3	—	0.5	—	—
18:3n-6	—	—	0.4	—
18:4n-3	—	—	1.8	0.9
20:3n-3	—	—	0.8	0.4
20:4n-6	—	—	—	—
20:5n-3	—	—	10.3	4.6
22:4n-6	—	—	0.5	—
22:5n-6	—	—	—	—
22:5n-3	—	—	1.8	2.6
22:6n-3	—	—	7.2	4.9
Fatty acid class profiles				
Total SFA	38.9	20.0	33.7	22.3
Total MUFA	31.5	29.2	24.0	50.6
Total PUFA	29.9	49.8	32.8	23.7
n-6 PUFA	29.9	49.3	10.9	10.3
n-3 PUFA	<0.6	0.5	21.9	13.4
n-6/n-3	>29.9	98.6	0.5	0.8

[a]Data are the means of values from analyses of 3-4 different food lots, each of which was analyzed in duplicate following the same procedure as that given for the tissue samples.
[b]PUFA, polyunsaturated fatty acids; CO, corn oil; MO, menhaden fish oil; SLO, harp seal oil; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids. Fatty acids are abbreviated as number of carbons:number of double bonds. The n-x refers to the position of the first double bond, relative to the methyl terminus of the fatty acid.

fiber), comparisons between them are not valid. However, data for the NP group are included in the tables and figures, as there are very few reports of lipid and fatty acid profiles for guinea pigs on standard laboratory diets. Based on the average food consumption per day and the proportions of fatty acids in the diets (Table 1), the MO- and SLO-fed guinea pigs ingested approximately 1.8 and 1.2 g n-3 PUFA/kg/d, respectively. Those fed CO consumed <0.05 g of n-3 PUFA/d. The levels of antioxidants and cholesterol consumed with each diet are shown in Table 2. The animals tolerated the oils well, and there was no evidence of diarrhea. The only difference in overall appearance between the groups was that the guinea pigs fed the seal oil-enriched diets had particularly healthy coats and long, hard nails. Under light microscopy, the hearts and livers showed no evidence of pathological effects attributable to the diets, including very little fatty steatosis. On autopsy, the only tissue that appeared to be affected by diet was the epididymal fat. There were significant differences between the relative weights of the fat pads, as those from the MO-fed guinea pigs were significantly lighter (0.35% of total body weight) than those from either the SLO- or CO-fed groups (0.44–0.49% of total body weights) ($P < 0.0001$). Physically, the MO fat pads were amorphous and very sticky, resembling those of the NP animals, whereas those of the SLO and CO groups were much more solid and cohesive.

Diet effects on plasma and organ TAG and TC levels. Only in the plasma were TAG and TC levels significantly altered by diet (Table 3). The TAG were significantly higher in the CO-fed guinea pigs than in those fed either MO ($P < 0.005$) or SLO ($P < 0.0001$). The highest levels of plasma TC were also in the animals fed CO, although differences were significant only relative to the MO group ($P < 0.02$). Values for plasma TAG or TC were not significantly different between the MO and SLO groups.

Effects of the diets on fatty acid class profiles. Fatty acid analyses were carried out on the total PL and/or TAG fractions of tissues and plasma (Table 4). The only significant difference in total saturated fatty acids was in the heart, where their proportions in the TAG of MO-fed animals were significantly (≈25%) higher than those in either the SLO or CO groups (data not shown). With the exception of the plasma PL, levels of monounsaturated fatty acids (MUFA) in the SLO-fed guinea pigs were all significantly higher than those in both MO and CO groups (Table 4). This may have reflected the higher levels of MUFA in the SLO diet (Table 1). Proportions of n-6 PUFA were consistently ≥2-fold higher in the animals fed CO, and differences between these and marine oil-fed animals were greatest in the TAG. In heart and adipose TAG, n-6 PUFA were significantly higher with MO than with SLO feeding ($P < 0.001$). Not surprisingly, total n-3 PUFA were elevated significantly in plasma and in all organs of marine oil-fed guinea pigs (Table 4), including brain (not shown). Levels of n-3 PUFA in the MO group were significantly higher than those of SLO-fed animals in the TAG of heart, plasma, and adipose tissue (all $P < 0.001$).

TABLE 2
Antioxidant and Cholesterol Contents, and Peroxide Values, of Dietary Oils

Dietary oil	Total tocopherol[a] (mg/g oil)	TBHQ (%)	Other antioxidants (mg/g oil)	Cholesterol (mg/g oil)	Peroxide value (meq O_2/g oil)
Corn	2.1	0.025	—	0	2.6
Menhaden	2.3	0.02	—	1.7	4.3
Seal	0.016	0.02	0.15	0.65	4.5

[a]Values for total tocopherol (α and γ) and TBHQ are based on content reported by distributor, analyzed by RGA, or by amount added. TBHQ, *tert*-butylhydroquinone.

TABLE 3
Effect of Dietary Oils on Triacylglycerol and Total Cholesterol Content of Guinea Pig Plasma and Organs[a]

Lipid class	Diet	Plasma (mmol/L)	Heart (mg/mg protein)	Liver (mg/mg protein)	Adipose[b] (mg/g wet wt)
Triacylglycerol					
	NP	0.78 ± 0.05	0.10 ± 0.02	nd	0.28 ± 0.05
	MO	0.53 ± 0.07^{a}	0.13 ± 0.03	0.14 ± 0.04	0.39 ± 0.07
	SLO	0.45 ± 0.05^{a}	0.17 ± 0.02	0.13 ± 0.02	0.38 ± 0.05
	CO	1.13 ± 0.14^{b}	0.10 ± 0.02	0.10 ± 0.04	0.29 ± 0.14
Total Cholesterol					
	NP	1.24 ± 0.08	0.064 ± 0.01	Not done	0.35 ± 0.07
	MO	1.31 ± 0.09^{a}	0.065 ± 0.01	0.042 ± 0.01	0.23 ± 0.10
	SLO	1.60 ± 0.11^{a,b}	0.074 ± 0.01	0.037 ± 0.01	0.19 ± 0.04
	CO	1.89 ± 0.18^{b}	0.060 ± 0.01	0.043 ± 0.01	0.24 ± 0.04

[a]Male albino guinea pigs, 10 to 14 d of age and weighing 120–240 g, were fed NP or NP supplemented (10%, wt/wt) with MO, SLO, or CO for 23 to 30 d. The animals were killed with an overdose of euthanol, and organs and blood taken as quickly as possible as described in the Materials and Methods section. Analyses of triacylglycerol and total cholesterol were carried out as described in the Materials and Methods section. Data are the means ± SEM of values obtained from 4 to 9 animals. For each data group in a column, differences in roman superscript letters indicate statistical significance. NP, nonpurified guinea pig diet; MO, NP + menhaden fish oil; SLO, NP + seal oil; CO, NP + corn oil.
[b]Epididymal fat.

TABLE 4
Effect of Dietary Oils on Monoenoic and Polyenoic Fatty-acid Class Profiles of Guinea Pig Plasma and Organs[a,b]

Fatty acid class	Diet	Plasma PL	Plasma TAG	Heart PL	Heart TAG	Liver PL	Adipose TAG
Total MUFA	NP	13.0 ± 0.6	28.6 ± 1.8	14.0 ± 0.7	33.5 ± 1.7	9.9 ± 0.3	31.3 ± 0.3
	MO	18.1 ± 4.3^{a,b}	25.9 ± 3.6^{a}	14.6 ± 0.3^{a}	27.6 ± 0.6^{a}	9.9 ± 0.3^{a}	30.7 ± 0.3^{a}
	SLO	23.8 ± 0.9^{b}	42.2 ± 1.6^{b}	18.7 ± 0.4^{b}	48.2 ± 0.4^{b}	17.1 ± 0.6^{b}	43.3 ± 1.4^{b}
	CO	12.3 ± 0.3^{a}	25.7 ± 0.5^{a}	11.6 ± 0.3^{c}	31.3 ± 0.9^{c}	9.9 ± 0.3^{a}	29.2 ± 0.6^{b}
Total n-6 PUFA	NP	37.1 ± 0.4	30.3 ± 2.5	39.1 ± 0.5	23.6 ± 1.8	38.1 ± 1.4	26.7 ± 1.4
	MO	15.2 ± 1.2^{a}	12.7 ± 0.7^{a}	21.2 ± 0.6^{a}	14.7 ± 0.3^{a}	16.6 ± 0.5^{a}	16.6 ± 1.0^{a}
	SLO	17.1 ± 0.8^{a}	14.4 ± 0.5^{a}	20.7 ± 0.8^{a}	12.3 ± 0.6^{b}	15.8 ± 0.7^{a}	13.6 ± 0.2^{b}
	CO	41.0 ± 1.4^{b}	49.2 ± 1.0^{b}	42.6 ± 0.9^{b}	38.8 ± 0.9^{c}	43.4 ± 0.9^{b}	47.5 ± 0.4^{c}
Total n-3 PUFA	NP	2.6 ± 0.2	5.8 ± 0.5	3.6 ± 0.1	4.1 ± 0.2	2.0 ± 0.3	4.9 ± 1.8
	MO	20.3 ± 1.9^{a}	34.2 ± 2.5^{a}	17.0 ± 0.6^{a}	20.1 ± 1.2^{a}	24.5 ± 0.9^{a}	13.4 ± 0.4^{a}
	SLO	15.1 ± 1.3^{a}	20.1 ± 1.1^{b}	16.7 ± 0.4^{a}	11.2 ± 0.7^{b}	23.5 ± 1.1^{a}	8.7 ± 0.b^{b}
	CO	1.5 ± 0.2^{b}	2.8 ± 0.1^{c}	1.9 ± 0.1^{b}	1.9 ± 0.1^{c}	1.2 ± 0.1^{b}	2.8 ± 0.1^{c}

[a]Data represent percent, relative to total fatty acids.
[b]Male albino guinea pigs, 10 to 14 d of age and weighing 120–240 g, were fed NP or NP supplemented (10%, w/w) with MO, SLO, or CO for 23 to 30 d. The animals were killed with an overdose of euthanol, and organs and blood were taken as quickly as possible as described the Materials and Methods section. Lipid classes were separated by thin-layer chromatography and methylated, and the fatty acid methyl esters were separated and quantitated as described in the text. Data are the means ± SEM of values obtained from 4 to 9 animals. For each data group in a column, differences in roman superscript letters indicate statistical significance. PL, polar lipid; TAG, triacylglycerol; Adipose, epididymal fat. For other abbreviations see Table 1.

Effects of the diets on major n-3 and n-6 PUFA. Literature reports have suggested that EPA, DHA, 22:5n-3DPA and arachidonic acid (AA, 20:4n-6) have distinct biologic functions (see Discussion). Accordingly, a major objective in this study was to determine whether the MO and SLO diets resulted in differences in the organ/plasma levels of each of these fatty acids. As shown in Table 5, MO-fed guinea pigs had maximal incorporation of EPA into plasma TAG, where levels were significantly higher than they were for SLO-fed animals ($P < 0.0001$). Indeed, EPA levels in all TAG fractions were consistently higher in the MO group than in the SLO group. In contrast, relative to the CO group, SLO-fed animals had the greatest increase in EPA in the heart PL. With both MO and SLO feeding, the greatest increases in DHA, relative to the CO group, were in plasma TAG, although liver PL had the highest DHA content (≈17% of total fatty acids) in both marine oil groups. As with EPA, proportions of DHA were consistently higher in the MO-fed group than those in SLO-fed animals in the TAG fractions of plasma

TABLE 5
Effects of Dietary Oils on Plasma and Organ Profiles of Major PUFA[a]

Fatty acid	Diet	Plasma PL	Plasma TAG	Heart PL	Heart TAG	Liver PL	Adipose TAG
18:2n-6	NP	31.4 ± 0.3	29.2 ± 2.3	20.1 ± 0.4	21.8 ± 1.9	31.2 ± 0.9	26.7 ± 1.4
	MO	7.7 ± 0.6^{a}	10.9 ± 0.6^{a}	9.1 ± 0.4^{a}	12.5 ± 0.4^{a}	8.3 ± 0.3^{a}	16.7 ± 0.3^{a}
	SLO	13.3 ± 0.9^{b}	13.5 ± 0.5^{b}	11.6 ± 0.6	11.4 ± 0.6^{a}	11.3 ± 0.4^{b}	13.4 ± 0.2^{b}
	CO	37.5 ± 1.1	49.4 ± 0.6	28.1 ± 0.4	35.4 ± 1.7^{b}	38.3 ± 0.9^{c}	46.6 ± 0.8^{c}
20:4n-6	NP	4.1 ± 0.3	0.5 ± 0.1	16.2 ± 0.4	0.9 ± 0.1	5.3 ± 0.6	nd
	MO	6.6 ± 1.1^{a}	1.1 ± 0.2^{a}	11.5 ± 0.3^{a}	1.2 ± 0.1^{a}	7.9 ± 0.3^{a}	0.4 ± 0.0^{a}
	SLO	3.5 ± 0.3^{a,b}	0.5 ± 0.05^{b}	8.9 ± 0.2^{b}	0.4 ± 0.03^{b}	4.3 ± 0.3^{a,b}	0.2 ± 0.0^{b}
	CO	2.3 ± 0.3^{b}	0.3 ± 0.02^{b}	12.4 ± 0.8^{a}	0.8 ± 0.2^{a,b}	3.8 ± 0.2^{b}	0.1 ± 0.0^{c}
20:5n-3	NP	0.2 ± 0.03	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.1	0.2 ± 0.03	nd
	MO	6.8 ± 0.7^{a}	13.6 ± 1.2^{a}	6.8 ± 0.3^{a}	4.1 ± 0.6^{a}	5.6 ± 0.2^{a}	3.8 ± 0.2^{a}
	SLO	5.6 ± 0.9^{a}	5.5 ± 0.4^{b}	6.2 ± 0.2^{a}	1.4 ± 0.2^{b}	4.6 ± 0.7^{a}	1.6 ± 0.1^{b}
	CO	0.2 ± 0.04^{b}	0.1 ± 0.02^{c}	0.1 ± 0.0^{b}	≤0.1	0.1 ± 0.01	Trace
22:5n-3	NP	0.8 ± 0.1	0.2 ± 0.03	1.8 ± 0.0	0.3 ± 0.0	0.5 ± 0.05	nd
	MO	2.0 ± 0.1^{a}	2.2 ± 0.1^{a}	1.6 ± 0.1^{a}	3.4 ± 0.3^{a}	1.0 ± 0.04^{a}	1.4 ± 0.04^{a}
	SLO	1.5 ± 0.1^{b}	2.5 ± 0.2^{a}	2.6 ± 0.0^{b}	2.6 ± 0.4^{a}	1.4 ± 0.05^{b}	1.7 ± 0.1^{a}
	CO	0.3 ± 0.1^{c}	0.1 ± 0.0^{b}	1.0 ± 0.03^{c}	0.2 ± 0.04^{b}	0.2 ± 0.00^{c}	0.1 ± 0.0^{b}
22:6n-3	NP	0.6 ± 0.04	0.2 ± 0.1	1.2 ± 0.1	0.3 ± 0.1	0.7 ± 0.1	nd
	MO	10.5 ± 1.7^{a}	14.9 ± 1.5^{a}	8.2 ± 0.4^{a}	9.6 ± 1.0^{a}	16.6 ± 0.5^{a}	3.8 ± 0.2^{a}
	SLO	7.4 ± 0.5^{a}	9.1 ± 0.7^{b}	7.6 ± 0.3^{a}	3.5 ± 0.6^{b}	17.2 ± 0.7^{a}	2.4 ± 0.2^{b}
	CO	0.5 ± 0.1^{b}	0.2 ± 0.1^{c}	0.5 ± 0.1^{b}	0.2 ± 0.0^{c}	0.5 ± 0.1^{b}	≤0.1

[a]Data represent percent, relative to total fatty acids. nd, Not detected; for other abbreviations see Tables 1 and 3.
[b]Male albino guinea pigs, 10 to 14 d of age and weighing 120–14 d of age and weighing 120–240 g, were fed NP or NP supplemented (10%, w/w) with MO, SLO, or CO for 23 to 30 d. The animals were killed, and organs and blood were taken as quickly as possible, flash frozen and stored at −86°C (see Methods section). Lipids were extracted as described in the Materials and Methods section, and lipid classes were separated by thin-layer chromatography. Lipids were transmethylated and the fatty acid methyl esters were separated by gas–liquid chromatography, and identified by comparison with authentic standards. Data are the means ± SEM of values obtained from 4 to 9 animals. For each data group in a column, differences in superscript roman letters indicate statistical significance.

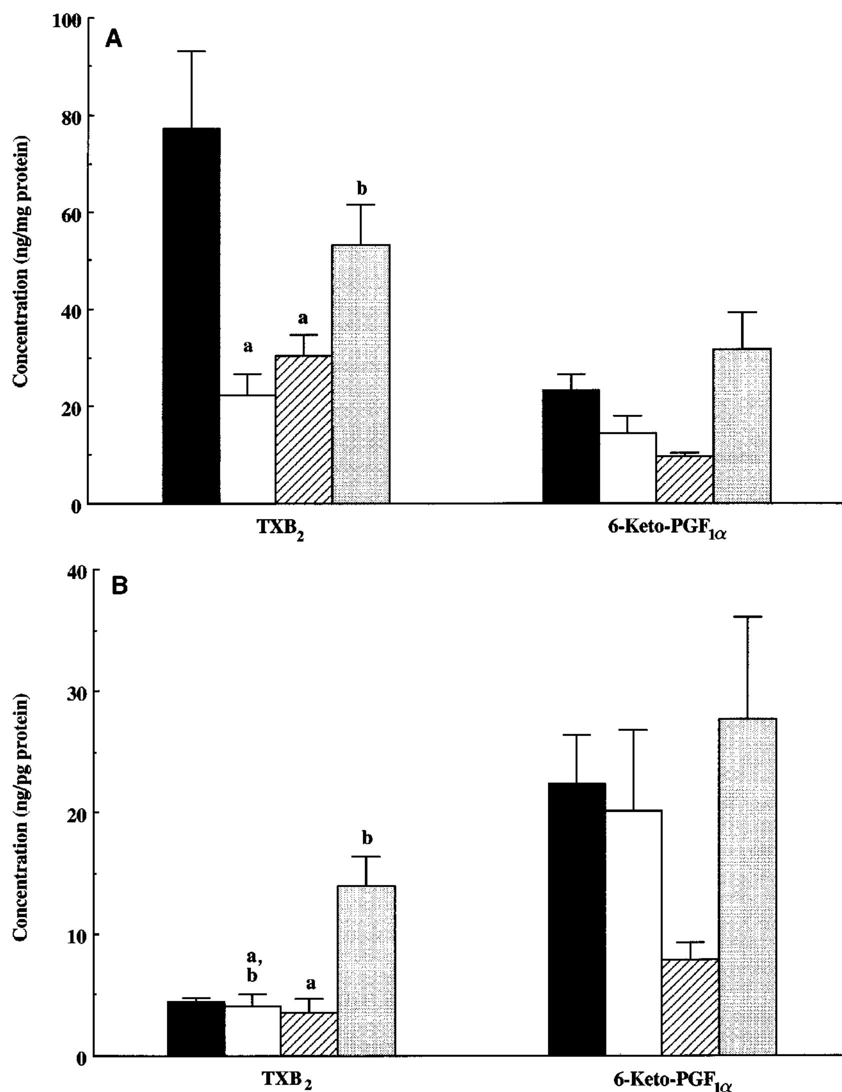

FIG. 1. Levels of thromboxane B_2 (TXB_2) and (6-keto-prostaglandin $F_{1\alpha}$ ($PGF_{1\alpha}$) in heart (A) and liver (B) of guinea pigs fed nonpurified diet (NP, solid block) supplemented (10%, wt/wt) with menhaden fish oil (MO, open block), seal oil (SLO, cross-hatched block) or corn oil (CO, stripped block). The guinea pigs were fed the diets for 23-28 d, after which they were killed. Organs were quickly harvested, flash-frozen, and stored at −86°C. Prior to analysis they were minced and incubated as described in the Materials and Methods section. The samples were purified by passage through Amprep™ minicolumns (Amersham Canada Ltd., Oakville, Ontario, Canada), and analyzed by enzyme immunoassay. The data are the means ± SEM of values obtained from 4–9 animals from two different dietary studies. Differences in letters above data bars indicate differences are statistically significant by student's *t*-test with Bonferroni correction factor.

($P < 0.05$), heart ($P < 0.005$), and adipose tissue ($P < 0.005$). Although its levels are often not reported, DPA can be present in appreciable amounts in mammals, particularly in heart and plasma. Remarkably, it was the major n-3 PUFA in heart PL of CO-fed guinea pigs. In both MO- and SLO-fed guinea pigs, the greatest increases in DPA were in the plasma TAG (22- to 25-fold increases, relative to CO), although its highest proportions were in the heart TAG. In all PL there were significant differences between levels of DPA in the MO vs. SLO groups, as exemplified in the heart PL, where values for the SLO animals were 63% higher than those for animals fed MO.

The effects of the marine oil diets on proportions of AA in plasma and organs were very surprising, as the only decrease in AA levels (relative to values with CO-fed animals) was in the heart PL of SLO-fed guinea pigs (Table 5). Otherwise, all samples and fractions had proportions of AA that were either unchanged or even increased; for example, the AA content of plasma PL and TAG of MO-fed animals was ≥3-fold higher than in the CO group. In all samples but plasma and liver, values for AA in the MO group were significantly higher than those for the SLO group.

Prostanoid levels in MO-, SLO-, and CO-fed guinea pigs. The effects of the diets on synthesis of TXA_2, PGI_2, PGE_2, and $PGF_{2\alpha}$ were determined in heart, liver, plasma, and brain. TXA_2 and PGI_2 were measured as TXB_2 and 6-keto-$PGF_{1\alpha}$, respectively. The only appreciable effects of the marine-oil diets on

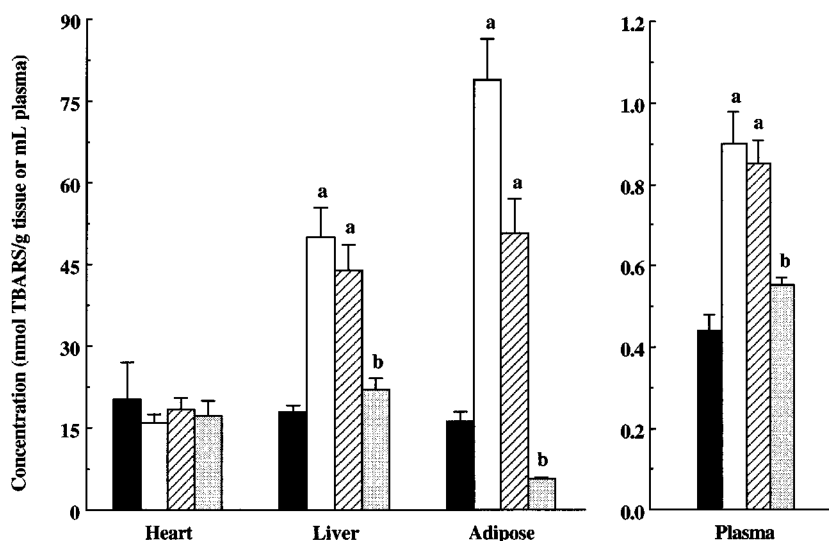

FIG. 2. Levels of thiobarbituric acid-reactive substances (TBARS) in organs and plasma of guinea pigs fed NP supplemented with MO, SLO, or CO (10%, w/w) for 23–28 d. The guinea pigs were killed with euthanol, after which organs were quickly harvested and plasma separated from whole blood. All samples were flash-frozen and held at −86°C prior to analysis. The color adducts were developed and TBARS were quantitated by high-performance liquid chromatography as described in detail in the Materials and Methods section. Data are the means (± SEM) of values obtained from 4 to 9 different animals. For each data bar, differences in letters above data bars indicate that differences are statistically significant.

the prostanoid synthesis associated with organ harvesting were in the hearts and livers. In the hearts (Fig. 1A), levels of TXB_2 in guinea pigs fed MO or SLO were consistently lower than those in CO-fed animals, although differences reached statistical significance only between the MO and CO groups ($P <$ 0.03). 6-Keto-$PGF_{1\alpha}$ measured in the heart preparations was also lower in the marine oil-fed animals, and was lowest in those fed SLO. As shown in Figure 1B, levels of TXB_2 were approximately threefold lower in liver homogenates from marine oil-fed than in CO-fed guinea pigs, although differences were statistically significant only between the SLO and CO groups ($P <$ 0.03). Liver homogenate 6-keto-$PGF_{1\alpha}$ was also lowest in the SLO-fed animals. There were no apparent differences in levels of PGE_2 or $PGF_{2\alpha}$ in preparations from either the hearts or livers (not shown). In plasma and brain, measured levels of prostanoidwere not altered significantly by dietary marine oils.

Effects of the diets on TBARS levels. One concern regarding ingestion of high levels of n-3 PUFA is that they could increase concentrations of potentially harmful LPO. As shown in Figure 2, there were no diet-related differences in levels of TBARS in the guinea pig heart homogenates. There also were no significant differences between TBARS in brain homogenates, although values for brain homogenates from SLO-fed animals were only about 75% of those in either the MO or CO groups (not shown). By contrast, homogenates of livers and adipose tissue, as well as plasma from guinea pigs fed either MO or SLO, were all higher in TBARS than were those from the CO-fed animals. There were no significant differences between values for the MO and SLO groups, although those for the SLO group were consistently lower, possibly reflecting a lower total dietary PUFA content. It was of interest that TBARS levels were appreciably higher in adipose homogenates from animals fed the NP diet than in those from the CO group.

DISCUSSION

This study provides a comprehensive analysis of the effects of dietary n-3 PUFA on guinea pig organ and plasma fatty acid and lipid profiles, and on generation of biologically active prostanoids and lipid peroxides in tissue homogenates. It confirms previous suggestions that when this species is maintained on standard laboratory diet it is relatively deficient in n-3 PUFA (11) and demonstrates that relatively short periods of marine oil feeding dramatically increase the n-3 PUFA content of major organs and plasma. It has been pointed out that relative n-3 PUFA deficiencies are appearing in an increasingly large sector of the Japanese population, and also in North Americans (23). The ability to reverse such deficiencies biochemically raises the question of the importance of the source of the marine oil, and this is one of very few studies to address this.

Much of the current interest in dietary marine oils concerns their effect on circulating levels of TAG and cholesterol. In the guinea pigs, only the plasma exhibited n-3 PUFA-dependent changes in quantities of these lipids (Table 3). The MO- and SLO-fed groups exhibited similar reductions in plasma TAG (≈55%) and TC (≈25%) levels, relative to levels in animals fed the CO diet. A significant hypotriglyceridemic effect has been reported to occur also in rats and swine but in few other species (see 24 for refs.), and the effect in rats may depend on the source of marine oil (4). Humans whose diets were supplemented with fish or flaxseed oils have been reported to have an average 25% reduction in circulating TAG (25,26), which is

less than that which we observed in the guinea pigs. There are several possible explanations for the more pronounced hypotriglyceridemic effect in the guinea pigs. First, we calculated the reductions relative to animals fed diets high in CO, and the comparator diets in humans probably did not contain similarly high proportions of n-6 PUFA. It may also reflect interspecies variability in the extent to which n-3 PUFA regulate metabolic events that determine circulating TAG levels, including the degree of inhibition of hepatic TAG synthesis (25). Data from the recent study of Ikeda *et al.* (26) support the idea of species differences, since rats fed either squid (fish-type oil) or seal oil had levels of liver TAG that were >20% lower than levels in safflower-fed controls (27), whereas in the guinea pigs, liver TAG levels actually increased slightly relative to the CO controls (Table 3). As with the TAG, discrepancies between humans and animals in the effects of n-3 PUFA on plasma cholesterol may reflect differences in comparator diets and/or interspecies differences. In humans, fish oils appear either to have no effect or even to increase TC slightly (24,26), whereas most commonly used laboratory species (4,24, and refs.), and the guinea pigs (Table 3) have reduced levels of TC with n-3 PUFA feeding. With respect to marine oil specificity in altering plasma lipids, we did not find any significant differences between the MO and SLO groups in terms of their effects on TAG or TC levels. By contrast, one of the first studies to compare the effects of seal and whale oils vs. fish oil in healthy humans found that, relative to control subjects who were not given oil supplements, serum TAG levels were reduced significantly by cod liver oil but not by SLO or whale oil (26). The authors proposed that the more pronounced effect of the fish oil was due to its specific *sn*-2 positioning of EPA, the n-3 PUFA that has been suggested as being specifically responsible for reducing circulating TAG (28). However, it could also reflect the 40% higher proportion of EPA in the cod liver oil. MO also has a higher proportion of EPA (relative to SLO) and it is in the *sn*-2 position, yet in the guinea pigs, plasma TAG levels were the same for both groups. Again, this could reflect a species-specific difference in the extents to which marine mammal oils and fish oils regulate lipid metabolism.

Data from two Norwegian studies indicated that the relative proportions of total n-3 PUFA in serum lipids of humans fed seal, whale, or cod liver oils reflect the relative content of these PUFA in the different diets (26,29). Similarly, the higher proportions of total n-3 PUFA in the plasma lipids of MO-fed guinea pigs (relative to SLO, Table 4) were consistent with the higher proportions of total n-3 PUFA in the MO diet (Table 1). This apparent dose response relationship for total n-3 PUFA was true only of the tissue TAG (heart, adipose), which we (5) and others (30) have proposed is a good indicator of dietary fat intake. This may not be true of rats, since Ikeda *et al.* (27) reported that, when two groups of rats were fed different marine oils that had equivalent levels of total n-3 PUFA, the resulting total n-3 PUFA levels were equivalent in liver phospholipids but were very different in liver TAG.

One n-3 PUFA that warrants brief mention is the frequently ignored DPA (22:5n-3). Although it constituted less than 3 and 2% of the SLO and MO diets, respectively (Table 1), DPA accounted for ≥10% of total n-3 PUFA of heart PL and ≥20% of total n-3 PUFA in the TAG of heart in animals fed these diets (Table 5). Even more surprising was the fact that it was quantitatively the most important n-3 PUFA in heart lipids of animals fed CO. This apparent concentrating of DPA in heart lipids was also seen in rats fed MO (4,31) or soybean oil (4), which tempts us to speculate that it plays a specific role in cardiac function. To date, however, the only property that has been attributed specifically to DPA is an antiatherogenic one (32).

Strong support for the concept that there are species-specific differences in regulation of fatty acid metabolism came from our observation that marine oil diets affected plasma and organ levels of AA in guinea pigs in a very different manner from that in rats. For example, although standard laboratory diets for rats and guinea pigs both contain high levels of n-6 PUFA, levels of AA in plasma phospholipids from rats (14–27 mol%) (4,27,33,34) are considerably higher than those of guinea pigs (4–8 mol%) (35; Table 5). Lower levels (4–10 mol%) are also reported for humans (26,29,33,36). Feeding fish-oil has been shown consistently to reduce AA levels in rats, whereas in humans and guinea pigs (e.g., Table 5) they either remain the same or even increase, as can be seen in the plasma PL of the MO-fed animals. However, we cannot rule out the possibility that reductions in plasma AA are dose and/or time dependent (37). The lack of change or increases in levels of AA in n-3 PUFA-fed guinea pigs occurred in all tissues with the exception of heart (Table 5). The consistently lower levels of AA in SLO-fed animals could be the result of a more effective inhibition by SLO of the Δ-6 desaturase that converts linoleic acid (18:2n-6) to AA (38). In some samples (e.g., plasma, liver) this is supported by the observation that levels of linoleate in the SLO group were significantly higher than they were in MO-fed animals, in spite of the fact that both marine oil diets had the same linoleate levels (Table 1). Regardless of mechanism, the importance of species- and/or oil-specific differences in effects on AA levels has been attributed their ability to modifiy endogenous eicosanoid production (39).

Although differences in experimental approaches make it difficult to compare literature data in a meaningful manner, it appears that dietary n-3 PUFA generally lower levels of TXA_2 in blood/vascular components of both rats (27,33,34) and humans (29,33,37,40) and have less effect on PGI_2 (34,40). In the present study, the fact that there were no appreciable diet-dependent differences in prostanoid levels in the guinea pig plasma (not shown) could be due to a true species-dependent difference in n-3 PUFA regulation of prostanoid synthesis, including an undetected compensatory increase in TXA_3 production. Previous studies have suggested that there is a species difference in ability to form PGI_3 from EPA (33). Alternatively, it could merely reflect the lack of difference between substrate availability in the marine oil and CO diets, since in the heart homogenates where AA levels were reduced by marine oil feeding, TXA_2 and PGI_2 were both reduced by 40–70% in the MO- and SLO-fed animals (Fig. 1A). Similar n-3 PUFA-dependent reductions in these

PG have been observed in marmoset monkey heart (20,41) and in rat heart (42,43) and cardiomyocytes (44). These demonstrations that n-3 PUFA modulation of cardiac eicosanoids may be physiologically relevant, as Leaf and colleagues recently demonstrated that TXA_2 induces arrhythmic activity in cultured neonatal rat myocytes, whereas PGI_2 attenuates beat rate and arrhythmic activity (45).

The role of dietary fat in tissue injury, due to increased LPO and free radical formation, has generated considerable interest and controversy, since the high degree of unsaturation of n-3 PUFA could theoretically promote cardiovascular disease and cancer (46,47). An interesting finding in the present study was that, although the marine oil-dependent increases in n-3 PUFA in the heart were of approximately the same magnitude as those in the plasma and adipose tissue (Table 4), there were no increases in TBARS in hearts of guinea pigs fed MO or SLO (Fig. 2). Similar types of discrepancies between organs and oxidative stress markers have been described in rats (48). Studies done with guinea pigs suggest that discrepancies may be related to numerous factors, including organ-specific differences in antioxidant enzyme levels (49) or activities (50), and/or in levels of antioxidants such as ascorbate and α-tocopherol (51). The effects of different dietary PUFA on any of these factors could also vary between organs (52), although data from the present study suggest that in organs (and plasma) where there are increases in TBARS levels in response to n-3 PUFA-rich marine oils (e.g., in liver), the increases are independent of the type of oil fed.

In summary, this study has provided new information regarding the effects of dietary marine oils on the lipid profiles and fatty acid metabolite levels in plasma and homogenates of major organs of the guinea pig. We found that in several respects, feeding seal oil produces metabolic profiles that differ from those produced with feeding fish oil. Equally significant, our data lend support to Harris' earlier suggestion (24) that there are potentially important species differences in fatty acid and lipid metabolism, and that in many respects responses of humans to dietary n-3 PUFA can be mimicked better in guinea pigs than in rats.

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Docosahexaenoic Acid in the Infant and Its Mother

Sir:

The potential role of 22:6n-3 (docosahexaenoic acid, or DHA) as a necessity for the development of the central nervous system, especially in the third trimester of gestation of the full-term human infant, is widely recognized (1–5). This has led to suggestions that either formulas (6) or maternal blood, and hence milk, should be enriched with DHA (7–9). Even a short period (2–4 mon) of supplementation may have long-term (~12 mon) benefits (10,11).

In many studies with fish oils, popular and reputable products such as MaxEPA® (Seven Seas Health Care Ltd., Hull, United Kingdom), with 180 mg/g of eicosapentaenoic acid (EPA) and 120 mg/g of DHA (12), have been used as sources of DHA. In one study the excess of EPA was presumed to have interfered briefly with the growth of preterm infants by competing with 20:4n-6 (arachidonic acid, or AA), also essential for infant health (2,13). Innis *et al*. (3) concluded, based on comparison with breast-feeding, that healthy term-gestation infants do not require DHA if the precursor 18:3n-3 (α-linolenic acid) is fed in a formula at 2.1% w/w (~10% of energy).

Carlson (2) mentioned that DHA and AA decline in infant plasma and erythrocyte phospholipids following birth. The work of Harzer *et al*. (14) clearly illustrates (Table 1) a similar phenomenon for whole human milk in Germany. Over 36 d AA dropped by nearly half, EPA 12-fold, and DHA by a third. The ratio of EPA to DHA at 36 d was 1:3. Table 2 gives comparable figures from Makrides *et al*. (4) for human milk in Australia; after 16 wk (112 d) the EPA to DHA ratio also stabilized at 1:3, and that ratio was maintained to 30 wk. Similar ratios are seen for milks from the United States collected at 42 d (15) and are also confirmed by Hoffman and Uauy (16) for human milk at day 200 and by Dotson *et al*. (17) for the mean of 30 samples collected at 4, 6, and 8 wk postpartum. An excellent collection of data by Jensen (18) provides other examples of this ratio in countries with dietary fatty acid patterns as diverse as France and Finland. Higher proportions of DHA in particular are recorded, for example by Boersma *et al*. (19) for milks on the island of St. Lucia, and by Jørgensen *et al*. (20) for both EPA and DHA in human milk in Denmark, although the approximately 1:3 ratio for EPA to DHA was observed in the Danish milks over 3 mon. The latter authors reported two cases of the relative concentration of DHA suddenly increasing during the study period for no obvious reason. Excluding these, the DHA proportion declined as expected, the median figures being 0.49, 0.40, and 0.32% at 1, 2, and 4 mon, respectively (*cf*. Table 2). It can be inferred that marine foods are involved in these instances, much as has been recently suggested for the fatty acids of human milks from different parts of China (21). It is of interest that this 1:3 ratio was also employed in the special preterm formula used by Carlson and Werkman (10).

The stabilization of the EPA to DHA ratio in the milk of mothers not receiving special diets may be compared to the data of Henderson *et al*. (9) condensed in Table 3. The mothers were given a diet supplement of a total of 1080 mg of EPA and 720 mg of DHA each day (six fish oil capsules), and this included 6 mg of vitamin E per day. The percentage of EPA increased sixfold in the fatty acids of milk and in the fatty acids of maternal and infant erythrocytes. DHA increased by about 25% in both erythrocyte samples, but only doubled in the milk. Possibly these two n-3 fatty acids displaced saturated acids from phospholipids in erythrocytes; importantly, the EPA increase did not seriously affect the AA level in those lipids. Fatty acid chain length may be a selection factor in the enzymatic processes involved (22).

TABLE 1
Total Fatty Acids (w/w%) of Human Milk[a]

	Day after parturition					
Fatty acid	1	3	5	8	22	36
18:2n-6	9.70	10.30	9.97	10.76	9.96	11.78
18:3n-3	0.60	0.70	0.62	0.69	0.70	0.71
20:4n-6	0.75	0.55	0.54	0.50	0.36	0.39
20:5n-3	0.64	0.43	0.18	0.22	0.14	0.05
22:5n-3	0.25	0.13	0.11	0.09	Trace	0.05
22:6n-3	0.22	0.21	0.29	0.26	0.15	0.16

[a]Average of 228 samples, from seven German mothers. Adapted from Harzer *et al.* (14).

TABLE 2
Fatty Acids (w/w%) of Breast Milk Supplied by Australian Mothers of Fully Breast-Fed Infants[a]

Fatty acid	Week 6	Week 16	Week 30
18:2n-6	13.56	13.92	13.56
18:3n-3	0.89	0.94	0.85
20:4n-6	0.45	0.40	0.39
20:5n-3	0.07	0.07	0.06
22:5n-3	0.16	0.16	0.16
22:6n-3	0.26	0.21	0.19

[a]Adapted from Makrides *et al.* (4).

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

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TABLE 3
Some Fatty Acid Changes (w/w%) in Human Milk and Erythrocyte Lipids, Before and After Maternal Fish Oil Intake[a]

	Human milk		Maternal erythrocyte		Infant erythrocyte	
Fatty acid	Day 0[b]	Day 21	Day 0[b]	Day 21	Day 0[b]	Day 21
18:2n-6	13.0	12.5	8.2	9.6	5.6	7.9
18:3n-3	0.77	0.76	0.09	0.03	0.11	—
20:4n-6	0.67	0.52	12.9	14.6	13.0	15.0
20:5n-3	0.08	0.50	0.24	1.4	0.11	0.70
22:5n-3	0.14	0.34	1.4	2.2	0.30	0.78
22:6n-3	0.37	0.70	4.5	5.6	4.5	6.1

[a]Recorded by Henderson *et al.* (9).
[b]Two weeks postpartum.

The constancy of the EPA to DHA ratio in normal human milk from different parts of the world and its relatively low level after approximately 30 to 40 d of lactation lead to the conclusion that the origins of EPA and DHA are specific and quite unconnected to an obligation on the part of the mother to supply preformed DHA to the infant. Tables 1–3 suggest that the EPA and DHA of human milk are derived from the same source, phospholipids. Neville and Walsh (23) clearly illustrated that the milk fat globule is secreted from the mammary alveolar cell by a "pinching off" process in which the triacylglycerol globule is encapsulated in actual cell membrane. This process is well understood (24,25). This cell membrane phospholipid, which amounts to at least 20% of the human milk fat globule membrane (26), presumably continues to incorporate EPA and DHA under normal conditions in a stable 1:3 ratio after lactation has been active for at least 30 d (Tables 1 and 2). Phospholipid content and some details about fatty acid of human milk globule membranes are shown in Table 4. The total for C_{22} acids is selective and somewhat higher, relative to AA, than the C_{22} acids in the detailed breakdown of whole milk lipids given by Jensen (18). However, in the same review, more of the C_{20} AA was present in the milk triacylglycerols, at about 0.3% of fatty acids. One can conclude that the long-chain n-3 fatty acids, both C_{20} and C_{22}, are derived from the maternal cell wall phospholipids. Bitman *et al.* (28) emphasized that class percentages of phospholipids in milks from mothers giving birth either prematurely or at term are remarkably constant. The origin of the higher levels of polyunsaturated fatty acids at earlier times of lactation (Table 1) is not readily explained, but the recent comments of Al *et al.* (29) that essential and long-chain polyunsaturated fatty acids (AA, DHA) in maternal plasma phospholipids increase considerably during normal gestation are possibly relevant. In maternal plasma phospholipids the total n-3 fatty acids then declined after the 18th wk of gestation (30), presumably as fetal brain development accelerated. Spear *et al.* (31) noticed no EPA at all in fatty acids of full-term human colostrum, but 0.32% of the fatty acids were DHA. These figures reflected respective maternal serum fatty acid EPA and DHA percentages of 0.06 and 1.80%. Colostrum figures are thus quite different from that of the milk at day 1 in Table 1. The subsequent decline in maternal DHA reserves (29) is of concern. Interestingly, the fatty acids of the infant plasma phospholipid fraction of preterm infants fed expressed breast milks had EPA to DHA ratios of 1:7 and 1:5 at the first and fifth week of life, respectively (6). This change was thought to reflect the development of the ability of the infant after 5 wk of life to convert α-linolenic acid to DHA, because maternal milk EPA to DHA should then be stabilized at 1:3.

TABLE 4
Phospholipid Classes and Long-Chain Polyunsaturated Fatty Acids of Human Milk Globule Membrane[a]

	Lipid class					
	PE	PC	SPH	LPC	PI	PS
Percentage of total phospholipids	36.6	29.7	26.2	1.9	4.6	1.0
20:4n-6 (w/w%)	1.0	1.7	1.6	—	—	—
C-22 (w/w%)	3.2	—	5.0	3.9	4.1	6.1

[a]As published by Bracco *et al.* (27). PE, phosphatidylethanolamine; PC, phosphatidylcholine; SPH, sphingomyelin; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine.

The ability of the young rat to convert α-linolenate to DHA is not influenced in the short term by linoleic acid (32), an interesting point in view of dietary research in human infants not involving preformed DHA (3,33). When rats were given human breast milk with an EPA/DHA ratio (actual w/w%) of 0.8:1.1, the lymph triacylglycerols had the same ratio (0.5:0.7) while the lymphatic phospholipids had a ratio of 0.8:1.8 (34). One would not expect this to have the 1:3 ratio of human milk, but the phospholipids of the lymph duct wall endoplasmic reticulum seem to show enrichment with respect to DHA relative to EPA when "pinched off" to enclose the basic triacylglycerol-rich part of the chylomicrons (35). The human milk given to the rats (34) came from a woman in the eighth month of lactation. Possibly the low proportion of DHA relative to EPA in her milk confirms the views of Al *et al.* on DHA depletion with lactation time (30).

Few of these concepts are new. Harris *et al.* (36) demonstrated enrichment of human milk with fish oil-derived polyunsaturated fatty acids in 1984. Hall (37) made the point that fatty acids of human milk collected during active feeding are generally uniform in composition (confirmed by Gurr [38]). Many changes outlined in Tables 1 and 2 are not peculiar to human milk. Bitman and Wood (39), for Holstein cows, may be quoted:

> The changes that occurred in milk total fatty acids were reflected in phosphatidyl phospholipid fatty acid compositions: an increase in medium-chain fatty acids and a decrease in polyunsaturated fatty acids of 18, 20 and 22 carbon atom chain lengths as lactation progressed. These changes are consistent with the theory that milk phospholipids are synthesized *de novo* entirely in the mammary gland.

That dietary fatty acids (including fish oil supplements) can modify total milk fatty acid composition through the triacylglycerol fatty acids is well known. In ruminants this ef-

fect is also demonstrable in the fatty acids of the milk fat globule membrane (25).

Bitman *et al*. (28) concluded for human milk that "core lipids are surrounded by a milk fat globule membrane which was identical in the milk of mothers of premature and term infants in all stages of lactation." However, modifying the maternal diet with regular amounts of supplemental DHA increased the amount of DHA in milk as shown in Table 3, counteracting the normal decrease with time shown in Tables 1 and 2. It is not known if this result reflects changes in fatty acids of triacylglycerols, smaller milk globules with a higher proportion of membrane phospholipid, or a modification of the fatty acids of that membrane. Effects of dietary fats on human milk are usually considered to be short-term. However, an important distinction has been noted for EPA and DHA. After fish oil ingestion these two fatty acids remain elevated in milk for up to 3 d (40). Because of uncertainty about temporary storage of EPA and DHA in adipose tissue and about mobilization (41), one cannot state with certainty that this retention of EPA and DHA is due to changes in the maternal cell phospholipids. The proportions and positions of additional DHA in milk triacylglycerols seemingly have not been reported, although obtaining this information is technically feasible.

The erythrocytes of both mother and nursing infant are accessible models for studying changes in DHA proportions in milk phospholipids (4,9). Recently Jørgensen *et al*. (20) compared EPA and DHA in the phosphatidylcholine and phosphatidylethanolamine of infant erythrocytes from 1 to 4 mon postpartum. These two fatty acids declined slightly, but in parallel, in the phosphatidylcholine from breast-fed and formula-fed groups. This confirms the report for the latter lipid published by Innis *et al*. (42) for very low birthweight infants. When term infants were fed α-linolenic acid (no DHA) at varying levels (33) DHA was observed to decrease in total erythrocyte phospholipids over 21 to 120 d, while EPA doubled in the same period. Because of controversies over digestion in the neonate, more comparisons of phospholipid fatty acids of erythrocytes with those of milk membranes are urgently required to determine how closely this parallel can be extended (5). Consideration should also be given to the route by which supplemental DHA in formulas is digested and distributed in the infant body because it may differ between triacylglycerols and phospholipids and because not all currently available fish oils or other sources of DHA supplementation are "natural" in the distribution of DHA on glycerol (43,44). Finally, it is now obvious that the emphasis on long-chain n-3 fatty acids for the development of the neonate cannot be separated from the nutritional aspects of the same fatty acids in the mother, particularly in view of the increasing prevalence of breast-feeding.

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SYMPOSIUM

PUFA in Infant Nutrition: Consensus and Controversies

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The AOCS-sponsored meeting on "PUFA in Infant Nutrition: Consensus and Controversies" was held November 7–9, 1996, in Barcelona, Spain. A total of 375 technical registrants from 39 countries attended. The focus of the meeting was on polyunsaturated fatty acid (PUFA) status of mothers and infants, PUFA metabolism, visual and neural development as related to long-chain (LC) PUFA status, safety, and other industry issues. One session included a review of the status of LCPUFA regulation and recommendations that have come from different committees. No attempt was made to reach a consensus on need, as such an attempt would have been inappropriate for a research meeting. Over 100 abstracts and posters were presented within the broad category of LCPUFA and infant development.

The meeting was conducted as an advanced workshop using a "new" concept that mandated more discussion than usual in scientific symposia. The regular sessions were 2 h long, and the time was divided equally between state-of-the-art lectures (to bring participants to some common ground on the topics) and discussion (to enhance communication on the topics among scientists from academia, government, and industry). By providing an opportunity for dialog among these groups, the intent was to encourage them to reach a consensus on the state of the field and to share perceived needs on directions for new work.

At the meeting, the first two sessions provided essential new information about brain development relative to LCPUFA and neonatal PUFA metabolism. These were followed by sessions devoted to studies of LCPUFA and visual and neurodevelopment, especially to critical analyses of the randomized trials that were published before the meeting. A session was devoted to biological effects of LCPUFA other than neural function during development. The consensus was that there is a need for balance between n-3 and n-6 LCPUFA for optimal biological function, with the understanding that the definition of optimal balance and the role that intake of essential fatty acids and LCPUFA play in achieving it may only be defined by further research. The last session was devoted to issues of industry and regulatory groups, especially the issue of safety.

In addition to the regular sessions, workshops provided technical direction for investigators and sponsors planning new work within the broad area of PUFA in infant nutrition. Some of these were directly related to regular sessions, but most of the workshops had a clinical orientation. The organizers were aware that larger trials with infants were a perceived need and realized that many working in the LCPUFA area had little experience with randomized clinical trials (RCT). For those planning new exploratory studies of LCPUFA and development, one workshop dealt with confounding and mediating variables in studies of human neurodevelopment. Another discussed issues essential to the conduct of RCT, particularly how to design studies with sufficient statistical power to reject or accept the null hypothesis. Other workshops provided directions for new studies to explore the basic or mechanistic effects of LCPUFA. Papers from the discussion leaders and workshop directors are included in this issue of *Lipids,* and they provide a state-of-the-art summary of the topics introduced above.

There is a need to look at more outcomes that can be related to n-3 fatty acid deficiency in human infants with different n-3 LCPUFA status. RCT designed to study outcomes affected by LCPUFA status in smaller clinical trials are also needed. Study designs, methods, and populations need to be carefully chosen in future clinical studies and related to the smaller published trials. A study that adheres strictly to RCT methodology, but that is poorly designed or based upon a lack of understanding of the biology of LCPUFA, may be technically excellent but have no biological meaning and value. Because the published effects of feeding LCPUFA on behavior have been small, an additional need for RCT is that they account for evaluator differences, which may mask differences in neural function if adjustments are not made in calculating the statistical power for the study. Because the design of better clinical trials was a major goal of the meeting, the organizers agreed that the true success of the meeting could not be evaluated until the design, methods, and results of new clinical trials are available.

Finally, it was noted that mechanistic studies of the effects of LCPUFA in neural development and in other physiological functions are needed. There appears to be an increased interest in conducting such studies since the meeting was held. We hope that some bases for the physiological actions of LCPUFA and new exploratory and clinical work can be available for presentation at the time of the second international meeting in the Fall 2000 in Kansas City, Missouri.

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Brain Development and Assessing the Supply of Polyunsaturated Fatty Acid

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ABSTRACT: Membrane lipids are necessary for structure and function of the developing nervous system. Rapid synthesis of brain tissue occurs during the last trimester of development of the human brain and the early postnatal weeks. This synthesis of brain structure involves the formation of complex lipids, many of which contain significant quantities of chain-elongated desaturated homologs of essential fatty acids. The present report discusses the implications of change in nutritional status on processes of brain development and metabolic events that involve lipids.

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EARLY STAGES

In adult vertebrates, the form of nervous system brain development at early stages is remarkably similar. The distinct character of the neural plate and neural tube, from which the nervous system originates in all vertebrates, suggests that development of the central nervous system occurs through similar overall mechanisms. The cells, regions, and various structures of the brain do not develop uniformly, as in other tissues and organs (1–3). Characteristic, well-defined stages of growth occur anatomically and biochemically (4,5) and result in significant growth spurts or critical periods during fetal and neonatal life. The development of any part of the brain occurs in stages: induction of the neural plate, localized proliferation of cells in different regions, migration of cells, formation of identifiable parts of the brain by cell aggregation, differentiation of immature neurons, formation of connections, selective cell death, and modification of connections (6). These changes within the developing nervous system proceed in a caudal (brain stem) to rostral sequence (7). Caudal brain structures include phylogenetically older brain structures, whereas rostral structures are phylogenetically newer (8). Structural changes in the brain during development result in an increase in weight and size. These increases are not necessarily parallel: the greatest growth in size occurs prior to the greatest gain in weight (9). Different parts of the brain grow at different speeds, and not all regions reach their fastest rates at the same time. In this respect, the "growth spurt" and velocity curves as defined by Dobbing (1,10,11) represent rates of change in total brain weight over time and not individual regions of the brain. The early concepts developed by Dobbing did not encompass the developmental processes that are now understood to be critical periods of growth and highly susceptible to insult. As well, they do not reflect the interrelationships of growth occurring in subregions of the developing brain (12).

Neurogenesis. Most neurons are generated in or close to the ventricular zone of the neural tube. A complex set of factors including neuron type, position in the mitotic gradients, and phylogenetic status determines the time of neuronal origin (7). The number of neurons initially formed in any brain region is determined by three factors: the duration of the proliferative period (which may last a few days to several weeks), the duration of the cell cycle (which in a young embryo is a few hours and increases to 4 or 5 d as development progresses), and the number of precursor cells (6).

Depending on the region of the nervous system (13) and neuron type (7), production of neurons occurs over varying lengths of time with different time schedules for formation. Neurons born first in any given region of the mature nervous system are the phylogenetically older neurons and the larger neurons (7). Large neurons apparently become postmitotic early because they have the longest axons and must reach their targets while the embryo is still small. Neurogenesis peaks around the 14th week of gestation and is completed by the 25th week when the adult number of neurons is present (14). In rats these developmental events occur at about 18 and 20 d of gestation, respectively. Known exceptions that continue to proliferate postnatally include some neurons of the hippocampus and the cerebellum. It is important to recognize that the timetable of neuronal differentiation cannot be simply deduced from its time of origin. The type of neuron, its regional situation, and the timing of the arrival of axons with which synapses will form are factors that also affect neuronal differentiation.

Gliogenesis. Glial cells tend to originate after neurons in any particular region of the brain. Formation of glia differs from neuronal formation in three main ways. First, many of the proliferative cells that generate glia lie outside the neuroepithelium, at or near the site where they will be located in the adult (15). Second, the production of glial cells continues throughout

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adult life (7). Gliogenesis is primarily a postnatal event (16); however, in some brain regions it is detected before birth (16,17). Early gliogenesis is completed by the 15th week of gestation in humans and the 16th day of gestation in rats (12). Third, damage to the glial population is rarely permanent (18–20). However, it is known that a qualitative unbalance or quantitative deficit of food intake produces alterations in the ontogeny and function of the nervous system (21–24).

Making connections with function. Because most nerve cells are generated at or close to the inner, proliferative surface of the neural tube, they subsequently migrate past other cells to their final locations. The temporal origin of neurons may be related to later establishment of anatomical connections (15). There are two spatiotemporal gradients for neuron migration. In nuclear regions of the brain (such as the thalamus and hypothalamus), the oldest neurons produced by the neuroepithelium are pushed farther out as the younger neurons are generated. In these regions, cells accumulate in an "outside-in" or "pushing" gradient (25). The "inside-out" or "passing" gradient occurs in regions of the brain that have a laminar structure (such as the cerebral and cerebellar cortices). Cell accumulation in these regions occurs as younger neurons migrate past the older neurons, which remain closest to the neuroepithelium (26). Despite the heterogeneity of the cell population in the nervous system, the development of the system's complex form is reliable. The developmental program of the nervous system must contain mechanisms for neurons to migrate to their proper destination. This arrangement of neurons must be completed successfully for normal functioning to result.

Functional connections in the nervous system are established through growth of neurites (axons and dendrites) and formation of synapses. Synaptogenesis is the contact between axons and target cells that starts before neurogenesis is completed (27) and follows a cell-specific, region-specific timetable. Most neurons generate and receive many processes, producing many more synaptic connections. A period of programmed cell death and synapse reorganization follows as the final stages of brain morphogenesis. Stages of brain morphogenesis ensue in each brain region in a specifically timed series of events. Each period becomes a critical basis for development of the next (28). Time scales occurring in each brain region are further complicated by migration of cells between regions. Due to the brain's general lack of regeneration potential and its dependence on specialized interactions, any misdirected, mistimed, or absent developmental cues can lead to structural aberrations (29). These structural changes are irreversible and result in functional deficits if future developmental changes are unable to compensate for these structural changes. Thus, insults that alter brain development in the fetus or neonate may permanently alter a myriad of functions in later stages of infant development.

BRAIN LIPIDS AND NUTRITION

Second only to adipose tissue, the brain is the most lipid-concentrated organ in the body. Nervous tissue contains 50% lipid on a dry weight basis, or 10% lipid on a weight/weight basis (30). This lipid plays a role in modifying the structure, fluidity, and function of brain membranes (31–36). A variety of complex lipids exists in the brain, and the composition and metabolism of these lipids change with development and age (37–39).

Fatty acid accretion in the brain. One approach to determining the fatty acid needs of the developing infant is to determine the fatty acid composition during infant growth and quantitate the accretion of fatty acids in body tissues. Although this approach seems simple, it may be hampered by not knowing whether fetal development is the optimal model for the assimilation of fat accretion during the extrauterine growth of infants born prematurely. It should be noted that a difference in the deposition and net absorption of fat exists between perinatal and postnatal growth (40). Accretion of essential fatty acids in adipose tissue during the last trimester of intrauterine development was estimated by using data on net fatty acid accretion and values for adipose tissue fatty acid composition (41). The average accretion rates determined are consistent with the fat deposition estimated by indirect calorimetry for preterm infants fed 120 kcal/kg/d (42). Based on the assumption that tissues not quantitatively analyzed have a total fat and essential fatty acid content similar to that of skeletal muscle (41), minimum values for essential fatty acid utilization in tissue synthesis during the last trimester of intrauterine growth were estimated. Estimates for utilization in *de novo* synthesis of tissues were approximately 522 mg/d of n-6 fatty acids and 67 mg/d of n-3 fatty acids (41). These values are minimal levels, as consideration was not made for the amounts of fatty acids oxidized to meet the energy requirements for tissue accretion. More information about the proportion of fatty acids used in energy metabolism and tissue synthesis is needed before information about body composition can be applied to a practical feeding situation. Proportions of major dietary fatty acids consumed may potentially affect the net contribution of fat oxidation to total energy production (43). This observation was made in adult males; however, it suggests that changing the balance of nonessential to essential fatty acids in the diet affects how fat oxidation is partitioned for energy production.

Analyses of whole-body fat content (44,45) indicate that preterm infants, with an appropriate weight for gestational age of 1300 g at birth, have a total body fat content of about 30 g compared with the term infant of 3500 g with a total body fat content of 340 g. Clandinin *et al.* (46) estimated that approximately 2783 mg of n-6 fatty acids and 387 mg n-3 fatty acids accrue in adipose tissue each week *in utero*. Birth after only a few more weeks of intrauterine development would dramatically increase the potential reserve of fatty acids in adipose tissue both for total fatty acids used for energy production and for essential fatty acids used for synthesis of structural tissues. These estimates also support the body of research by Van Houwelingen *et al.* (47) suggesting that the growing fetus represents a large draw upon maternal essential fatty acid stores and perhaps that a limitation in the size of the maternal essential fatty acid stores may be critical to fetal growth and development.

TABLE 1
Fatty Acid Accretion Rates in Infant Brain and Cerebellum (mg/wk)[a]

	Intra-uterine (26–41 weeks: preterm)	Extra-uterine (0–10 weeks: term)
Total n-6	32.8	82.4
Total n-3	14.6	5.5
Total n-9	31.2	65.5

[a]Data from References 48 and 49.

During the third trimester of human development, n-3 and n-6 fatty acids accrue in fetal tissues as an essential component of structural lipids, and rapid synthesis of brain tissue occurs. This rapid synthesis causes increases in cell size, cell type, and cell number (48). Brain lipid levels increase rapidly during this period. Levels of 18:2n-6 and 18:3n-3 were consistently low in the brain during the last trimester of pregnancy (48). However, accretion of long-chain essential fatty acid desaturation products 20:4n-6 and 22:6n-3 occurred, and the absolute accretion rates of the n-3 fatty acids, specifically 22:6n-3, were greater in the prenatal period compared with the postnatal period (Table 1; 48, 49). It is apparently critical that the developing fetus obtain the correct types and amounts of fatty acids to ensure complete and proper development of the brain. Timing of the availability of these fatty acids is also a factor. Collectively, this quantitative information indicates that large amounts of docosahexaenoic acid (22:6n-3) and arachidonic acid (20:4n-6) are required during development of neural tissue when cellular differentiation and active synaptogenesis are taking place.

Development of the visual system. The entire visual system spans caudal to rostral regions of the brain and includes some lateral areas of the brain. The visual pathway involves a chain of visual processing events that only begin with the retina in the eye. During visual processing, incoming light strikes the photoreceptors and generates electrical signals, which are sent to bipolar cells and ganglion cells. These networks in turn send visual information along the optic nerve to the visual cortex. “Funneling” of information within the eye is the result of an individual neuron receiving, converging, and combining impulses from several incoming nerve fibers. Thus, the separate signals of each nerve fiber are integrated into an entirely new message based on all the inputs. The retinal content of 22:6n-3 normally increases during development, and this change in lipid composition is apparently important to the function of the visual system although it cannot be easily distinguished functionally from the simultaneous increase in 22:6n-3 in the entire visual pathway.

SOURCES OF ESSENTIAL FATTY ACIDS FOR THE FETUS AND NEONATE

The developing fetal brain can synthesize saturated and monounsaturated fatty acids (50,51). High rates of lipogenesis in fetal liver (52) may act as a source of fatty acids for the fetus. Initially the placenta, by controlling the passage of 20:4n-6 and 22:6n-3, determines the amount of these fatty acids available to the fetus, but then the fetus (by its own metabolism) starts to synthesize its own 20:4n-6 and 22:6n-3 (53). The age at which this progression may begin and be completely achieved is not defined. As synthesis of long-chain polyenoic fatty acids by fetal liver has not been clearly demonstrated under physiological conditions, this progression remains hypothetical (53). Thus, the contribution of fatty acid synthesis to 20:4n-6 and 22:6n-3 accumulation by the neonate would be affected by the age of the infant at birth. While *in utero*, it is clear that the fetus relies on the mother for its supply of many fatty acids, particularly the essential fatty acids.

Biomagnification. Crawford *et al.* (54) describe a process in which, compared with parent essential fatty acids, the relative percentage of 20:4n-6 and 22:6n-3 increases in phosphoglycerides progressively from maternal blood to placenta and to fetal blood, liver, and brain. By this process, termed biomagnification, specific mechanisms within the placenta hypothetically result in sequestration and release of specific fatty acids to the fetal circulation. Neuringer *et al.* (55) also reported that, in monkey and human fetuses, the levels of 22:6n-3 and 20:4n-6 are higher in fetal blood compared with maternal blood, whereas the opposite is true for their precursors. Thus, the importance of these long-chain polyunsaturated fatty acids is evident in their preferential, active transfer across the placenta to the fetus in a lipid form normally impermeable to the placental barrier. It has recently been proposed that a more likely mechanism of biomagnification is selective sequestering of long-chain polyunsaturated fatty acids on the fetal side of the placenta (56). King *et al.* (57) found that, when comparing adipose tissue triglyceride between infants and mothers, infants have greater levels of palmitic and palmitoleic acids. The predominance of these two fatty acids in newborns indicates that glucose plays an important role in fetal fat synthesis. Embryonic and fetal lipids in early gestation are derived from maternal fatty acids that cross the placenta, but with advancing gestational age, there is a gradual shift to *de novo* synthesis from glucose in fetal tissue (58).

It is evident that some fatty acids are transferred to the fetus across the placenta. It also appears that the degree of placental and fetal synthesis of fatty acids varies with gestational age (58,59). Clandinin *et al.* (48) reported that 80% of human fetal brain 22:6n-3 accrues between 26 and 40 wk of gestation. These authors also observed that infants born prior to 32 wk gestation have low concentrations of brain 22:6n-3. Early in pregnancy, there is apparently a great dependence on maternal fatty acids to provide the fetus with lipids. This may have important implications for the low-birthweight, premature infant and for the shift of essential fatty acid from maternal stores to fetal tissues during fetal growth.

Altering n-6 to n-3 fatty acid balance in premature infants. Human milk contains both n-6 and n-3 fatty acids. One percent 18:3n-3 and 10 to 15% 18:2n-6 are typical amounts of essential fatty acids in the breast milk fat of North American and European women (41,60). The need to balance n-6 and n-3 fatty acids fed to infants to reflect the overall fatty acid

balance in human milk was proposed by Clandinin *et al.* (61). Moreover, a balance between the levels of very-long-chain C20 and C22 n-6 and n-3 polyunsaturated components was recommended to be 1.4 (61). Based on quantitative analysis of 24-h milk collections, the n-3 long-chain polyenes are present at 0.3–0.6% and n-6 long-chain polyenes at 0.5–1.5% (41,60). Recommended ratios of n-6 to n-3 fatty acids in infant formula have also been suggested to be within the range of 4 to 1 and 10 to 1 (Health and Welfare Canada, 1991) or 5 to 1 and 15 to 1 (62). The concern surrounding an appropriate n-6 to n-3 fatty acid ratio stems from the competition that exists between the n-6 and n-3 series for the Δ-6 and Δ-5 desaturase enzymes. There is also the desire to mimic the composition of fats present in human milk and the effect of feeding this fatty acid balance on the fatty acid composition of various phospholipids in the developing neonatal brain or tissues (61). Studies that have attempted to increase only the n-3 intake by adding marine oil sources to diets have reported a predictable decrease in arachidonate levels in various cellular phospholipids (63–67).

Arachidonic acid status is an important factor in the growth of the fetus and premature infant (41,68). In the fetus and newborn, birthweight is significantly correlated to plasma triglyceride content of arachidonic acid (69). An important consideration in using marine oil sources is that fish oil contains both eicosapentaenoic and docosahexaenoic acid and results in higher levels of n-3 long-chain fatty acids compared with n-6 long-chain polyenes. This produces an unbalanced n-6 to n-3 ratio. Eicosapentaenoic acid may have a greater effect than docosahexaenoic acid on the reduction of arachidonate levels (63,70), which may be due in part to the inhibition of Δ-5 desaturation and competition for insertion into phospholipids. Mohrhauer and Holman (71) reported that the precursor n-3 fatty acid, 18:3n-3, also reduces arachidonate levels. Other groups studying both humans and rats (66,72,73) have since reported this observation. Dyer and Greenwood (32) examined neural long-chain fatty acids in weanling rats in response to a range of 18:2n-6 and 18:3n-3 fatty acid ratios between 1.8 and 165 and concluded that the dietary 18:2n-6 to 18:3n-3 ratio affected membrane fatty acid profile. The level of brain arachidonic acid did not appear to be significantly affected by lower 18:2n-6 to 18:3n-3 ratios.

Effect of dietary 18:2/18:3 ratios on long-chain polyene content of brain. Research on the effect of dietary fat on brain development has generally been limited to undernutrition, malnutrition, or essential fatty acid deficiency and study of animal models. Within what are believed to be essential fatty acid-adequate diets, studies that have focused on altering n-6/n-3 fatty acid ratios have been limited to analyzing the brain as a whole or to considering the response at only one time period. Research in this area has not examined the effect of changes in dietary fat content on lipid composition of different brain regions or different cell types during one of the initial developmental periods. To date, most research has examined the effects of diet on brain composition and inferred effects on neuronal function from behavior. Much less is known about glial cell responsiveness to alterations in nutrient supply in the absence of malnutrition (22). Studies of brain regions have focused primarily on the cerebrum, brainstem, and cerebellum. Few studies have examined the hippocampus, despite the relative ease of isolation and removal of this structure and the fact that it completes development during the postnatal period.

In a recent experiment (74), the fatty acid composition of rat brain cells in different brain regions was examined over time in response to feeding diets varying in fat composition. Diets of similar 18:2n-6 and 18:3n-3 content, with and without small amounts of arachidonic and/or docosahexaenoic acid, were fed to nursing dams. The n-6 to n-3 fatty acid ratios were within the recommended range for infant formulas and varied between 4 to 1 and 7.3 to 1. The cerebellum and the frontal and hippocampal brain regions were excised from rat pups at birth and at 1, 2, 3 and 6 wk of age. Neuronal and glial brain cells were isolated from each brain region. By analysis of the fatty acid composition of ethanolamine-, choline-, serine- and inositolphosphoglycerides, it was apparent that the diets fed altered neuronal and glial cell composition differently and in a region- and time-specific manner. It is thus clear that analysis of whole brain cannot reflect the complexity of changes occurring during development of individual brain regions.

It can be concluded that the temporal development of brain regions is different—the cerebellum and the hippocampus are among two of the regions to complete development postnatally; the timing of development differs between the brain cells—the majority of neurogenesis is complete prior to completion of gliogenesis; and physiological changes in dietary fat affect the fatty acid composition of brain regions and of cell types. The functional implications of these changes in brain structural lipids are still unknown. However, it is certain that feeding small amounts of 20:4n-6 and/or 22:6n-3 will produce remarkable transitions in the fatty acid composition of individual membrane phospholipids during the postnatal growth and development of the brain.

SPECULATIVE RELATIONSHIPS AND COGENT QUESTIONS

The brain is an incredibly complex organ. Connections within the nervous system may exhibit a high degree of variability between individuals and change during the lifetime of each individual (75). These anatomical connections influence learning and memory and ultimately affect how an individual interacts with and adapts to an environment. Confounding variables influencing an individual's interactions are genetics and environmental factors, including nutrition. In addressing possible later behavioral differences resulting from a nutritional insult, such as low 20:4n-6 or 22:6n-3 status during development, consideration of both genetic and environmental factors is relevant. Comparisons of one without the other will not clearly reveal consistent insights, as both factors affect the development of an individual and the expression of traits in later life.

It is difficult to establish conclusive links between the functional and behavioral effects of nutritional insults when many of the methods for testing behavior cannot be clearly or easily related to human functioning (76). Other methods attempt to correlate the appearance of a behavior with the development of specific indices, such as neurotransmitter systems. However, to prove cause-and-effect relationships in this way would be limiting, as no one event occurs independently in the developing brain or whole animal. Thus, the appearance of a behavior, or the lack thereof, may also be correlated with other events, such as postnatal development and maturation of cells, myelination, endocrine system maturation, or skeletal muscle development. Subsequently, modifications produced by behavior or cognition involve not just a single cell and its connections but many cells and their connections. In this regard, it may not be reasonable to expect to identify a clear relationship between n-6 and n-3 fatty acid imbalance and the exhibition, or lack thereof, of a single behavior or functional impairment.

One important challenging question that remains to be addressed is the effect(s) arising from nutritional imbalances or deficiency in availability of long-chain essential fatty acids. If long-chain n-6 and n-3 fatty acids are so important in function and development of the brain, why are specific characteristics not evident in large populations? To answer this question, we need to understand how the lack of or imbalance of 20:4n-6 and 22:6n-3 during development may be manifested in terms of characteristics or behaviors in later life. These "effects" have not been defined, although suggestions have been made that depression, visual development, and perhaps "intelligence" are adversely affected, at least initially. It has been demonstrated that visual function is slow to develop if adequate docosahexaenoic acid is not supplied. However, visual function is improved with the addition of this fatty acid. This does not imply that the individual will never catch up or adapt, nor does it imply that the individual will not be as "smart." Visual function is not related to an individual's intellectual capacity, as evidenced by visually impaired individuals who are not intellectually impaired. Intellect is the "faculty of knowing and reasoning; understanding." Intelligence is acquired through exposure to the environment and experience, which occur in many forms. In this respect, testing intelligence by present methods is limiting and perhaps inappropriate in some situations. These views regarding vision and intellect are no doubt provocative, but until further research is conducted in this area, and until appropriate measures of intelligence are developed and confounding variables carefully analyzed, care should be taken in drawing conclusions and making speculations. Many questions remain unanswered.

Future directions should focus on establishing functional and/or behavioral implications caused by varying the n-6 to n-3 fatty acid ratio within the ranges recommended by experts and by including or omitting long-chain polyunsaturated fatty acids, namely, docosahexaenoic and arachidonic acids. A discrepancy remains regarding the degree to which a newborn human infant is capable of desaturating and elongating 18:2n-6 and 18:3n-3 to their longer-chain homologs. That is, immediately after birth there is no significant accretion of chain elongation–desaturation products. This suggests that the limiting factor may initially exist in liver synthesis of these products or that mobilization of these long-chain polyunsaturated fatty acids from the liver exceeds the capacity of the liver to synthesize them from dietary precursors (61). If the capability for desaturation exists, is it sufficient to support the requirements of the developing nervous system? If it is sufficient, in what instances, diseases, or dietary circumstances may it be compromised? Premature or very-low-birthweight infants are likely candidates in this category. Is the full-term infant also a likely candidate in certain circumstances? What factors are likely to affect this activity? Maternal nutritional status during pregnancy may be one example. Another example is the occurrence of fatty acids in the diet. Fatty acids do not occur individually but with other fatty acids and are associated with vitamins and other nutrients. These clusters of nutrients likely also affect how individual fatty acids are metabolized.

Little quantitative evidence is available to indicate the sources of 20:4n-6, 22:4n-6, 22:5n-3, and 22:6n-3 in the fetus. During intrauterine development, does the fetus rely entirely on placental synthesis and a transfer mechanism to obtain these essential structural long-chain fatty acids or are they synthesized by the fetus or in specific tissues? Because development of the brain is not uniform and many vulnerable periods exist prenatally and postnatally, the use of whole-brain analyses is conceptually quite limiting. In the same regard, because phospholipids vary in fatty acid composition, analyses of total brain phospholipids are also limiting. In terms of correlating functional deficits with morphological alterations, Vitiello and Gombos (21) noted that these can occur only in some cases. These authors point out that relationships between structure and function in brain are not always well defined. Thus, a single morphological structure cannot always be identified as the structure essential for a certain function (21). The need exists to focus our efforts on developing innovative tests to dissect out functions of the central nervous system that are interpretable in terms of brain regions, groups of neurons, or transmitter activity and function. These tests would need to be applicable in some form for use in infants and children and would then lead to the means to develop meaningful markers of essential fatty acid status during fetal and infant development.

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Neonatal Polyunsaturated Fatty Acid Metabolism

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ABSTRACT: The importance of n-6 and n-3 polyunsaturated fatty acids (PUFA) in neonatal development, particularly with respect to the developing brain and retina, is well known. This review combines recent information from basic science and clinical studies to highlight recent advances in knowledge on PUFA metabolism and areas where research is still needed on infant n-6 and n-3 fatty acid requirements. Animal, cell culture, and infant studies are consistent in demonstrating that synthesis of 22:6n-3 involves C24 PUFA and that the amounts of 18:2n-6 and 18:3n-3 influence PUFA metabolism. Studies to show that addition of n-6 fatty acids beyond Δ6-desaturase alters n-6 fatty acid metabolism with no marked increase in tissue 20:4n-6 illustrate the limitations of analyses of tissue fatty acid compositions as an approach to study the effects of diet on fatty acid metabolism. New information to show highly selective pathways for n-6 and n-3 fatty acid uptake in brain, and efficient pathways for conservation of 22:6n-3 in retina emphasizes the differences in PUFA metabolism among different tissues and the unique features which allow the brain and retina to accumulate and maintain high concentrations of n-3 fatty acids. Further elucidation of the Δ6-desaturases involved in 24:5n-6 and 22:6n-3 synthesis; the regulation of fatty acid movement between the endoplasmic reticulum and peroxisomes; partitioning to acylation, desaturation and oxidation; and the effects of dietary and hormonal factors on these pathways is needed for greater understanding of neonatal PUFA metabolism.

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The fatty acids currently recognized as essential dietary components (nutrients) are linoleic acid (18:2n-6) of the n-6 series and α-linolenic acid (18:3n-3) of the n-3 series. Once consumed, these fatty acids can be converted to longer-chain, more highly unsaturated fatty acids of the same series, including arachidonic acid (20:4n-6) from 18:2n-6 and docosahexaenoic acid (22:6n-3) from 18:3n-3 (1). Considerable controversy exists over how much and, in particular, which n-6 and n-3 fatty acids are needed in the diet of young infants. Indeed, the literature contains many studies to suggest that groups of infants who are breast-fed may have a developmental advantage and subsequently higher scores on psychometric tests than infants who are not breast-fed (2). These observations have fostered interest in the possible role of human milk nutrients in providing for optimal neurodevelopment. The presence of high concentrations of 20:4n-6 and 22:6n-3 in select regions of the brain and retina (3,4), and of small amounts of these same fatty acids in human milk (5) has raised questions about the potential need for a dietary source of 20:4n-6 and 22:6n-3 during development. Adequate levels of 20:4n-6 and 22:6n-3 in membrane phospholipids (PL) are crucially important to many different functions, for example, 20:4n-6 as a precursor for formation of eicosanoids and 22:6n-3 in normal visual function (2).

This session on neonatal polyunsaturated fatty acid (PUFA) metabolism was designed to review current information, specifically focusing on important unanswered questions about the synthesis and pathways of tissue assimilation of 20:4n-6 and 22:6n-3 in the newborn. Information about the pathways involved in the biosynthesis of 20:4n-6 and 22:6n-3 in liver; the steps involved in committing fatty acids to acylation, partial (peroxisomal) or complete oxidation or further desaturation and elongation, as well as potential differences between the metabolism of n-6 and n-3 fatty acids; and the regulation of n-6 and n-3 fatty acid metabolism in brain, eye, and other organs compared with that in the liver are being actively explored. The findings of recent research on these topics were summarized as described below. Several groups have used recent advances in stable isotope technology to provide evidence that term- and preterm-gestation infants are able to convert 18:2n-6 to 20:4n-6 and 18:3n-3 to 22:6n-3 (6–8). One important aspect of these studies, which are based on measures of isotopic enrichment of fatty acids in plasma lipids, is how to use the data to obtain quantitative information on the effects of various diets on 20:4n-6 and 22:6n-3 synthesis in relation to the tissues' needs for optimal function. A summary of studies focusing on the effect of age and dietary 18:2n-6 and 18:3n-3 quantity and ratio on conversion of 18:2n-6 and

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; GCC-IRMS, gas chromatography-combustion-isotope ratio mass spectrometry; NCI, negative chemical ionization; PFB, pentafluorobenzyl; PL, phospholipids; PUFA, polyunsaturated fatty acids; ROS, rod outer segments; RPE, retinal pigment epithelium; TG, triglyceride.

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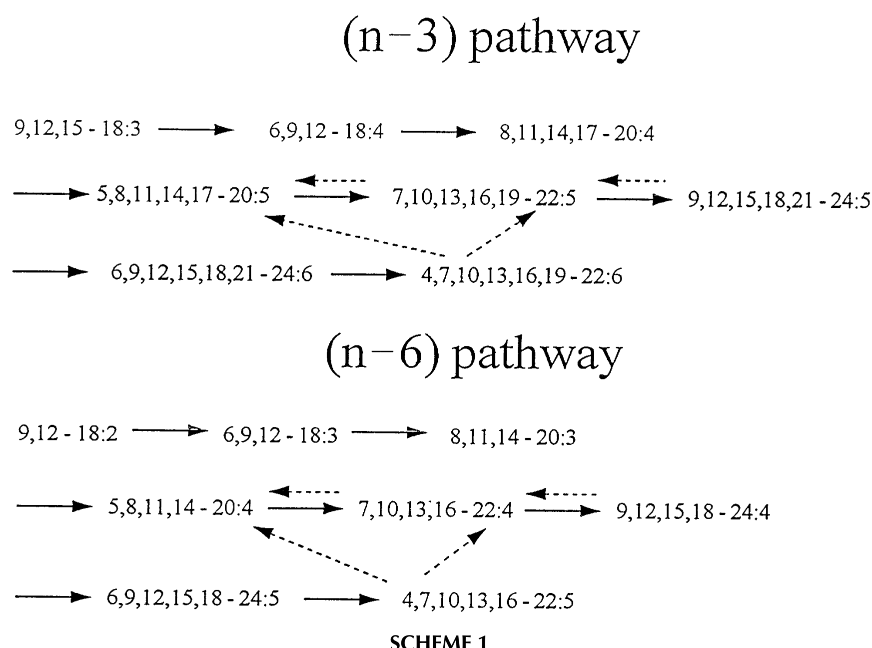
18:3n-3 to 20:4n-6 and 22:6n-3, respectively, forms the second part of this review. This theme is continued in a review of studies using the gastrostomy-reared rat fed with lipids containing stable isotopes to explore the highly selective ways by which the brain controls the supply of saturated and unsaturated fatty acids and cholesterol during growth and development. This is followed by discussion of the unique aspects of PUFA metabolism and of specific pathways for conservation of 22:6n-3 in retina. Although these important studies illustrate unique aspects of PUFA metabolism in the central nervous system, many basic questions about the digestion and absorption of dietary fatty acids by young infants remain unanswered. The consideration of some of these issues forms the final section of this review.

DIFFERENCES IN REGULATION OF BIOSYNTHESIS OF 5,8,11,14-ARACHIDONIC ACID AND 4,7,10,13,16,19-DOCOSAHEXAENOIC ACID

The biosynthesis of 20:4n-6 from dietary 18:2n-6 takes place in the endoplasmic reticulum. The liver is the major site for the production of PUFA, both for synthesis of its own membrane PL and for export and uptake by most other cells. The incorporation of unsaturated fatty acids into membrane PL is also a process that is localized primarily in the endoplasmic reticulum. Most PL contain large amounts of 18:2n-6 and 20:4n-6 but only relatively small amounts of 18:3n-6 or 20:3n-6. Studies with rat liver microsomes have shown that desaturation at position 6 is rate-limiting for the conversion of 18:2n-6 to 20:4n-6 (9). This finding implies that the addition of fatty acids to the diet beyond the reaction catalyzed by a 6-desaturase (e.g., 18:3n-6) should increase the amount of 20:4n-6 that is produced and incorporated into tissue lipids. To address this, a series of cross-over feeding studies was conducted in which unlabeled or deuterium-labeled 18:3n-6 or 20:3n-6 was added to a diet containing a constant amount of either labeled or unlabeled 18:2n-6 (10). These studies found that the addition of fatty acids beyond the 6-desaturase step did not markedly increase the amount of 20:4n-6 that was esterified in heart, liver, or kidney PL. However, there was an increase in the specific activity of esterified 20:4n-6 when the contributions of deuterium-labeled 18:2n-6 plus the other deuterium-labeled n-6 fatty acid were calculated. It was also observed that the specific activities of 20:4n-6 esterified in heart, kidney, and liver PL were all modified differently when n-6 fatty acids were added to a diet with a constant amount of 18:2n-6. The results of these studies showed that individual tissues process unsaturated fatty acids in different ways. Another important conclusion was that simply measuring the amount of 20:4n-6 in a tissue PL did not completely describe how a dietary fat supplement modifies fatty acid metabolism. In addition, the results also implied that measuring one pool of lipids (for example, liver phosphatidylcholine) does not adequately describe what takes place in any other pool [for example, heart phosphatidylcholine or liver triglyceride (TG)]. It is also important to stress that much of what we know about the regulation of PUFA biosynthesis has come from studies with rats. It remains to be determined if there are major differences among species and which animal most closely mimics what takes place in humans.

The evidence to show that 22:4n-6 (7,10,13,16-22:4) and 22:5n-3 (7,10,13,16,19-22:5) are not converted to 22:5n-6 (4,7,10,13,16-22:5) and 22:6n-3 (4,7,10,13,16,19-22:6), respectively, by a microsomal acyl-CoA-dependent 4-desaturase has been summarized recently (1). Instead of a microsomal 4-desaturase, the pathways, as shown in Scheme 1, re-



SCHEME 1

quire that when they are produced in the endoplasmic reticulum, 24:5n-6 and 24:6n-3 must move to a site for partial β-oxidation. Solid arrows denote reactions taking place in the endoplasmic reticulum, dashed arrows show fatty acids that are partially degraded, with subsequent esterification of the indicated chain-shortened metabolites into membrane lipids. This site is most likely peroxisomes. These pathways raise several new questions about both microsomal fatty acid biosynthesis and the intracellular movement of fatty acids between microsomes and peroxisomes. Two fatty acids in each of the n-6 and n-3 fatty acid pathways are desaturated at position 6 of the carbon chain. Although there is no conclusive evidence for multiple forms of a Δ6-desaturase (11), it seems highly unlikely that the enzyme previously assumed to catalyze the rate-limiting step, i.e., desaturation of 18:2n-6 and 18:3n-3, would also desaturate 24:4n-6 and 24:5n-3 at position 6. How many chain-elongating enzymes are present in the endoplasmic reticulum is also unknown (12). The revised pathways of fatty acid biosynthesis require three, rather than two, chain elongation steps, and two of these reactions now take place in sequence. It remains to be determined whether a single chain-elongating system can accept three n-6 and three n-3 fatty acids as substrates.

The pathways in Scheme 1 imply that when 24-carbon unsaturated fatty acids are produced in the endoplasmic reticulum they move to a site for partial β-oxidation. It is generally accepted that, once initiated, mitochondrial β-oxidation proceeds to completion (13). It is also generally recognized that peroxisomes partially degrade fatty acids (14). What regulates the transfer of fatty acids to a site for partial β-oxidation, rather than to mitochondria for complete oxidation, and whether they move as free fatty acids or acyl-CoA are not yet known. Some insight as to what terminates peroxisomal β-oxidation has been gathered from studies comparing the rates of β-oxidation of [3-^{14}C]-labeled 9,12,15,18,21-24:5 (24:5n-3) with 6,9,12,15,18,21-24:6 (24:6n-3). When [1-^{14}C] 4,7,10,13,16,19-22:6 (22:6n-3) was produced from [3-^{14}C] 24:6n-3, it was preferentially transported out of peroxisomes for use in membrane lipid biosynthesis by microsomes, rather than serving as a substrate for continued β-oxidation. The next cycle of β-oxidation of 22:6n-3 requires NADPH-dependent 2,4-dienoyl CoA reductase, which has been shown to be a slow step. Conversely, two cycles of β-oxidation of 24:5n-3 require only the enzymes of saturated fatty acid degradation, and indeed, the preferred metabolic fate of [1-^{14}C]-labeled 7,10,13,16,19-22:5 (22:5n-3) was continued β-oxidation rather than transfer from the peroxisomes back to microsomes for esterification (15).

This short review of current information on the regulation of 20:4n-6 and 22:6n-3 is confined to metabolism in the liver. Other types of controls are operative in other tissues. For example, brain and other tissues contain small amounts of very long chain unsaturated fatty acids with up to 40 carbon atoms with four, five, and six double bonds (16). When 24-carbon chain n-6 and n-3 fatty acids are produced in these tissues, they do not simply move to peroxisomes for partial degradation; they may also serve as precursors for the production of very long chain fatty acids. In addition, the differences in PL fatty acid composition, for example the presence of very long chain n-6 and n-3 fatty acids in some tissues, suggest that the activity and/or specificity of chain-elongating enzymes in some tissues may differ from those of liver.

STABLE ISOTOPE TRACERS AS PROBES TO STUDY PUFA BIOCHEMISTRY AND PHYSIOLOGY

The human infant requires an adequate supply of n-6 and n-3 fatty acids in order to sustain development of neurological structures of the brain and retina and to support normal growth and development of other tissues. The goals of the stable isotope studies were to validate the role of 24-carbon PUFA in the synthesis of 22:6n-3, to quantify levels of PUFA metabolites present in serum PL, to measure the enrichment of various PUFA isotopomers, and to devise kinetic models to quantify rates of synthesis of 20:4n-6 and 22:6n-3.

The role of 24-carbon chain PUFA in the metabolism of 18:2n-6 and 18:3n-3 to 22:5n-6 and 22:6n-3, respectively, is widely accepted. Whether these pathways occur in humans, and what the capacity is for conversion of dietary 18:2n-6 to 20:4n-6, and particularly of 18:3n-3 to 22:6n-3, are of particular interest with respect to the lipid composition of infant formulas and the effect of age and postnatal development on PUFA metabolism. However, the very low concentration of some n-6 and n-3 fatty acids, especially the 24-carbon PUFA, in tissue lipids hinders the design of metabolic studies aimed at quantifying the elongation and desaturation of n-6 and n-3 fatty acids. Several studies have now been reported on human infants, neonatal piglets, and pregnant baboons (6,17,18). The consensus is that infants are able to elongate and desaturate 18-carbon precursors to form 20:4n-6 and 22:6n-3. However, to establish whether the capacity of these metabolic systems proceeds at a sufficient rate to meet tissue needs for 20:4n-6 and 22:6n-3 will require developmental tests with a finer capability to discriminate between populations of normal infants on various diets.

In one series of studies, [U-^{13}C]-18:2n-6 (25–40 mg/kg) and [U-^{13}C]-18:3n-3 (15–25 mg/kg) were emulsified in formula and given orally to 4-mon-old infants. Blood was collected at 8, 12, and 24 h (8,18). In a study in neonatal piglets, [U-^{13}C]-18:2n-6 (25 mg/kg) and [U-^{13}C]-18:3n-3 (15 mg/kg) were emulsified in intravenous lipid and given i.v. *via* an indwelling catheter; 15 blood samples were then collected over a 3–5 d period. Plasma lipids were extracted with hexane/2-propanol, separated into TG and PL fractions on silica columns, and saponified with methanolic KOH. Pentafluorobenzyl (PFB) esters were then prepared. Samples were analyzed by methane negative chemical ionization (NCI)–gas chromatography–mass spectrometry (GC–MS) on both 60 m × 0.32 mm Omegawax and 60 m × 0.32 mm SP-2380 capillary columns to completely resolve all of the putative metabolites.

Several approaches have been employed recently to quantify the stable isotopic enrichment of PUFA. Emken and coworkers used methyl esters and positive chemical ionization GC–MS to study fatty acid metabolism in adults (19,20). Their studies were among the first to show direct synthesis of 20:4n-6 and 22:6n-3. More recently, Carnielli *et al.* (17), Demmelmair *et al.* (6), and Greiner *et a*l. (21) used high-precision, GC-combustion-isotope ratio mass spectrometry (GCC-IRMS) for analysis of fatty acid methyl esters. The chief advantage of GCC-IRMS is its ability to quantify much lower levels of enrichment accurately than is possible with GC–MS. However, GCC-IRMS is unable uniquely to detect and to quantify minor fatty acids present at very low concentrations in a complex biological matrix. To overcome these limitations, Sauerwald *et al.* (8,18,22–24) and Salem *et al.* (7,25) employed the PFB ester and NCI GC–MS to study PUFA metabolism. The PUFA-PFB esters have three desirable attributes: this derivative affords extraordinary attomole sensitivity, it possesses good chromatographic resolution of *cis/trans* and positional isomers, and it gives unambiguous assignment of chain length and degree of unsaturation (8,24). Moreover, the dominant ion in the mass spectrum is the carboxylate anion caused by scission of the PUFA-PFB ester bond, thus $[^{13}C]$- and $[^{2}H]$-PUFA are readily quantified because the carbon skeleton remains intact during ionization (18,22). The molar response factors were determined using a commercial standard with 31 fatty acids of C10 to 24. The concentration of various PUFA in infant plasma PL was determined by comparison of GC–MS peak areas relative to the concentration of 18:3n-3 and 22:6n-3 determined by capillary GC. The mole percentage and concentration of n-3 series PUFA in plasma PL from infants fed a formula with 16% 18:2n-6 and 3.2% 18:3n-3 are summarized in Table 1. The C24 PUFA were present in plasma PL at 0.003 to 0.012 mol% of total fatty acids, and the plasma concentration was 0.087 to 0.38 nmol/mL.

Figure 1 shows a total ion chromatogram for the C24 PUFA region and the selected ion recordings for the [M + 18] isotopomers of the elongation products (18). The n-3 series lipids are clearly enriched, whereas neither the C22 nor the C24 n-6 series had detectable levels of enrichment, although 20:4n-6 was labeled. The enrichment data for one infant, including both n-6 and n-3 series lipids, are shown in Figure 2. The peak enrichment in the precursor PL was achieved in under 8 h, consistent with studies in adult humans (19,20). Simulation of kinetic tracer studies reveals that enrichment flows from high to low as the isotope traverses the metabolic path of the system (26,27). These data show that the C24 n-3 PUFA possess a greater enrichment than 22:6n-3, thus they must arise at an earlier stage of biosynthesis than 22:6n-3. The results are also consistent with *in vitro* studies which show that the final steps in synthesis of 22:5n-6 and 22:6n-3 involve elongation to 24:5n-6 and 24:4n-3, rather than direct 4-desaturation of 22:4n-6 and 22:5n-3, respectively (1). The extent to which the 4-desaturase alternate pathway, rather than other pathways, is utilized for synthesis of 22:5n-6 and 22:6n-3 *in vivo,* however, cannot be determined unambiguously from the tracer data.

Studies employing NCI GC–MS with term and preterm infants have also assessed the effect of age and dietary 18:2n-6 and 18:3n-3 amounts and ratios on the endogenous conversion of 18:2n-6 and 18:3n-3 as well as variability among individual infants (7,18,24). The studies were performed in term infants fed formulas with 16% of total fatty acids as

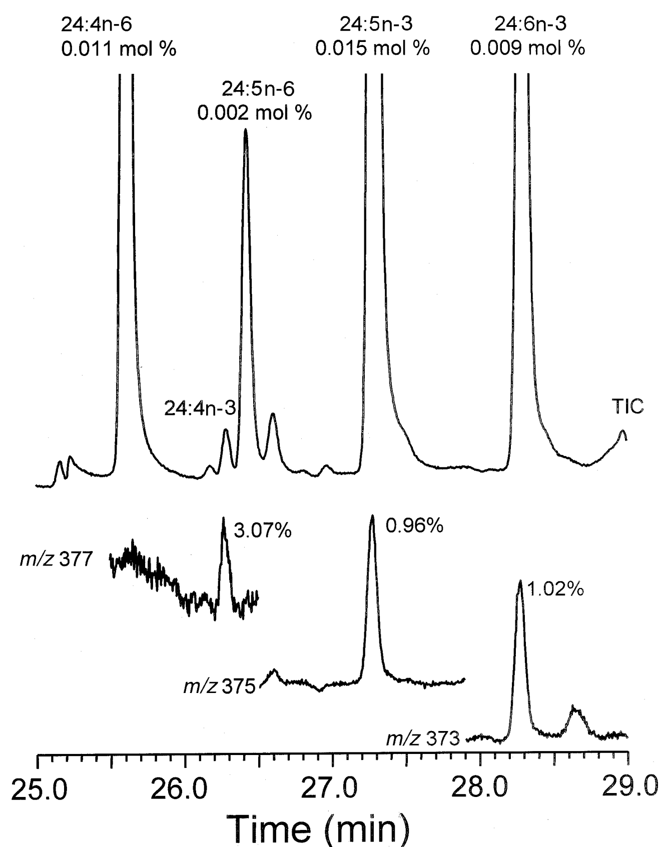


FIG. 1. Selected ion chromatograms showing incorporation of $[U-^{13}C]$-18:2n-6 and $[U-^{13}C]$-18:3n-3 into serum 24-carbon phospholipid polyunsaturated fatty acids.

TABLE 1
Composition and Concentration of Selected Plasma Phospholipid PUFA Determined by Capillary GC and NCI–GC-MS

PUFA	Mol%	Concentration (nmol/mL)
18:3n-3[a]	0.32 ± 0.12	9.95 ± 4.23
18:4n-3	0.002 ± 0.001	0.067 ± 0.070
20:4n-3	0.62 ± 0.76	18.4 ± 20.1
20:5n-3	1.69 ± 1.23	46.1 ± 35.8
22:4n-3	0.023 ± 0.022	0.69 ± 0.60
22:5n-3	0.81 ± 0.64	25.0 ± 17.7
22:6n-3[a]	2.93 ± 0.82	93.3 ± 31.4
24:5n-3	0.003 ± 0.002	0.087 ± 0.047
24:6n-3	0.012 ± 0.011	0.38 ± 0.29
24:5n-6	0.006 ± 0.004	0.14 ± 0.11

[a]Determined by capillary gas chromatography (GC). PUFA, polyunsaturated fatty acid; NCI, negative chemical ionization; MS, mass spectrometry.

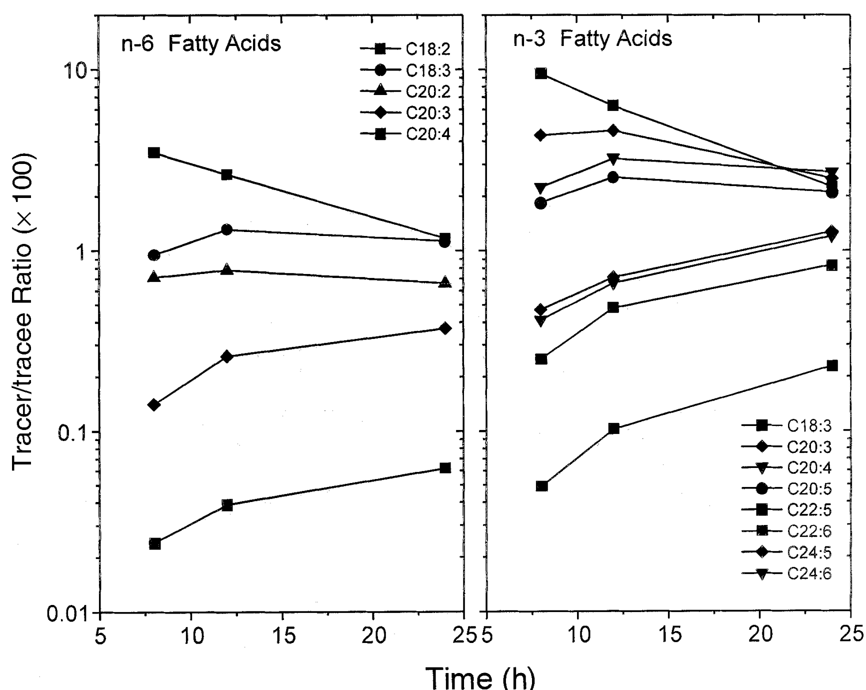


FIG. 2. Incorporation of [U-^{13}C]-18:2n-6 and [U-^{13}C]-18:3n-3 and various elongation and desaturation products into serum phospholipids in formula-fed infants over 24 h.

18:2n-6; with 0.4, 1.0, 2.0, or 3.2% 18:3n-3; and in premature infants fed formulas with 1 or 3.2% 18:3n-3. The formulas were fed from shortly after birth until 56 wk postmenstrual age. Bolus doses of [UL-^{13}C]-18:2n-6 and [UL-^{13}C]-18:3n-3 were given with a feeding at 43 and 56 wk postmenstrual age. Blood samples were taken 8, 12, and 24 h later for determination of concentrations and isotopic enrichments of all detectable n-6 and n-3 fatty acids in plasma lipids. Fractional rates of conversion of 18:2n-6 and 18:3n-3 as well as fractional rates of synthesis and incorporation of 20:4n-6 and 22:6n-3 into plasma lipids were calculated from the data using a simple precursor–product compartmental model (Fig. 3). Precursor–product models are widely used in kinetic studies and have been systematically validated (27,28). These studies confirm that both term and preterm infants synthesize 20:4n-6 and 22:6n-3 and that synthesis of 22:6n-3 proceeds *via* C24 PUFA metabolites, which utilizes a Δ6-desaturase. The fractional rates of endogenous conversion of 18:2n-6 and 18:3n-3, however, were highly variable among individual infants and were not higher in term than in preterm infants. Furthermore, rates of conversion did not increase with increasing postnatal age. These studies also found that increasing 18:3n-3 intake from 0.5 to 3.2% fatty acids increased the incorporation of 22:6n-3 into plasma lipids but inhibited the fractional conversion of 18:2n-6 and incorporation of 20:4n-6 into plasma lipids. Across all infants, the fractional rates of conversion of 18:2n-6 and 18:3n-3, as well as the fractional rates of 20:4n-6 and 22:6n-3 incorporation, were highly correlated ($r^2 = 0.5$). This suggests marked individual differences in desaturase and elongase activities.

The physiology and biochemistry of PUFA are sufficiently complex that they demand more invasive studies than are possible in human infants. A series of studies in neonatal piglets quantified individual steps in the elongation and desaturation of 18-carbon PUFA precursors using the techniques described

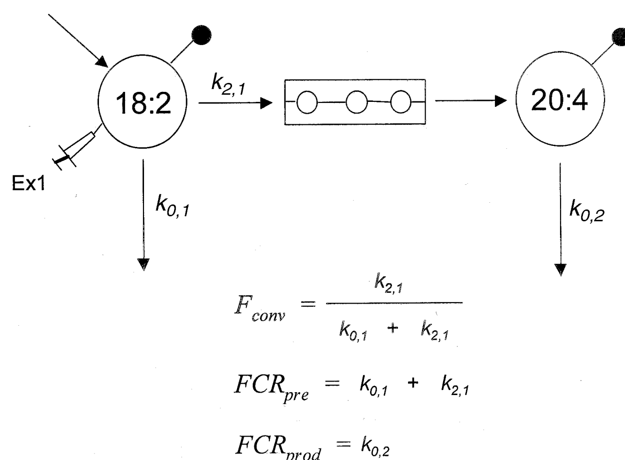
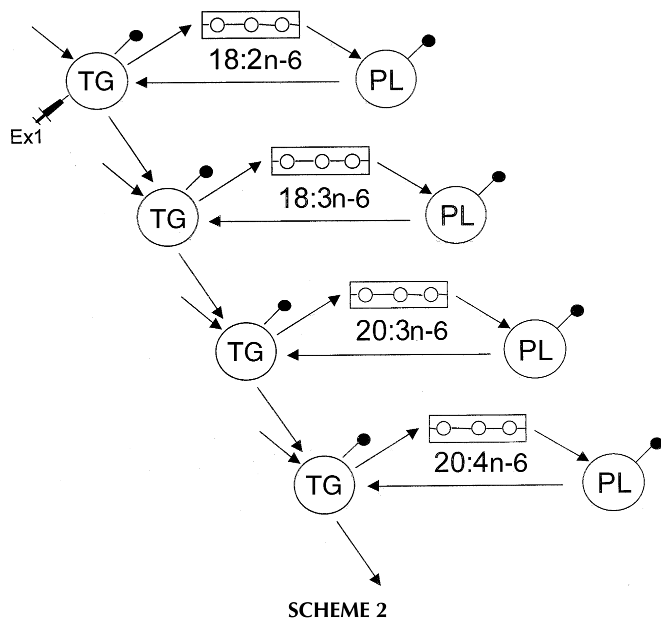


FIG. 3. Simple precursor product compartmental model used to estimate rates of synthesis of arachidonic acid and docosahexaenoic acid in human infants. The compartments are labeled "18:2" and "20:4" inside the large circle. The symbol (•—) emanating from the side of the large circles is the kinetic notation for the site of sample measurements. The subscripts 1 and 2 refer to the precursor compartment 18:2 and product compartment 20:4, respectively. Ex1 is the "external input of tracer" into compartment 1; F_{conv} is the fraction of compartment 1 converted to product in compartment 2; FCR_{pre} is the fractional catabolic rate of material in precursor compartment 1; FCR_{prod} is the fractional catabolic rate of material in product compartment 2.



SCHEME 2

above (22,23). A tentative model for the synthesis from [U-^{13}C]-18:2n-6 of 20:4n-6 in a neonatal piglet is shown in Scheme 2 (22), where FCR = fractional catabolic rate. This model incorporates two aspects of physiology and biochemistry not possible with the simple precursor–product model. The model partitions the fatty acids into separate TG and PL compartments, and it incorporates separate compartments for each step in the desaturation and elongation sequence. To model this system adequately, four pieces of information are required; separate concentration measurements of the various PUFA in TG and PL fractions, and measurement of isotopic enrichment in each polyunsaturate in TG and PL. The studies are demanding in terms of the number of analytical measurements required, especially for the n-3 series fatty acids, which have twice the number of metabolites as the n-6 series fatty acids. A model determined to fit the data is shown in Figure 4. The median fractional error estimate of the rate constants in the model was 7% (range: 2.5–35%). The model confirms that the rate-limiting step is the initial Δ6-desaturase (18:2n-6 to 18:3n-6) and that accumulation of 20:4n-6 is due to a slow fractional catabolic rate. As intricate as this model is, it does not reflect the true complexity of the system, e.g., no attempt was made to quantify cholesterol esters nor to incorporate transport *via* lipoproteins. Moreover, the model does not consider that biochemical conversions take place *via* unesterified fatty acids in intracellular compartments.

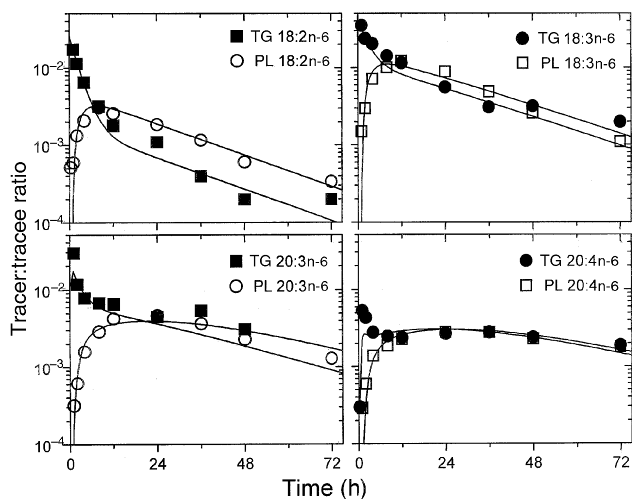


FIG. 4. Incorporation of [U-^{13}C]-18:2n-6 into n-6 series polyunsaturated fatty acid metabolites in a neonatal piglet. Model parameters and fit were obtained using the model shown in Scheme 2. TG, triglycerides; PL, phospholipids.

In the neonatal piglet (22), the rate of conversion was extraordinarily rapid compared to what was anticipated from studies in the human infant. Indeed, high levels of enrichment were observed in 20:4n-6 in TG as early as 60 min after the tracer was administered, whereas the 20:4n-6 in PL did not peak until 24 h, as observed in the infant studies. Thus, the kinetics observed in the infant studies may have been dominated by slow rates of PL synthesis and probably do not reflect the underlying kinetics of desaturation and elongation. Refinements in the model may identify kinetic parameters with a finer ability to discriminate between subject populations and thus permit more reliable evaluation of the effects of diet on PUFA metabolism. Kinetic analysis of the PUFA system is in an early stage of development compared to that of other complex kinetic systems, such as cholesterol metabolism or the lipoprotein transport system (29). The kinetic tools are available to model complex physiologic systems, but investigators working in this area should come to some consensus on the most appropriate model structure in order to design and implement tracer studies.

The studies described above, together with those of others (6,17,18), provide firm evidence that term and preterm infants synthesize 20:4n-6 and 22:6n-3 from their respective dietary precursors, 18:2n-6 and 18:3n-3. The relative amounts of 18:2n-6 and 18:3n-3 in the diet, however, appear to be important determinants of this conversion. In summary, NCI GC–MS measures of stable isotopically labeled fatty acids can be used to study (i) pathways and (ii) the effects of diet, age, or various clinical conditions on the conversion of n-6 and n-3 fatty acids and their incorporation into plasma and tissue lipids. Results of studies completed with term and preterm infants confirm that 18:2n-6 and 18:3n-3 compete for desaturation and elongation, and that n-6 and n-3 fatty acid metabolism involves 24-carbon chain intermediates. The results obtained dispel the assumptions that 18:2n-6 and 18:3n-3 conversion is lower in preterm- than term-gestation infants and increases with postnatal age. The results also show that dietary 18:2n-6 and 18:3n-3 intake determines the conversion 18:2n-6 and 18:3n-3 to 20:4n-6 and 22:6n-3, respectively. Further studies employing these techniques are likely to provide important information on the effects of dietary supplementation with 20:4n-6 and 22:6n-3 in term and preterm infants.

METABOLISM, SOURCES, AND UTILIZATION OF PUFA BY THE DEVELOPING BRAIN: LESSONS LEARNED FROM AN ANIMAL MODEL

The brain is a unique organ and not subject to the same conditions as nearly all the other organs of the body. The management of lipids for the brain must be a highly specialized process equated to its development, structure, and function. Studies with rat pups reared on defined diets *via* gastrostomy tube feeding (gastrostomy-reared rat) have provided basic evidence that the brain is autonomous in providing the bulk of its lipids, entirely by *de novo* synthesis (30–32). Neither cholesterol nor 16:0 nor 18:0 nor 18:1 is supplied from exogenous (diet/plasma) sources. All are provided within the brain. Because of this, synthesis can be directed to supply needs in the specified regions and compartments as required to meet the demands for their dynamic growth and specialized development. The essential features are that this commitment to lipid synthesis is site-directed, efficient, and avoids problems of redundancy in supply that an organ like the brain must avoid.

Studies using gastrostomy-reared rat pups have taken advantage of lipids containing stable isotopes with characteristic mass to study which fatty acids can enter the brain at the most dynamic period in brain development, the primary brain growth spurt and onset of white matter formation (34,35). The artificially reared rat pups were pulse-fed *via* gastrostomy (36–38) for 20 or 30 min in each hour throughout each 24-h day beginning at day 6 postnatal with a rat milk subtitute containing fats of defined composition. The rat pups were fed over 7–10 d periods, long enough to ensure that labeled lipids reached all organs to which they have access. Results of these studies showed that deuterated cholesterol, D_7 -cholesterol (mw 393, compared to 386 for D_O-cholesterol), even when fed to produce a hypercholesterolemic state, does not enter the brain (30). In contrast, other tissues, for example, liver, red blood cells and lung, contained D_7-cholesterol in excess of 55% of their total cholesterol. Subsequent studies using the same methodology demonstrated that deuterated 16:0, either the D_3 or D_{31} perdeuterated form (mw 273 and 301, compared to mw 270 for D_0-16:0 on quantitation as their methyl esters), when fed in the fat blend of the milk substitute, did not gain entry to the brain; organs such as liver and lung contained both isotopomers in the relative proportions in which they were fed (31).

The above studies have now been expanded to determine the transport and utilization of dietary essential and nonessential fatty acids. In these more recent studies, gastrostomy-reared rat pups were fed a rat milk substitute containing the perdeuterated (97 atom% D) fatty acids 16:0, 18:0, 18:1, 18:2n-6, and 18:3n-3 from day 7 to day 14 after birth (32,33). Fatty acids in lipid extracts of the liver, lung, kidney, and brain were subjected to GC–MS analysis to determine their content of each of the deuterated fatty acids (301 vs. 270, 16:0; 333 vs. 298, 18:0; 329 vs. 296, 18:1; 325 vs. 294, 18:2n-6; and 321 vs. 292,18:3n-3), and were quantitated from the abundance of these respective ions; the exception was oleate, which was determined as stearate (331 vs. 298) after oleate was separated from the fatty acid mixture and converted to stearate by hydrogenation. The uptake and metabolism of perdeuterated fatty acid leads to the appearance of three distinct groups of isotopomers: the intact perdeuterated, the newly synthesized [with recycled deuterium from the oxidation of fatty acids to the water-soluble metabolites acetoacetate and hydroxybutyrate, which are used for *de novo* lipogenesis (39)], and the natural unlabeled (D_0) fatty acid. The quantitation of these isotopomers permits the estimation of uptake and *de novo* synthesis of these fatty acids. Intact perdeuterated 16:0, 18:0, and 18:1 from the diet were found in liver, lung, and kidney but not in brain. By contrast, perdeuterated 18:2n-6 was found in all these organs; in the brain the perdeuterated 18:2n-6 equaled the natural form in its abundance. Isotopomers of fatty acids from *de novo* synthesis as measured by the increase in mass over natural abundance in their respective (M + 1) and (M + 2) ions were observed in 16:0, 18:0, and 18:1 in all tissues. The highest enrichment of isotopomers with recycled deuterium was found in the brain. As an example, from the data it could be calculated that the relative rate of synthesis of 18:1 in brain was sixfold greater than the relative rate of 18:1 synthesis in the liver at this age (33). The data indicate that during the brain growth spurt and the prelude to myelination, the major nonessential fatty acids (and cholesterol) in lipids in brain are exclusively produced locally by *de novo* biosynthesis. These results suggest essential fatty acids must be transported and delivered to the brain by highly specific mechanisms capable of distinguishing between, for example, 18:1 and 18:2n-6. In the studies discussed in this section it was found that 18:1 is excluded from brain whereas 18:2n-6 passed readily into brain, indicating the mechanism for transport/transfer has the sophistication and selectivity to distinguish between 18-carbon monocarboxylic acids which differ by one double bond.

The conclusions from the studies with the gastrostomy-reared rat are that there are essentially no contributions of saturated or monounsaturated fatty acids or cholesterol to brain from a plentiful extraneural supply of these lipids. By extrapolation, this means that those fatty acids that do need to enter the brain, especially the n-6 and n-3 fatty acids, must be processed in a unique manner. From studies to date one can hypothesize that there must be a stringent approach to how the brain deals with its need for the PUFA. An extension of the information gathered on saturated and monounsaturated fatty acids, and cholesterol is that the brain exercises exclusive control on the content of these PUFA, and by analogy, this control would dictate that the brain produces 20:4n-6 and 22:6n-3 as needed in the specific regions and compartments on demand, as required to meet the needs for dynamic growth and specialized development. These conditions would imply that the optimal arrangement calls for a controlled and selective uptake of their precursors, 18:2n-6 and 18:3n-3, which are delivered (uniquely again) for *in situ* conversion to the products, or for specific and regulated uptake of 20:4n-6 and

22:6n-3 as commissioned by, and specifically required within, the brain.

SYNTHESIS, UTILIZATION AND CONSERVATION OF PUFA IN THE CENTRAL NERVOUS SYSTEM

Tissues of the nervous system and reproductive organs are unique in that their membrane PL contain large amounts of n-3 fatty acids. The rod outer segments (ROS) of the retina have up to 50% n-3 PUFA, most of which is 22:6n-3. These membranes contain light-sensitive photopigments that absorb light and initiate visual excitation. Biochemical events that lead to the generation of the electrical signals that are transmitted to the occipital cortex occur in these membranes on a millisecond time scale. These rapid changes evidently require the presence of 22:6n-3, as evidenced by changes in retinal function in rats fed n-3 fatty acid-deficient diets (40–42). Similar functional changes have been demonstrated in guinea pigs (43), monkeys (44), and human infants (45). Given the important role of 22:6n-3 in retinal function, it is not surprising that the nervous system has developed mechanisms for conservation of n-3 fatty acids during times of dietary deprivation. In fact, it is rather difficult to reduce the retinal levels of 22:6n-3 in a rat, even when the diet is started at weaning (46). The largest changes in retinal 22:6n-3 levels can be made by depriving female rats of n-3 fatty acids during the last trimester of pregnancy and throughout the nursing period (47). ROS are dynamic structures whose membrane components are constantly being replaced. The distal 10% of mammalian ROS is shed daily and phagocytized by the retinal pigment epithelium (RPE); new membranes are synthesized and incorporated into the base of the ROS to maintain a constant length of the outer segment. This process is not affected by n-3 fatty acid status (48). Since 22:6n-3 is present in the shed tips (49,50), the RPE must metabolize it and return it to the retina for incorporation into new membranes. This is accomplished by a shuttle system that is not well understood but involves transient storage of 22:6n-3 in the RPE as TG and possibly 22:6n-3 specific-binding and transport proteins. The efficiency of 22:6n-3 conservation by the retina was demonstrated (48). Labeled 22:6n-3 injected into the vitreous cavity was rapidly incorporated into retinal PL, but no label could be detected in 22:6n-3 in the circulation (51).

In addition to its efficient uptake and conservation mechanisms, the retina is also supplied with 22:6n-3 derived from synthesis from appropriate n-3 precursors by cells within the eye. The RPE can convert 18:3n-3 and 22:5n-3 to 22:6n-3 *via* the Sprecher pathway (52). Recently, it was demonstrated that retinal capillary endothelial cells can also synthesize 22:6n-3 from 22:5n-3 (53). Interestingly, these cells released 24:5n-3, 24:6n-3, and 22:6n-3 into the medium, suggesting that they may be responsible for supplying these long-chain PUFA to retinal cells.

In summary, the retina conserves 22:6n-3 during n-3 fatty acid deprivation by recycling between the retina and RPE. In addition, RPE and capillary endothelial cells can synthesize 22:6n-3 from precursors and release it into the intercellular space. Both mechanisms of conserving 22:6n-3 ensure that the retina maintains a high level of this fatty acid in ROS membranes.

UNANSWERED QUESTIONS ON THE DIGESTION AND ABSORPTION OF PUFA

Breast-fed infants receive a dietary source of 20:4n-6 and 22:6n-3, and the fetus probably receives 20:4n-6 and 22:6n-3 by placental transfer. One important as yet unanswered question is whether the dietary essential fatty acid requirements of infants born early in the third trimester of gestation are the same as those of infants born after full-term gestation. Several studies on the essential fatty acid requirements of young infants have been done with premature infants, generally of birthweight less than 1500 grams. Infants born early in the third trimester of gestation are often more vulnerable to nutritional deficiencies, including essential fatty acid deficiency, than healthy term infants (54). For example, premature infant formulae with higher concentrations of protein and minerals than in term infant formulae produce a major advantage in neurodevelopment (55). The much earlier stage of brain maturation at birth, suggesting greater developmental need, as well as minimal stores of n-6 and n-3 fatty acids in adipose tissue (which often represent only 1–2% bodyweight in a 1,000 g premature infant), indicate that n-6 and n-3 fatty acid requirements may be higher than, or different from, term infants. Results of recent studies with stable isotopes of 18:2n-6 and 18:3n-3, however, suggest that premature infants do not have a lower ability (immaturity) to form 20:4n-6 and 22:6n-3 when compared with their term counterparts (6,17,18). Despite this, it is important to recognize that there are many differences in the clinical care, rate of growth, and composition of developing tissues in premature infants which could influence fatty acid metabolism and thus requirement and tissue assimilation. As a result, caution is needed before extending results of studies with premature infants to the needs of term gestation infants and vice versa.

Because breast-fed infants and the developing fetus receive preformed 20:4n-6 and 22:6n-3, it seems reasonable to consider providing an exogenous (dietary) source of 20:4n-6 and 22:6n-3 for infants who are bottle-fed or fed intravenously. Several potential sources of 20:4n-6 and 22:6n-3 are available for supplementation of infant formula. These include fish and other marine oils, egg total lipids or PL, and oils derived from micro-algae and fungi (single-cell organisms). These oils provide 20:4n-6 or 22:6n-3, as well as other fatty acids, unusual TG configurations, and potentially sterols and pigments native to the oil. Simply matching the composition of the major fatty acids in a formula with that in human milk, however, may not provide assurance that individual fatty acids are absorbed and assimilated in the same way as from human milk. For example, a recent study with rats found that 22:6n-3 from fish oil was preferentially transported in lymph TG, rather than in lymph PL as when fed as human

milk (56). Unfortunately, very little is as yet known about the pathways of digestion, absorption, and transport of n-6 and n-3 fatty acids from human milk TG and PL. Some studies have led to the suggestion that the milk enzyme, bile salt-stimulated lipase, which is present in human milk but not in infant formula, may be involved in the digestion of long-chain PUFA (57). Studies on the increase in blood lipid 20:4n-6 and 22:6n-3 in infants fed formulae supplemented with egg lipids and/or fish oils, however, clearly show 20:4n-6 and 22:6n-3 are digested and absorbed (58–60), even in the absence of this enzyme.

Current information suggests that 20:4n-6 and 22:6n-3 in human milk TG are distributed between the 2- and 3-positions of the glycerol (61). Studies with TG with varying saturated and unsaturated fatty acid distributions have suggested that dietary TG fatty acid distribution is important in determining the efficiency of fatty acid absorption, the pathway of absorption (as unesterifed fatty acids or as 2-monoglycerides), and subsequent routes of intravascular transport and tissue clearance (62–65). A better understanding of the physiological significance of milk fat fatty acid distribution seems fundamental to the design of fat blends which more closely mimic human milk lipids.

Some studies have found that adding fish oils with 22:6n-3 with either high or low 20:5n-3 to formula can reduce growth in premature infants (66,67) and possibly some aspects of neural function related to language in term infants (68). The levels of 22:6n-3 used in these studies did not, however, exceed those usually found in human milk. The absence of information on the usual pathways of digestion and absorption, transport, and tissue uptake of long-chain n-6 and n-3 fatty acids and on the specific roles these fatty acids play in development makes it difficult to understand the mechanism of possible adverse effects on growth and development. Studies with animals, however, have found evidence of reduced visual function (43) and decreased brain weight and performance on some tasks (69) as a result of feeding large amounts of fish oil with 20:5n-3 and 22:6n-3, or of single cell TG sources of 22:6n-3. Potential reasons for these problems could include oxidative problems in membrane lipids or effects secondary to reduced 20:4n-6. The findings, however, suggest that the balance of 20:4n-6 and 22:6n-3 is an important nutritional consideration for optimal growth and neural development.

Another important question is whether the composition of saturated and monounsaturated fatty acids, and the levels of 18:2n-6 and 18:3n-3 in a formula, influence the metabolism of either 18:2n-6 or 18:3n-3, or of any supplemental 20:4n-6 and 22:6n-3. Studies with formula-fed piglets have shown that dietary saturated fatty acid chain length influences tissue levels of 22:6n-3, 20:4n-6, and 18:2n-6 as well as other fatty acids (70,71). Recent studies in preterm infants also reported that the chain length of saturated fatty acids in formula influences plasma lipid levels of 22:6n-3 (72). Whether this is due to changes in β-oxidation of n-6 and n-3 fatty acids for energy vs. direct acylation or desaturation and elongation is not known. However, the findings suggest that studies on the appropriate levels of n-6 and n-3 fatty acids for infant diets should consider the composition of nonessential fatty acids as well as the amounts and balance of 18:2n-6 and 18:3n-3 levels and of any other n-6 and n-3 fatty acids in the diet fed.

This review has provided information from basic science and clinical studies that should be considered in future work on neonatal PUFA metabolism, and has indicated areas where research is needed to advance understanding on infant n-6 and n-3 fatty acid requirements. Animal, cell culture, and infant studies are consistent in demonstrating that synthesis of 22:6n-3 involves C24 PUFA and that the amounts of precursor n-6 and n-3 fatty acids influence PUFA metabolism. Studies have shown that addition of n-6 fatty acids beyond the Δ6-desaturase step alters n-6 fatty acid metabolism, with the effect differing among different tissues and with no marked increase in tissue 20:4n-6; this underscores the limitations of measures of tissue fatty acid compositions with respect to the effects of diet on fatty acid metabolism. The studies that show the brain has highly selective pathways for uptake of n-6 and n-3 fatty acids and that the retina has efficient pathways for conservation of 22:6n-3 further emphasize the differences in PUFA metabolism among different tissues and unambiguously confirm that many unique features are present to allow the brain and retina to accumulate and maintain high levels of n-3 fatty acids. Greater understanding of n-6 and n-3 fatty acid metabolism will be obtained with further elucidation of the Δ6-desaturases involved in the synthesis of 24:5n-6 and 22:6n-3, and the biochemistry of regulation of movement of fatty acids between the endoplasmic reticulum and peroxisomes, partitioning to acylation, desaturation–elongation and oxidation, and the effects of other dietary and hormonal factors on these pathways.

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Assessment of Long-Chain Polyunsaturated Fatty Acid Nutritional Supplementation on Infant Neurobehavioral Development and Visual Acuity

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ABSTRACT: The aims of this paper are (i) to consider how best to examine effects of long-chain polyunsaturated fatty acid nutritional supplementation or deficiency on infant neurobehavioral development, after controlling for other factors that might influence outcome, including maternal demographic, intellectual, and personality characteristics, and (ii) to present new findings on the relation between visual acuity and processing speed and the effects of prenatal alcohol exposure and visual acuity on infant information processing. The following topics are also addressed: (i) breastfeeding and intelligence, (ii) criteria for the selection and control of potential confounding variables, and (iii) new infant information processing measures.

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The aims of this paper are twofold: (i) determination of how best to examine effects of long-chain polyunsaturated fatty acid (LCPUFA) nutritional supplementation or deficiency on infant neurobehavioral development, after controlling for other factors that might influence outcome, including maternal demographic, intellectual, and personality characteristics, and (ii) presentation of new findings on the relation between visual acuity and processing speed and the effects of prenatal alcohol exposure and visual acuity on infant information processing.

Much of the data presented is drawn from one of two prospective longitudinal studies: our Detroit study on effects of prenatal exposure to alcohol on infants (1) and our Michigan study on effects of pre- and postnatal exposure to polychlorinated biphenyls (PCBs) (2), an environmental contaminant found at elevated levels in contaminated Lake Michigan sportsfish. Findings from these studies are reviewed because they generated data about the sensitivity and predictive validity of new information processing and sensory measures also used in LCPUFA supplementation studies. These assessments include the Fagan Test of Infant Intelligence (FTII) (3) and the Teller Acuity Card Procedure (4). The findings on alcohol-related deficits on FTII processing speed and visual acuity may be particularly relevant for researchers who have found enhanced fixation and acuity effects of LCPUFA on these instruments. Recent studies linking chronic alcohol exposure to altered levels of LCPUFA concentrations in the feline brain and retina suggested that infants of women who abuse alcohol during pregnancy may be potentially at risk for reduced levels of these essential fatty acids (5).

LCPUFA STUDIES

The research design used to study the effects of adding LCPUFA to formula in many studies of preterm and full-term infants involves a clinical trials approach in which nonbreast-fed newborns are randomly assigned to different supplementation groups (e.g., 6–10). Comparison with a breast-feeding reference group is also often included in the study; and between-group differences in infant growth, visual acuity, and cognitive development are subsequently assessed. In that event, strictly speaking, random assignment is no longer complete because the mothers self-select on whether or not to breast-feed, a decision known to distinguish them (e.g., 11). Although comparisons of the randomly assigned newborn groups with the breast-feeding reference group could be omitted, this comparison seems useful in order to determine how well bottle-fed infants fare in contrast to those receiving maternal milk. Similar comparisons have been made in other developmental research areas, such as in longitudinal studies of low birthweight preterms or substance-exposed newborns in comparison with normal, full-term, nonexposed newborns. Intervention studies can randomly assign newborns to different experimental conditions, but subsequent follow-up studies have assessed group performance in comparison with full-term or nonexposed infants to determine degree of catch-up.

BREAST-FEEDING AND IQ

There has been considerable interest in the finding by Lucas *et al.* (12) that children who had been breast-fed as infants

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Abbreviations: AA/d, absolute alcohol per day; FTII, Fagan Test of Infant Intelligence; GA, gestational age; HOME, Home Observation for Measurement of the Environment; LCPUFA, long-chain polyunsaturated fatty acid; MDI, Mental Development Index; PCBs, polychlorinated biphenyls; PDI, Psychomotor Development Index; PPVT-R, Peabody Picture Vocabulary Test-Revised; RT, reaction time; SES, socioeconomic status; VexP, Visual Expectancy Paradigm; WISC-III, Wechsler Intelligence Scale for Children—III.

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had significantly higher IQ scores at 7.5 to 8 yr of age than those not breast-fed. This finding and the high rate of accumulation of LCPUFA in the retina and cortex during the last trimester of pregnancy and postnatally have been used to suggest that bottle-fed infants may miss some of the nutritional benefits of exposure to LCPUFA found in mother's milk (e.g., 6,8). However, human milk contains many components not present in infant formula, and comparisons of these groups cannot provide evidence for a single dietary component. Similarly, although an IQ difference remained after control for social class and maternal education in the Lucas *et al.* study, we found that the decision to breast-feed may reflect other factors, such as parenting skills, maternal personality characteristics and IQ, that may be responsible for the higher IQ scores in the children.

In an earlier study, we compared cognitive and personality correlates of breast-feeding in two independent cohorts: (i) 137 African-American inner-city mothers and (ii) 50 predominantly white lower-class mothers receiving WIC (Women, Infants, and Children, a U.S. government program to provide dietary assistance to financially disadvantaged families with small children) support (11). The decision to breast-feed and the duration of breast-feeding were positively related to maternal verbal IQ on the Peabody Picture Vocabulary Test-Revised (PPVT-R) (13) and ego development (14) in both samples (see Table 3 in Ref. 11). Two earlier studies also reported that breast-feeding mothers had higher verbal IQs and provided a more enriched home environment (15,16). We have since found that differences in maternal IQ and intellectual stimulation may account for the reported association between breast-feeding and childhood IQ (17).

Since 1980 we have been conducting a longitudinal study on the effects of prenatal exposure to PCBs (18). The data presented here are from this Michigan cohort of 323 white, predominantly middle-class 4-yr-old children. Although prenatal PCB exposure is associated with subtle cognitive deficits, no PCB-related cognitive deficits have been found in relation to postnatal exposure from breast milk (2,19,20). Data on feeding were collected at 2, 4, 5, and 7 mon, and infants were classified in one of five categories ranging from exclusively breast-fed to exclusively bottle-fed. These detailed estimates were highly correlated with duration of nursing ($r = .94$) and suggest that weeks of nursing can be used to indicate amount of breast milk consumed (21).

The children were administered the McCarthy Scales of Children's Abilities and the PPVT-R in the child's home at a mean age of 4.1 yr. The McCarthy Scales yield a General Cognitive Index that is highly correlated ($r = .81$) with IQ. Socioeconomic status (SES) was assessed on the Hollingshead Scale (22), an index that is more strongly related to early childhood cognitive functioning than other indices of SES (23). Maternal verbal IQ was assessed on the PPVT-R. Quality of parenting was evaluated on the Home Observation for Measurement of the Environment (HOME; 24), a semistructured maternal interview and observation of mother–child interaction. (The PPVT-R and HOME will be described in greater detail later.)

The majority (73.4%) of the mothers breast-fed for an average of 28.1 wk (SD = 27.7). As in the two other cohorts described above (11), breast-feeding was related to higher social class and education in this cohort, and mothers who breast-fed scored higher on the PPVT-R and had better parenting skills on the HOME.

Children who were breast-fed scored significantly higher on the McCarthy and PPVT-R tests at 4 yr, confirming the findings of the Lucas *et al*. (12) study (Table 1). The beneficial effect of breast-feeding remained significant after inclusion of social class and education in the regression analyses, but addition of maternal IQ and the HOME parenting score in the next step reduced the breast-feeding, social class, and educational influences to nonsignificant levels.

When the Michigan cohort was assessed at 11 yr on the Wechsler Intelligence Scale for Children-III (WISC-III) (21), the same pattern of effects was detected, namely, that breast-feeding was associated with higher IQ scores in the older children, but the effect disappeared again when maternal IQ and the HOME score were included in the regression analyses (25). At 11 yr, achievement tests were also administered to the children. No between-group differences were found on the Wide Range Achievement Test-Revised (WRAT-R) Spelling and Arithmetic subtests (Ps > .6). By contrast, achievement scores on the Woodcock Word, Passage, and Reading Comprehension tests were higher for breast-fed than bottle-fed children. As with the IQ scores, the relations between breast-feeding and achievement scores were significant after inclusion of social class and maternal education but not after inclusion of maternal IQ and the HOME scores.

These findings confirm those of the Lucas *et al*. (12) study regarding the IQ advantage shown by children who were breast-fed as infants and extend them to a full-term sample. However, our study included direct measures of maternal IQ and parenting skills. After these more direct measures of parental input were controlled statistically, breast-feeding no

TABLE 1
Effects of Breastfeeding, After Adjustment for Maternal Characteristics[a]

	McCarthy GCI[b] (n = 321)		PPVT-R (child)[b] (n = 319)	
	r	β^b	r	β^b
Step 1: Breastfeeding	.21***	.21***	.24***	.24***
Step 2: Breastfeeding	.21***	.12*	.24***	.14**
Social class	.34***	.21**	.36***	.17**
Education	.32***	.17*	.36***	.22***
Step 3: Breastfeeding	.21***	.06	.24***	.08
Social class	.34***	.06	.36***	.05
Education	.32***	.04	.36***	.10
Maternal IQ	.39***	.14*	.42***	.19**
HOME score	.51***	.38***	.46***	.29***

[a]From a hierarchical multiple regression analysis.
[b]Standardized regression coefficient: adjusts for influence of other variables at that step in the model. *$P < 0.025$; **$P < 0.01$; ***$P < 0.001$. Abbreviations: GCI, General Cognitive Index; PPVT-R, Peabody Picture Vocabulary Test-Revised; HOME, Home Observation for Measurement of the Environment.

longer appeared to affect childhood IQ. The mother's decision to breast-feed presumably reflects her concern with her infant's health and her motivation and ability to stimulate and enrich her child's subsequent development, which are at least partly independent of social class and education.

POTENTIAL CONFOUNDING VARIABLES

As already indicated, ethnic, socioeconomic, parenting, and intellectual differences associated with incidence of breast-feeding are known to influence infant outcome (11). In laboratory animal studies, random assignment can control for individual differences in genetic make-up, and cross-fostering for poor parenting. Given that complete random assignment will not necessarily occur in LCPUFA supplementation studies, it is important to compare experimental and breast-feeding reference groups in order to rule out any other group differences that might account for the observed differences. In fact, failure to control for measures, such as maternal IQ or quality of parenting, may underplay the positive role of the nutritional supplement and result in Type II error, the failure to recognize a true effect or, in this case, to understate the magnitude of the effect. If, as shown above, offspring of breast-feeding mothers consistently test at slightly higher developmental or IQ levels, a lower score for a supplementation group may be due to lower maternal IQ scores that are transmitted genetically and/or environmentally to their offspring rather than to a lack of an LCPUFA effect. Maternal IQ in these instances acts as a suppressor of the true effect.

Socioenvironmental influences. Measures selected to represent the potential confounders must be both reliable and valid because measurement error in the control variables can threaten the validity of any causal inferences drawn from the data. Although SES and maternal education have been used to assess socioenvironmental influences in some studies, these variables assess environmental effects on child performance less adequately than direct measures of quality of parenting and parental intelligence (17). A standard instrument for evaluating quality of parenting is the HOME Inventory (24), which combines a semistructured parental interview with informal observation of parent–child interaction to assess quality of parental intellectual stimulation and emotional responsiveness. The validity and sensitivity of the HOME as an indicator of socioenvironmental influences on development have been demonstrated by evidence that it explains significant variance in childhood cognitive functioning over and above that attributable to socioeconomic status (SES) and maternal education (17,25,26). Its validity for use with African-American samples has also been established (27). Although designed to be administered in the home, considerations of personal safety may preclude home visits in certain neighborhoods. The validity of administering the HOME in the laboratory has been demonstrated at 12 and 24 mon (28,29).

Because it is often not possible to perform a full IQ test on parents and because vocabulary is the strongest single correlate of IQ, a standardized vocabulary test, such as the PPVT-R (13), can be used to assess parental intelligence. The PPVT-R is a brief vocabulary recognition test that (i) is strongly correlated with more comprehensive measures of adult IQ, such as the Wechsler Adult Intelligence Scale, median $r = .72$, (ii) is highly stable over a 1-yr period, median $r = .72$ (13), and (iii) has been shown to be valid for use with various populations (11).

A parental vocabulary test assesses two sources of extraneous influence on child cognitive performance, genetic endowment and quality of stimulation, both of which may need to be controlled. Standardized vocabulary tests may not be valid, however, for minority or at-risk populations. Adult vocabulary scores are very low in most economically disadvantaged samples, averaging 75 on tests standardized to a mean of 100 (SD of 15), for example, in both inner-city Cincinnati (30) and Detroit (11). In Detroit, more than one-third of the mothers scored below 70 on the PPVT-R, even though few if any appeared to be functionally retarded. The mothers' scores were higher on two Performance IQ scales from the Wechsler Adult Intelligence Score; scores normalized to a mean of 100 (SD of 15) averaged 82.5 (SD = 9.6) and 85.4 (SD = 9.7) for Block Design and Picture Completion, respectively. The PPVT-R was, nevertheless, more strongly correlated with other relevant maternal demographic and personality variables than the Performance IQ scores (11). Although the low vocabulary scores were misleading, presumably owing to the limited educational opportunities available to these inner-city mothers, the PPVT-R apparently rank-ordered them appropriately within the cohort.

SAMPLE SELECTION AND ATTRITION BIAS

Exclusionary criteria. In evaluating the effects of LCPUFA supplementation or deficiency, infant exclusionary criteria should include major chromosomal anomalies or neural tube defects or multiple births, conditions known to have a major impact on cognitive outcome. Inclusion of a cognitively impaired child in one of the experimental groups may bias the findings and result in another instance of Type II error. Similarly, unless maternal instruments are translated into the mother's primary language and there are interviewers who are fluent in the mother's language, inclusion of mothers who do not speak English may lead to a considerable amount of missing or poor data. Data from infants or children who are "untestable" or refuse or are unable to cooperate may also need to be excluded from the data set or from analyses of specific outcomes for which most of the data are missing. An analysis should, however, be conducted to see whether such children are found more often in one experimental group or another, since a specific type of supplement may be responsible for their inability to perform.

It is important to test that random assignment to condition has been successful by comparing maternal demographic, intellectual, and parenting characteristics, as well as gestational age, gender, parity, and birth size of the infant for the supplementation groups at the outset of the study. Similarly, in any

long-term follow-up, it is important to test for attrition bias since it is possible that demographic or other socioenvironmental differences that may influence neurobehavioral or physical outcome may affect which infants are brought back for reassessment. For example, working mothers or mothers whose infants are ill are sometimes less likely to return for follow-up, thereby affecting outcome. If such a systematic difference between groups is detected, it can be controlled in the multivariate analyses.

It is also important to ensure that there is sufficient power (a large enough *n*) in each experimental group both at the onset and at follow-up to assess the desired outcomes (31). In our alcohol research, we demonstrated that an insufficient number of moderately to heavily exposed children in a follow-up cohort can result in a lack of alcohol-related effects on the Bayley Scales of Infant Development (see below for description) that would otherwise be found were enough such children represented in the long-term sample (32). In the case of alcohol exposure, we found that 45 moderately to heavily exposed infants proved sufficient to detect a subtle alcohol effect in our Detroit cohort, whereas another large study conducted in Cleveland with only seven moderately to heavily exposed infants did not. When all but seven of the infants whose mothers drank above a moderate threshold (5 oz. AA/d or more, where AA is absolute alcohol) were randomly deleted from the Detroit data, the zero-order correlation of alcohol with the Bayley dropped from −.17 to −.05, similar to the −.06 correlation reported in the Cleveland study. If we had not overrepresented moderate to heavy alcohol users in our cohort, we would have failed to detect a true relation between prenatal exposure and infant outcome.

Statistical analysis. In multivariate analysis, the coefficient assessing the magnitude of the experimental effect is likely to be unreliable if the ratio of subjects to independent variables is too low; a minimum of 20 subjects per independent variable is recommended (33). In addition, the inclusion of control variables unrelated to the developmental outcome is likely to increase the error term, that is, make the statistical analysis less accurate, which can make it more difficult to detect significant effects (34).

For these reasons, control variables are usually prescreened to determine which ones to include in the multivariate analyses. This prescreening is based on the premise that a control variable cannot be the true cause of an observed deficit unless it is related to both predictor and outcome (35). In our Detroit alcohol study, all control variables that were at least weakly related to the outcome being evaluated (at $P < .10$) were included as potential confounders. The $P < .10$ criterion is conservative in this context because it includes even weak potential confounders. Control variables are usually selected based on their relation to outcome because their inclusion will reduce the error term, thereby improving the chances of detecting real effects (34). Control variables can be selected either by examining their univariate relations with each outcome or simultaneously by stepwise multiple regression (e.g., 30). A group effect is inferred only if the relation between the predictor and outcome is significant at $P < .05$ after controlling for the potential confounders.

Confounders that are too highly correlated with each other ($r = .70$ or more) should also not both be included in multivariate analyses at the same time since they may be too confounded to determine which is the true predictor of the outcome being studied (36). For example, since pre- and postnatal drinking by the mother are moderately correlated, statistical analyses that include both may obscure true effects of prenatal exposure and lead to the conclusion that only the postnatal social environment is influencing the child's performance. A similar problem in LCPUFA research occurs when both docosahexaenoic and eicosapentaenoic acids, two highly correlated fatty acids, are included in the same multivariate analysis with the former usually obscuring the role of the latter.

NEW INFANT INFORMATION PROCESSING OUTCOME MEASURES

Virtually all prospective studies of the effects of teratogenic exposure or medical or socioenvironmental at-risk conditions have used the Bayley Scales of Infant Development (37) to assess neurobehavioral function in infancy. The Bayley is an apical, multidomain test, which provides both a Mental Development Index (MDI) and a Psychomotor Development Index (PDI) score. In our research, we have tried to include infant information processing tests with better predictive validity than the more traditional assessments, such as the Bayley. We hoped to detect effects early in development and expected that more focused, narrow-band tests would provide more information about the specific domain affected. We found, as predicted (3), that these information processing tests were less influenced by socioenvironmental factors than the Bayley measures (see Table 2 in Ref. 1).

Several new paradigms for assessment of infant information processing have been advanced in recent years (see Ref. 8 for an excellent review of infant tests used in LCPUFA supplementation studies). Traditional infant tests, such as the Bayley Scales, focus on rate of progress through developmental milestones, an approach also taken by measures from the Uzgiris-Hunt (38) Scales and complexity of play tasks (e.g., 39,40). Other tests based on habituation and paired comparison paradigms focus more directly on information processing capabilities (41). Some investigators (3,42) have emphasized measurement of novelty preference in the paired comparison paradigm, whereas others have recommended assessment of duration of attention and inattention during familiarization (43). Newer infant information processing tests, such as the FTII, appear to be especially sensitive to specific deficits, enabling researchers to detect deficits in memory, attention, or processing speed previously seen only in older children (e.g., 1,2).

Furthermore, novelty preference has been found to have markedly better predictive validity for later performance on standardized intelligence tests than the Bayley (3,44), and three studies have shown that shorter fixation duration during

infancy also predicts better childhood performance (45–47). Shorter fixation duration, which has been related to superior performance on numerous infant tasks (e.g., 43), may indicate more efficient information processing (48), which is often measured by reaction time (RT) paradigms in older children and adults. We have recently reported a direct relation of fixation duration on the FTII and cross-modal transfer test to infant RT using this paradigm (49).

DETROIT PROSPECTIVE LONGITUDINAL PRENATAL ALCOHOL EXPOSURE STUDY

We have been studying the effects of prenatal alcohol and cocaine exposure in a cohort of 480 inner-city infants. In this study visual recognition memory was assessed at 6.5 and 12 mon using the FTII novelty preference test developed by Fagan and Singer (3). The infant was seated on the mother's lap in front of a portable observation chamber containing a pivoting stimulus presentation "stage," on which were located two stimulus plaques. Infant fixation to the stimuli was judged from corneal reflections of the stimulus targets observed through a 0.64-cm peephole located halfway between the two plaques and recorded on a Zenith PC computer. The stimuli consisted of 10 pairs of faces, measuring approximately 14.5 cm from crown to chin and mounted on a 20.0 × 17.5 cm white posterboard. In the 6.5-mon test, the familiar targets were shown until the infant had fixated them for a total of 20 s. The familiar target was then paired with a novel target for two 5-s periods, reversing left-right positions from one period to the next. The same pairs of photos were used in the 12-mon test, but the initial study time was shortened to 12 s and the test period to 3 se on each side.

Preference for novelty on the FTII is computed separately for each problem by dividing duration of fixation to the novel stimulus by total time fixating the novel and familiar stimuli and then averaging across the 10 problems. Mean preference for novelty was calculated by averaging percentage of novelty preference for the 10 problems at each age and then, to increase reliability, across the two ages (see 43,50).

Following Colombo *et al*. (43), mean fixation length or duration was computed for each problem by dividing the total duration looking time by the number of looks and averaging these across the 10 problems and then across the two ages. It is also possible to compute length of look for the familiarization or test period only by dividing duration looking time for that period by number of looks during that period. Since we found basically the same pattern of results when looking at mean duration looking time for the total or for the familiarization period only, we have usually presented the findings in terms of the mean duration fixation for the combined familiarization and test periods. FTII data were used in our study only if available for both visits and were omitted for a few infants who completed fewer than three problems, whose mothers interfered throughout testing, who were sick during the visit or refused to look at the photos, or because of computer failure.

Age at time of testing was adjusted for infants born at less than 38 wk gestational age. For the FTII, for example, infants should be seen for the 6.5-mon assessment within 1 wk of the conceptual rather than the chronological age. Since the Bayley Scales are standardized, a broader testing window is permissible, and age at testing does not need to be as closely adjusted.

Prenatal alcohol exposure sample. The sample described here consists of the 403 black infants (233 males, 170 females) for whom there are complete FTII data. They were born to women recruited antenatally based on their consumption of alcohol during pregnancy. The women were recruited at their first visit (mean = 23.3 wk of pregnancy, SD = 8.0) to the prenatal clinic of a large, inner-city maternity hospital serving primarily (92%) black women. In using the protocol described in Jacobson *et al*. (51), each gravida was interviewed regarding her alcohol consumption both currently and at conception. All women reporting alcohol consumption at conception averaging at least 0.5 oz of AA/d (about one drink per day), a random sample of approximately 5% of the women who drank at lower levels or abstained, and 57 high-cocaine (at least 2 d/wk), low alcohol (<0.5 oz AA/d) users were invited to participate. The high-cocaine/low-alcohol users were included to reduce the risk that alcohol would be confounded with cocaine exposure in this sample.

The sample was predominantly lower lower class (mean = 19.8, SD = 8.5 on the Hollingshead Four Factor Index (22), with 84.4% receiving welfare. Mean maternal age was 26.9 yr (SD = 6.1), with 3.5% of the women between 14 and 17 yr of age. Only 10.7% were married. Maternal education was low (M = 11.7 yr, SD = 1.6, range = 6–18); 42.7% had not completed high school. Mothers averaged 5.3 prenatal clinic visits (SD = 3.2), with 13.2% having only one prenatal visit. Infant exclusionary criteria included birthweight less than 1500 g, gestational age (GA) less than 32 wk, major chromosomal anomalies or neural tube defects, and multiple births. About one-third of the infants (35.0%) were first-borns. Mean birthweight was 3088 g (SD = 530); mean GA was 39.4 (SD = 1.6). Seven newborns (1.8%) weighed less than 2000 g. Age of testing was corrected for the 11.4% of the infants born between 32.5 and 38.0 wk gestation by testing them at the conceptual age equivalent to that of the full-terms.

Processing speed effects. The most consistent neurobehavioral effect of prenatal alcohol exposure in older children relates to attention. Poorer accuracy and slower reaction times have been reported in a Seattle cohort of 4- and 7.5-yr-old children exposed prenatally to alcohol at moderate levels (52,53), an effect also detected in children with fetal alcohol syndrome (54). These findings led Streissguth (52,53) to suggest an alcohol-related deficit in "speed of central processing."

In our Detroit longitudinal study we included three tests that assess processing speed in infancy: the FTII, a test of cross-modal transfer, and Haith's Visual Expectancy Paradigm (VExP; 55), which for the first time assesses RT in infancy. Processing speed was assessed in the FTII recognition memory and cross-modal tests in terms of length of visual fix-

ation. Short looks on the FTII and cross-modal tests are associated with more rapid information processing and are predictive of higher childhood IQ (43,47,56).

We found that prenatal alcohol exposure, like LCPUFA (9), is unrelated to novelty preference in these infant tests (Table 2). Prenatal alcohol was, however, related to slower processing speed on the FTII and cross-modal transfer test and longer latencies to respond on the VExP (1,57). This pattern of impairment seems specific to alcohol exposure and is different from that seen in infants exposed to environmental contaminants, such as PCBs (2,58), or other prenatal substance exposure, such as heavy maternal cocaine use (59). These deficits, which can already be detected in infancy, were consistent with speed of processing deficits seen in older alcohol-exposed children (52,53). Early deficits in play and symbolic development were also related to prenatal alcohol exposure (1).

By contrast to pregnancy drinking, postpartum drinking by the mother or primary caregiver did not relate significantly to any of the outcomes tested (all $P > .15$, after adjustment for the potential confounders) (Table 2), suggesting that these effects are related to prenatal exposure rather than a consequence of being raised by a drinking mother.

Predictive validity. Several studies have documented the predictive validity of new infant information processing measures by looking at the association between the infant measures and childhood IQ assessed on the WISC-III or other tests (56,59–62). In addition to a Full Scale IQ, the WISC-III provides a Verbal IQ score which summarizes performance on the more verbally mediated subtests, including Vocabulary, Information, and Similarities, and a Performance IQ, which is based on subtests that assess more perceptual organization tasks, such as Block Design, Object Assembly, and Picture Organization. Analyses of the data from the first 94 Detroit children seen at follow-up visits (47) indicate that the FTII recognition memory measure appears to be a stronger predictor of 7-yr Verbal IQ on the WISC-III than the Bayley MDI, and 12-mon fixation duration was related to poorer Performance IQ scores (Table 3). Whereas the Bayley PDI was not significantly related to either Verbal or Performance IQ at 7 yr, level of play, assessed on the Belsky play scale (39), was moderately predictive of Verbal IQ.

A regression analysis indicated that the MDI did not contribute independently to Verbal IQ (Table 4). By contrast, fixation duration, a measure of processing speed, and the 13-mon MDI, which is composed largely of fine-motor manipulation and spatial ability items, were independently associated with 7-yr Performance IQ. When play was included in the Verbal IQ regression, it provided an additional independent dimension of verbal cognitive function, probably because it assesses early symbolic development. These effects remained virtually unchanged when the Infant HOME and maternal verbal IQ from the PPVT-R were included in the regressions. These data are among the first to demonstrate these associations in a highly disadvantaged, inner-city sample. These

TABLE 2
Effects of Drinking During Pregnancy on Infant Cognitive Outcome

		Maternal drinking[a] (oz AA/d)			
		During pregnancy		Postnatal	
	n	*r*	β[b]	*r*	β[b]
Fagan Test of Infant Intelligence (FTII)					
Novelty preference	315	.08	.07	−.01	−.02
Fixation duration	315	.15**	.12*	.12*	.08
Cross-modal transfer					
Novelty preference	362	.04	−.01	.11	.08
Fixation duration	362	.16**	.12*	.10	.03
Visual Expectancy Paradigm					
Median reaction time (ms)	103	.25**	.27***	.02	.02
% Fast (201–300 ms)	103	−.31****	−.36****	−.07	−.09

[a]*$P < .05$; **$P < .01$; ***$P < .005$; ****$P < .001$.
[b]Standardized regression coefficient: adjusts for influence of potential confounders related to outcome at $P < .10$. AA, absolute alcohol.

TABLE 3
Correlations of Infant Measures with 7-Year IQ

	WISC III[a]		
	Verbal IQ	Performance IQ	Full IQ
Bayley Scales of Infant Development			
Mental Development Index	.18†	.36***	.30**
Psychomotor Development Index	.15	.16	.17
Fagan Test of Infant Intelligence			
Novelty preference[b]	.24*	.13	.22†
Mean fixation duration			
6-mon	.00	.05	.02
12-mon	−.15	−.27**	−.23*
Highest level of play[c]	.34**	.13	.27*

[a]*Note:* Values are Pearson product-moment correlation coefficients. †$P < .10$; *$P < .05$; **$P < .01$; ***$P < .001$.
[b]Averaged across 6 and 12 mon.
[c]Based on Belsky play measure. Abbreviation: WISC III, Wechsler Intelligence Scales for Children—III.

TABLE 4
Effects of Infant Measures on 7-Year IQ

	WISC III[a]		
	n	*r*	β
Verbal IQ			
Novelty preference[b]	66	.33**	.27*
Bayley Mental Development Index	66	.15	.01
Highest level of play[c]	66	.35**	.30*
Performance IQ			
Mean fixation duration[d]	82	−.27**	−.23*
Bayley Mental Development Index	82	.32**	.29**
Full IQ			
Novelty preference[b]	66	.28*	.22†
Mean fixation duration[d]	66	−.21†	−.24*
Bayley Mental Development Index	66	.27*	.14
Highest level of play[c]	66	.26*	.21†

[a]†$P < .10$; *$P < .05$; **$P < .01$. For abbreviations see Tables 2 and 3.
[b]Averaged across 6- and 12-mon FTII assessments.
[c]Based on Belsky play measure.
[d]12-mon FTII assessment.

findings provide further support for the hypothesis that memory/attention (as assessed by novelty preference) and processing speed (fixation duration) represent separate underlying components of intellectual function in infants and children. These data are consistent with findings from two studies of infant monkeys, which also found evidence for independent effects on visual recognition memory/novelty preference and fixation duration of taurine and LCPUFA, respectively (63,64). In addition, symbolic play, assessed on the Belsky play scale (39), appears to reflect yet a third independent component of intellectual function (Table 4).

VISUAL ACUITY

Procedure. To ensure that the alcohol-related infant deficits detected in our research reflect cognitive rather than perceptual difficulty, infants were screened at 6.5 and 12 mon on the Teller Acuity Card Procedure (4), a test commonly used in LCPUFA studies to assess the effects of LCPUFA supplementation on visual acuity in infants. The infant is seated on the mother's lap in front of a gray screen designed by Vistech, Inc. (Dayton, Ohio). A shield suspended in front of the screen prevents the mother from viewing the stimuli and inadvertently influencing the infant's gaze. In this procedure, based on infant fixation of patterned stimuli, the infant is shown a visual target with vertical black and white stripes, which appears alternately on the right and left side of the gray screen. An examiner notes side preferred by observing the infant's gaze through a peephole in the center of the screen. Over successive trials, progressively narrower stripes are displayed. Acuity is defined by the smallest grating size (spatial frequency) the infant fixates and the distance of the infant from the stimuli.

Visual acuity effects. All but four infants in the cohort scored in the normal range at both ages. Two with low scores at 6.5 mon scored within the the normal range at 12 mon; two with marginal scores at 12 mon performed well on the cognitive tests at 12 and 13 mon. There was therefore no evidence that visual acuity problems interfered with the assessment of cognitive performance.

Although acuity was unrelated to prenatal exposure to marijuana, opiates, or smoking (*r* ranged from .03 to .08, n.s.), alcohol exposure was associated with poorer infant visual acuity, after control for potential confounders (Table 5). This relation was not mediated by GA or birth size since acuity was unrelated to GA, birthweight, length, or head circumference (*r* ranged from .01 to .08, n.s.). As can be seen in Table 5, the alcohol effect on acuity was particularly strong for infants of mothers 30 yr or older. This effect is consistent with our finding that maternal age appears to be a moderator for several of the alcohol-related neurobehavioral deficits we have detected in infancy (65). Performance on the Bayley Scales, processing speed on the FTII and cross-modal transfer, and elicited play were more severely affected in infants born to older moderate-to-heavy drinking mothers (at least 30 yr of age).

A Pearson correlation analysis indicated a significant relation between visual acuity and the FTII mean length of gaze and infant reaction time on the Haith VExP (Table 6). Poorer acuity was associated with slower processing speed. Regression analyses show, however, that, although correlated, the effect of prenatal alcohol exposure and visual acuity on mean fixation duration and the infant RT measures are independent, even after control for potential confounders (Table 7). Thus,

TABLE 5
Effects of Prenatal Alcohol Exposure on Visual Acuity by Maternal Age

	n	*r*	β[a]
Maternal age (yr)			
<30	239	–.02	–.02
≥ 30	79	–.30**	–.30**
Total sample	318	–.12*	–.12*

[a]Standardized regression coefficient: adjusts for confounders. *$P < .05$; **$P < .01$.

TABLE 6
Correlation of Visual Acuity with Infant Outcome[a]

	n	*r*
Bayley Scales of Infant Development		
Mental Development Index	341	–.03
Psychomotor Development Index	334	.07
Fagan Test of Infant Intelligence		
Novelty preference (6- and 12-mon)	311	–.02
Mean fixation duration		
6-mon	317	–.18***
12-mon	357	–.17***
6- and 12-mon	311	–.20***
Haith Visual Expectancy Paradigm		
Median reaction time (ms)	82	–.18†
% Fast (<300 ms)	82	.26*
% Quick (anticipations + fast)	82	.25*

[a]†$P < .10$; *$P < .05$; ***$P < .001$.

TABLE 7
Relation of Prenatal Alcohol Exposure and Visual Acuity to Infant Outcome

		Quantity of absolute alcohol consumed per day		Mean acuity[a]	
	n	*r*	β	*r*	β
Novelty preference					
Fagan visual recognition memory[a]	309	.06	.07	.10†	.11†
Cross-modal transfer	326	.03	.00	.12	.02
Mean look duration[b]	300	.16*	.12*	–.16*	–.14*
Visual Expectancy Paradigm					
Reaction time (ms)	82	.26*	.27**	–.26*	–.23*
% Fast responses (201–300 ms)	82	–.30**	–.34***	.34***	.30**
% Quick (anticipation + fast)	82	–.30**	–.34***	.30**	.27**

[a]Average 6.5- and 12-mon assessments.
[b]Average 6.5- and 12-mon FTII and 12-mon cross-modal scores. †$P < .10$; *$P < .05$; **$P < .01$; ***$P < .001$. For abbreviation see Table 2.

the alcohol-related processing speed deficits were not mediated by acuity.

CONCLUSIONS

There has been considerable recent interest regarding the advisability of adding LCPUFA to infant formula. The issue was first examined in studies such as those conducted by Neuringer and colleagues. The findings of Lucas *et al*. (12) that children who had been breast-fed as infants had higher IQ scores than those not breast-fed, and the concern that preterms and bottle-fed infants may lack some of the nutritional benefits of exposure to LCPUFA found in mothers' milk, have also been noted. The findings presented here suggest that any comparisons between breast-fed and supplemented groups should include measures of maternal IQ and quality of parenting on which these groups tend to differ. Failure to control for maternal IQ or quality of parenting may underplay the potentially positive role of the nutritional supplement.

Although our data raise doubt about whether higher IQ in full-terms can be attributed to the nutritional benefits of breast-feeding, they have no bearing on evidence of the medical and psychological benefits of breast-feeding, which have been extensively demonstrated in other studies, or on the need to provide nutritionally adequate formula for preterm or other nutritionally-deprived or at-risk infants. Nor do our findings detract from the potential benefits of LCPUFA supplementation to infant formula. By contrast to prenatal alcohol exposure, which is related to poorer acuity and longer fixations on the FTII, animal (63,64) and human studies (7–9) suggest that LCPUFA supplementation may result in enhanced acuity and shorter, more optimal fixation duration patterns, which have been found to be predictive of more optimal cognitive function and higher IQ in children.

Given the alcohol-related deficits in infant visual acuity and fixation duration found in our Detroit sample, maternal report of prenatal alcohol use should also be considered a potential confounding variable for human studies of LCPUFA supplementation on these outcomes. A brief screening of alcohol use during pregnancy at the time of recruitment may help identify women whose infants may be affected. Failure to control for the impact of moderate to heavy prenatal alcohol exposure (>.05 oz AA/d) in an LCPUFA study may mask true effects of the supplement on these outcomes.

As noted earlier, chronic alcohol exposure has recently been linked to altered levels of LCPUFA concentrations in the feline brain and retina, leading the authors to note that women who abuse alcohol during pregnancy may be especially at risk for reduced levels of essential fatty acids and may need to supplement their diets in order to ensure an adequate neural supply of 22:6n-3 (5). In our research, we found a relation between visual acuity and processing speed. Our findings suggest, however, that although prenatal alcohol exposure is related to both poorer infant visual acuity and processing speed, these appear to be relatively independent alcohol-related deficits. Thus, animal and human studies indicating a relation between LCPUFA supplementation and enhanced visual acuity and shorter visual fixations may, in fact, represent relatively independent effects of supplementation on both acuity and cognitive processing speed.

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Effects of Dietary Polyunsaturated Fatty Acids on Neuronal Function

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ABSTRACT: Diets deficient in linoleic acid (18:2n-6), or that have unusual ratios of linoleic acid to α-linolenic acid (18:3n-3) induce changes in the polyunsaturated fatty acid (PUFA) composition of neuronal and glial membranes. Such changes have been linked to alterations in retina and brain function. These functional effects are presumed to follow from the biochemical consequences of modifying membrane PUFA content; known effects include modifications in membrane fluidity, in the activities of membrane-associated, functional proteins (transporters, receptors, enzymes), and in the production of important signaling molecules from oxygenated linoleic and α-linolenic acid derivatives. However, despite the demonstration that central nervous system function changes when dietary PUFA intake is altered, and that in general, membrane PUFA content influences membrane functions, little work has focused specifically on brain and retina to reveal the underlying biochemical bases for such effects. This review examines this issue, looking at known effects of dietary PUFA on neurons in both the central and peripheral nervous systems, and attempts to identify some approaches that might promote productive investigation into the underlying mechanisms relating changes in dietary PUFA intake to alterations in neuronal and overall nervous system functioning.

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Over the past 30 yr, evidence has accumulated showing that modification of the dietary intake of linoleic (18:2n-6) acid or of the proportion of linoleic to α-linolenic (18:3n-3) acids, particularly during development, influences the content of these and related polyunsaturated fatty acids (PUFA) in the membrane lipids of cells (1,2). Modification of the PUFA content of cell membranes has a large impact on membrane fluidity and the functioning of a variety of membrane-associated proteins (transporters, enzymes, receptors) (3,4). As a result, cell functions are altered, such as glucagon-stimulated adenylate cyclase activity in liver (5) and insulin binding and insulin-stimulated glucose transport and lipogenesis in adipocytes (6).

The central nervous system (CNS) is not immune to such effects. Indeed, modifications in the dietary intake of linoleic acid or in the proportion of linoleic to α-linolenic acids were repeatedly shown to influence neuronal and glial membrane contents of several PUFA, including arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) (1,2,7,8). The most carefully studied CNS effects of dietary PUFA relate to the visual system, where DHA is a major constituent of photoreceptor membranes (2). Photoreceptor DHA abundance in these (as well as in retinal and occipital cortex) membranes is modified by dietary PUFA content and is associated with changes in the electroretinogram and in visual acuity tests in humans and nonhuman primates (2,9–12). These findings indicate that functional effects occur at several levels of the visual system, including the photoreceptor, the retina, and the visual areas of the brain (13). [A separate literature source also describes dietary PUFA effects on learning and memory, but the complexity of the models examined and the effects obtained suggest that further work is required before a clear linkage emerges (14).]

Given reported effects of dietary fat intake on the nervous system, both with regard to membrane fatty acid composition and complex CNS functions, it would be of interest to identify underlying mechanism(s) in brain and retina that link these effects. (Presumably, the functional changes are causally related to the alterations in membrane composition.) Apart from studies that reach to the molecular level to illuminate DHA function in phototransduction (15,16), few attempts have been made to evaluate the potential connections. Occasional studies have looked for effects of dietary PUFA on brain membrane-associated activities that influence neurotransmitter function, such as acetylcholinesterase (17), or the presynaptic norepinephrine (NE) reuptake transporter (which is embedded in the presynaptic membrane, and rapidly removes the amine from the synaptic cleft, once it has been released) (18). Conceivably, dietary PUFA-induced changes in membrane functions such as these could modify neurotransmission across affected synapses in visual circuits in retina and/or brain and contribute to the observed visual changes.

A priori, to the neuropharmacologist, no obvious reason is apparent why studies have not progressed in brain and retina. The starting points for hypotheses are clear: diet-related

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Abbreviations: CNS, central nervous system; cAMP, cyclic AMP; DHA, docosahexaenoic acid; DA, dopamine; NE, norepinephrine; PUFA, polyunsaturated fatty acid; 5HT, serotonin.

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changes in membrane PUFA composition should modify membrane (and thus cellular) function, based on changes in (i) membrane fluidity, which could be expected to influence membrane-associated cellular functions (e.g., transporters, enzymes, receptors) (3) and, (ii) the production of second messengers (notably prostaglandins), which influence many important cell-associated functions in response to specific, external signals (4,19). Indeed, reported effects of dietary PUFA on acetylcholinesterase activity (17) and NE reuptake (18) in brain are consistent with such a hypothesis. Given the broadness of the postulated PUFA effects (to modify an array of membrane-associated functions in essentially any neuron), it is remarkable that exploratory studies identifying dietary PUFA effects on neurotransmitter systems in brain/retina (20,21) were not followed up to elucidate the biochemical mechanisms of such effects. As one example, given the existence of dopamine (DA) interneurons in the retina, their known function, the wealth of biochemical tools for studying DA neurons and receptors, and the known actions of several DA drugs on the electroretinogram (22–28), it is surprising that biochemical studies of retinal DA were not conducted under dietary conditions that alter retinal membrane PUFA content. If reuptake mechanisms, receptors, and second-messenger systems for neurotransmitters are targets of dietary PUFA action in brain and retina, many such neurotransmitter-specific effects should be awaiting discovery.

Peripheral nerves and synapses: a known site of dietary PUFA action. Although few data exist regarding dietary PUFA actions on neuronal functions inside the brain, data describing effects on neurons outside the CNS do exist. While these studies show some dietary naiveté by present standards (while they focus on manipulation of linoleic acid, they do not appreciate that α-linolenic acid content is also being modified), they are neurochemically and neuropharmacologically thoughtful, and thus offer insight into general approaches that can be taken to examine this dietary-neuronal linkage inside as well as outside of the brain.

Diet-related changes in neuronal (and glial) membrane PUFA content could affect either (or both) of two broad categories of neuronal function: conduction or transmission. Conduction describes the movement of nerve impulses along the axon from cell body to nerve terminal, while transmission is the transfer of an electrical impulse across the synapse from one neuron to the next. Both processes are complicated membrane functions: conduction requires a continuous depolarization and repolarization process to move the signal along the axon and utilizes membrane-associated enzymes (e.g., Na^+,K^+ATPase) and ion pores. Transmission first involves an electrical–chemical transduction, during which the electrical signal, on reaching the nerve terminal, causes a chemical (neurotransmitter) to be released into the synapse (the small space between a nerve terminal and an adjacent neuron). The neurotransmitter molecules move into the synaptic space, where some quickly occupy specific receptors on nearby neurons. Finally, a chemical–electrical transduction occurs, for the appropriate receptor type, and depolarization of the neuron occurs. (Some receptors do not cause depolarization, when occupied, but instead initiate other functions, such as the activation of enzymes and various cellular processes.) Like conduction, transmission thus involves many membrane-associated events, including the release of transmitter through the terminal membrane (or from vesicles that join with the membrane), its association with membrane-bound receptors (and attendant ion pores or second-messenger systems), and its rapid removal from the synapse either by a process of reuptake into the terminal (involving membrane-associated transporters) or destruction by membrane-associated enzymes (e.g., for acetylcholine, acetylcholinesterase) (Fig. 1). Events such as these occur in most neurons (although some neurons make direct membrane contact, and thus do not require neurotransmitters to share electrical signals), suggesting that dietary PUFA-associated changes in neuronal membranes and functions might be widespread.

A few studies have examined the influence of the diet on peripheral nerve membrane PUFA profile and on conduction. Yao *et al.* (29) fed weanling rats 5% fat diets consisting of either hydrogenated coconut oil (n-3 and n-6 fatty acid-deficient) or corn oil (n-3 fatty acid-deficient). After 8 mon, most phospholipids in sciatic nerve myelin of rats fed the coconut oil diet contained considerably lower levels of the n-6 fatty acids than was the case in rats ingesting the corn oil diet. Moreover, following nerve crush, the regenerating sciatic nerve in coconut oil-fed rats did not experience the large increase in AA observed in the regenerating nerves of corn oil-fed rats (perhaps suggesting that the nerves would not regenerate normally). In a functional study of like dietary design, similar effects were observed on myelin phospholipids, but no impact of these differences was noted on nerve conduction velocity, the compound action potential, or on the morphological features of nerve regeneration that follow injury (30). Conceivably, functional effects on nerve conduction properties might emerge in animals made linoleic acid-deficient during pre- and postnatal development, rather than postweanling, but no data are available that evaluate this possibility.

Bourre *et al.* (31) also looked at peripheral nerves under conditions of dietary PUFA deficiency but focused solely on membrane fatty acid composition (there were no functional measures). Nevertheless, it is an interesting study, showing a marked membrane deficiency in DHA (and a large excess of 22:5n-6) in rats fed 1.5% sunflower oil for three–four generations, when compared to animals ingesting 1.5% soybean oil. Such PUFA abnormalities were corrected by changing n-3-deficient rats (sunflower oil-fed) to a soybean oil diet (high in 18:3n-3), but the reversal required nearly 2 mon. An evaluation of nerve conduction properties in this paradigm would be interesting.

In contrast to the scarcity of studies of nerve conduction in animals fed diets varying in n-3 and n-6 fatty acids, more information is available showing clear changes in neurotransmission in the peripheral nerves of animals consuming a linoleic acid-deficient diet. The nerves most carefully studied are the postganglionic sympathetic neurons (for which NE is

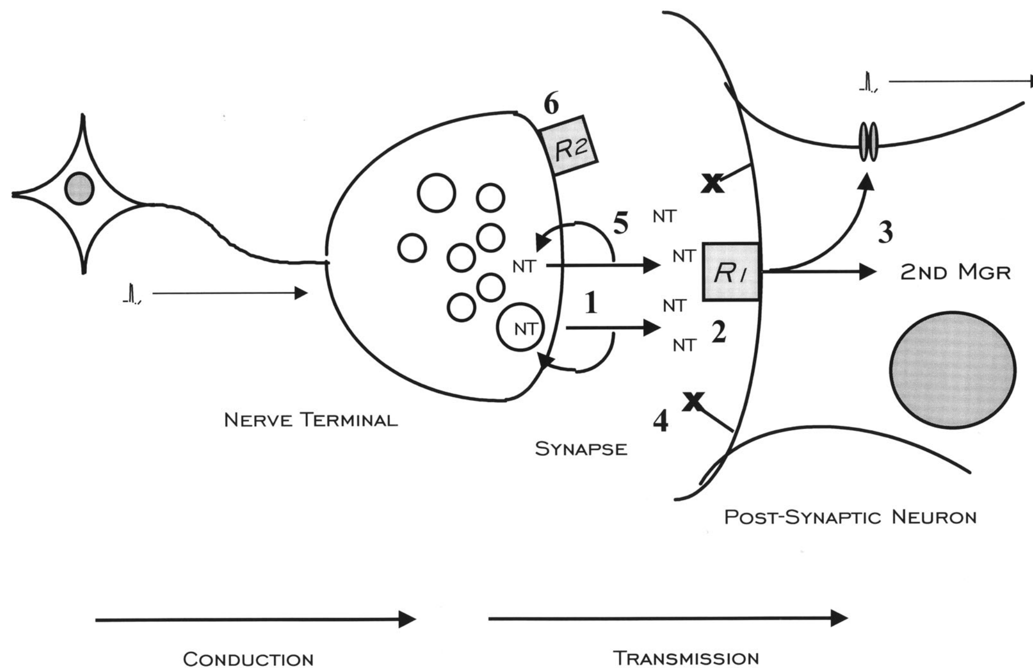

FIG. 1. Neuronal conduction and transmission. The depolarization wave moves along the axon from cell body to nerve terminal (conduction). On reaching the nerve terminal, neurotransmitter (NT) is released into the synapse (1), where it quickly diffuses, some molecules reaching postsynaptic receptors [R_1] (2), with which they bind, leading to postreceptor events that promote neuronal depolarization and/or second-messenger-mediated cellular events (3). Other NT molecules interact with catabolic enzymes (4) and are destroyed, with presynaptic reuptake transporters (5), or with presynaptic receptors [R_2] (6). X = membrane-bound catabolic enzyme; 2nd mgr = second messenger (e.g., cyclic AMP).

the neurotransmitter) that innervate the heart. The heart rate (beats/minute) and force of contraction are stimulated by the firing of the sympathetic nerves that innervate it, effects that are mediated primarily *via* β adrenergic receptors on the heart (pacemaker and muscle cells). When most sympathetic nerves fire, therefore, they release NE, which interacts with postsynaptic (i.e., heart cell) β-receptors to increase the heart rate (chronotropic action) and force of contraction (inotropic action). Since β-receptors are adenylate cyclase-linked (32), the chronotropic and inotropic effects on the heart are cyclic AMP-mediated. Once the sympathetic nerve has released NE, the transmitter is removed from the synapse by a reuptake mechanism located on the nerve terminal. This mechanism ensures that the synaptic dwelling time of NE will be short, thus enabling the synapse to be quickly "reset" for the next firing. One other relevant feature of sympathetic nerve endings is that their surface contains a "presynaptic" α_2 receptor (also adenylate cyclase-linked), which can be occupied by NE, once released. Activation of this receptor exerts a negative feedback effect on nerve firing and NE release. These relationships are summarized in Figure 2.

The dietary paradigm involved the *ad lib* feeding of pregnant rats (from day 10 of gestation) a semipurified, casein-based diet containing either 16% (by weight) coconut (n-3 and n-6 fatty acid-deficient) or sunflower oil (n-3 fatty acid-deficient) (18). [A third group remained on the standard laboratory rat diet (4–5% fat).] The rats continued receiving their respective diets at parturition, and the pups were weaned onto the same diets. The newborn animals were studied at intervals from birth to young adulthood (postnatal days 60–65), a time during which the sympathetic innervation of the heart continues to develop and mature (33). Since the sunflower oil and coconut oil diets were of similar composition, containing equivalent amounts of fat, while the regular diet was of different composition and much lower fat content, the most meaningful comparisons are between the two experimental diets. Because the heart can be removed with the sympathetic nerve trunk and endings intact, all biochemical and physiological measurements could be made using just the heart, greatly simplifying experimental design and interpretation of results (since complex physiological feedback loops were no longer present).

Several interesting dietary effects were found to occur on both the sympathetic nerve terminals and the heart tissue itself. When the sympathetic endings to the heart were electrically stimulated *in vitro,* NE release was much lower from the hearts of rats that had ingested the coconut oil diet compared to those that had consumed the sunflower oil diet. This effect was noted from postnatal day 14 into adulthood. A reduction in NE release from nerve endings could follow from several effects: the nerve endings might contain less NE (and thus release less), the activity of the reuptake carrier for removing

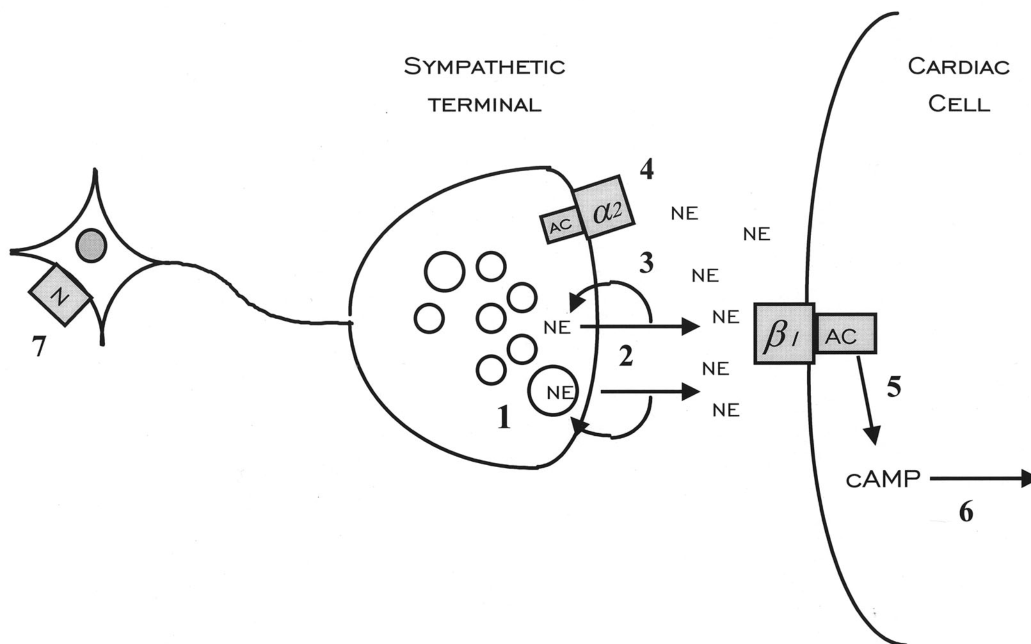

FIG. 2. Postganglionic norepinephrine synapse on heart tissue: modification by dietary polyunsaturated fatty acids (PUFA). Norepinephrine (NE) is released into the synapse, where it can interact with β (β_1) receptors on cardiac cells (leading to stimulation of cyclic AMP (cAMP) production, and thus enhanced cellular functions), with α_2 receptors on the presynaptic terminals [the receptor is also adenylate cyclase (AC) linked and causes a reduction in NE release when stimulated] or with presynaptic reuptake carriers. The postganglionic NE neuron is stimulated by preganglionic sympathetic neurons, which release acetylcholine onto nicotinic receptors (N). Changes in the NE neuron caused by dietary PUFA modifications include: (i) an increase in stored NE; an apparent reduction in NE release; (ii) (see text), an enhancement of NE reuptake, and (iii), an increase in the sensitivity of presynaptic NE α_2 receptors and increase in cAMP production (5), and (iv) an enhanced sensitivity of cardiac cells to cyclic AMP (6). Some data also indicate that PUFA can influence the sensitivity of nicotinic receptors (7), which might also modify sympathetic neurotransmission; not shown, changes in prostaglandin release by cardiac cells, which may also influence NE synaptic functions (see text).

NE from the synapse might be greatly increased, and/or the sensitivity of the inhibitory presynaptic α_2 receptors may have increased. A difference in NE content did not account for the difference in release. However, NE reuptake was greater from birth until adulthood in rats consuming the coconut oil diet vs. that in animals ingesting the sunflower oil diet (34). In addition, α_2 receptors were more sensitive to NE in coconut oil-fed than in sunflower oil-fed rats (35). Hence, the relatively lower rate of NE release from sympathetic nerve endings of coconut oil-fed rats vs. sunflower oil-fed animals could have been produced by an enhancement in the reuptake of released NE and a reduction in the release of NE secondary to the release-inhibiting action of α_2-receptor stimulation.

The heart's responsiveness to NE was also altered by diet. First, the number of β receptors on the heart was found to be greater at postnatal day 60 in rats ingesting the coconut oil diet than in those ingesting the sunflower oil diet (36). In addition, the inotropic and chronotropic efficacies of NE and isoproterenol (a β agonist) became greater as adulthood approached in the coconut oil-fed vs. the sunflower oil-fed rats (36,37). Finally, NE-stimulated cyclic AMP (cAMP) formation in heart membrane preparations was greater in coconut oil-fed than in sunflower oil-fed rats (36). Together, these results suggest that the heart's sensitivity to NE becomes greater in rats ingesting the coconut oil diet than in animals consuming the sunflower oil diet. The effects emerge by the time the animals reach postnatal days 60–65, and thus follow temporally the changes in NE release by sympathetic nerve endings. This sequence suggests a causal link, namely that in animals releasing less NE (coconut oil-fed rats), the heart compensates by becoming supersensitive to the reduced exposure to neurotransmitters. If correct, this assessment indicates that the primary effects of the dietary difference in fat source are on the nervous system (possibly the NE neuron), not the heart. However, Wince *et al.* (37) obtained data indicating that cAMP efficacy in promoting the heart's contractile force was directly affected by the diets: when hearts were exposed to dibutyryl cAMP (which crosses cell membranes) or agents that directly increase endogenous cAMP levels, the tension developed was greater in tissue from coconut oil diet-fed than sunflower oil-fed rats, indicating that the proportion of saturated to unsaturated fat in the diet directly influences cellular sensitivity to the second messenger. From these data one might suggest that the primary effect of the diets is to modulate cAMP action. If the coconut oil diet produces an enhanced cAMP response in the end-organ (the heart), and

the heart therefore pumps blood more efficiently, possibly feedback circuits that monitor and control blood pressure would adjust, in part by reducing sympathetic activity to the heart. Arguing against this possibility, however, is the view that to envision a reduction in sympathetic firing enhancing NE reuptake is difficult. Hence, the change in dietary fat may influence more than one biochemical point in the sympathetic neurons and cardiac cells. By way of summary, some of the possible sites of action for these dietary effects are indicated in Figure 2.

Only a few other studies have examined the heart's sensitivity to β-receptor stimulation. Hoffmann *et al.* (38) fed postweanling rats for 10 wk, diets either rich [12% (weight) sunflower oil, 2% lard] or poor (14% palm oil) in linoleic acid. While the strength of contraction of the heart muscle from the linoleic acid-adequate rats exceeded that from the linoleic acid-deficient rats, the inotropic effect of isoproterenol (a β agonist) was lower in linoleic acid-adequate vs. the linoleic acid-deficient heart muscle. This latter effect is thus similar to that described above. However, Rulka and Hamm (39) fed weanling rats for 4–5 wk diets containing 10% fat (weight), either as corn oil, butterfat, or a 1:9 corn oil/butterfat mix. They observed that the *in vivo* responsivity of the heart to a β agonist or antagonist was greater in the corn oil-fed than the butterfat-fed rats. This finding thus seemingly contrasts with the results above, although, as the authors point out, the diets in these studies had quite different fatty acid compositions. This latter point reveals a significant impediment to more rapid progress in this area, namely, the absence of a standardized dietary paradigm. In addition, the dietary treatment periods in all of the above studies only partly overlapped. Regardless, the important point should not be obscured that in all cases the difference in dietary fat produced a difference in the responsivity of a postsynaptic receptor for a neurotransmitter, as well as to specific receptor agonists and antagonists.

The heart represents only a single terminus for the sympathetic nervous system. Various other organs receive sympathetic fibers, including the blood vessels. Panek *et al.* (40) examined the impact of feeding weanling rats coconut oil- vs. sunflower oil-containing diets on NE release from sympathetic terminals to blood vessels, and on the vessels' responsivity to NE. Using the tail artery preparation, they observed that, like the heart, the sympathetic terminals of coconut oil-fed rats contain more NE than their counterparts in sunflower oil-fed rats, and release less NE on stimulation. When the sympathetic fibers to the tail arteries were stimulated electrically, the increments in pressure were smaller in the arteries from coconut oil-fed rats than in those from sunflower oil-fed rats, a finding consistent with diminished sympathetic NE release in the coconut oil-fed rats. However, unlike the heart, which developed enhanced sensitivity to NE under like conditions in coconut oil-fed rats, the tail artery blood vessels in these animals became less sensitive to NE (relative to that seen in the sunflower oil-fed rats). This difference appears not to have been resolved and suggests that the changes in the sensitivity of sympathetic end organs to NE that accompany alterations in dietary PUFA intake are not uniform.

Together, these studies of peripheral nerve function indicate that (i) the fatty acid composition of neuronal/myelin membranes differs markedly as a function of dietary PUFA content, (ii) nerve conductance does not appear to be influenced by differences in dietary PUFA intake, and (iii) the transmission properties of at least one class of peripheral nerve (NE-containing sympathetic neurons) are remarkably modified by changes in dietary PUFA intake.

Several points seem worthy of comment regarding these conclusions. First, too few data are available to conclude convincingly that nerve conduction is not modified by alterations in membrane PUFA profile. Further work is clearly warranted, since, for example, the activity of a key enzyme in the maintenance of the sodium and potassium gradients across neuronal membranes, Na^+K^+ATPase, has been shown by some to be altered by membrane PUFA profile (3,4,41). Second, the results described for NE (i.e., sympathetic) transmission in the heart, while indicating the possibility of quickly reaching significant biochemical detail in studies of dietary PUFA effects on neurotransmission, do not adequately investigate the range of possible mechanisms. It should be possible to use the heart preparation (or indeed, other sympathetic end-organ models) to go into greater detail in identifying dietary PUFA actions on NE neurotransmission. For example, the prostaglandins have notable effects on the sympathetic nervous system and on end-organ responsivity to NE: NE release by sympathetic nerve terminals can be inhibited by prostaglandins, which are released during nerve stimulation (by both nerve and end organ); the response of the heart and blood vessels to NE is also influenced by prostaglandins (42,43); and nicotinic cholinergic receptors that reside on and activate postganglionic sympathetic neurons may be influenced by local PUFA availability (44). Because prostaglandin release in the hearts of rats fed high- or low-linoleic acid diets differs substantially, both basally and in response to isoproterenol stimulation (38), possibly some (or all) of the NE effects on cardiac function discussed above relate ultimately to dietary PUFA-induced changes in the membrane content of relevant prostaglandin precursors and/or in their rate of conversion to prostaglandins. This issue has not been examined, although some data are available, using patch-clamping techniques, showing that PUFA (AA, DHA) directly modify voltage-activated sodium currents and membrane electrical excitability in cardiac myocytes (45,46). This technique might be productively applied to the study of cardiac excitability and responsivity to NE in several dietary PUFA paradigms.

Insights gleaned from studies of the peripheral nervous system should stimulate and guide studies on retinal, brain, and other peripheral neurons. But how might such studies proceed? As indicated above, if NE neurotransmission can be affected by dietary PUFA differences, there is no reason to think that other catecholamine neurons might not also be affected. The most immediate example would be retinal DA neurons, because of the known effects of dietary PUFA manipulations on retinal and visual function. A very interesting series of studies could be undertaken to determine, among

other things, if light-induced activation of retinal DA neurons (and tyrosine hydroxylase), the functional state of retinal DA receptors (and second-messenger responsiveness), and the modification of the electroretinogram by DA drugs (agonists, antagonists) are modified by dietary PUFA treatment. As another example, one could simply use the results obtained from the sympathetic nerve–heart studies discussed above as a guide to similar biochemical studies in brain (are NE receptors, reuptake transporters, and biosynthetic enzymes modified?). Interesting findings might also result from the application of a well-studied neurophysiological paradigm for measuring the sensitivity of primary motor neurons in brain to NE boutons that terminate on them [the electrical activity of neurons of the facial motor nucleus and their responsivity to glutamate are influenced by local NE terminals (47)]. Variations in dietary PUFA intake might be found to alter this relationship in a manner similar to that observed for the sympathetic NE neurons innervating the heart.

A related approach would be to study neurons that utilize noncatecholamine neurotransmitters, such as serotonin (5HT). If changes in dietary PUFA intake modify membrane functions in catecholamine neurons (transporters, receptors, biosynthetic enzymes), they might also have similar effects in other neurons. Exploratory studies to identify biochemical effects on brain 5HT neurons would be straightforward. If positive results were obtained, good models exist to examine functional consequences [e.g., 5HT neurons, like NE neurons, project to the facial motor nucleus and influence motor neuron function (47); they also project into the hypothalamus, and predictably modify the secretion of pituitary hormones such as prolactin, which is readily studied (48,49)].

In general, this focus of study begins with postulated membrane effects of dietary PUFA manipulations, attempts first to identify specific neurochemical consequences (i.e., changes in how the neuron "handles" NE or 5HT), and then, based on much literature linking CNS functions to such transmitters, searches for specific functional consequences. Such an approach may more quickly identify links between alterations in dietary PUFA intake and CNS function than an approach that begins with an effect on a global brain function (e.g., visual function), for which few neurochemical/neuropharmacological links are currently known, and attempts to identify underlying mechanisms (although the hopeful exception may be the retinal DA neurons). If so, the findings would at least establish the principle and ultimately aid in the search for underlying explanations for dietary PUFA-induced changes in very complex CNS functions, such as vision.

PUFA models. Several current ambiguities of experimental design no doubt impede neurochemists, neuropharmacologists, and neurophysiologists who might otherwise become interested in exploring for CNS effects of dietary PUFA. First and foremost is the diet. No commonly-agreed-upon dietary formulations exist that the nonnutritionist can easily identify for study. The neurophysiological, neurochemical, and neuropharmacological paradigms that can be brought to bear on this issue are often complicated, time- and labor-intensive, and are unlikely to be applied to this area of investigation if the "treatment" is not clear. Reference to a selection of nervous system-focused studies reveals diets of a wide range of fat contents (e.g., 1→20% by weight) and fat sources (e.g., sunflower oil, safflower oil, corn oil, lard, butter, red fish oil, "fish oil," coconut oil, hydrogenated coconut oil, soybean oil, rapeseed oil, menhaden oil), almost all of undescribed purity (7,11,17,18,29,31,38,39,50,51). The occurrence of such an extensive variety of fat sources makes it difficult (for the neuroscientist) to know which is (are) the accepted standard(s) in the field. The most important contemporary issues relevant to brain PUFA composition and function suggest that the specification of perhaps five standardized diets might eliminate this dietary confusion. Such diets might be (i) a linoleic acid/α-linolenic acid "control" diet, (ii) a linoleic acid-deficient diet, (iii) an α-linolenic acid-"deficient" diet, (iv) a "breast-milk" diet (i.e., one containing appropriate amounts of longer-chain PUFA, such as arachidonate, eicosapentaenoate, and docosahexaenoate), and (v) an "infant formula" diet (which lacks the longer chain PUFA) (1). In the absence of such recommendations, for example, a neuroscientist might simply refer to the work of Yao *et al.* (29) or Brenneman and Rutledge (18) (discussed above) and choose a corn oil or sunflower oil diet as a "control" diet. The results of studies using such a "control diet" might later be discounted by some investigators, since the control diet would not be accepted as a true control (though containing adequate amounts of linoleic acid, it would be deficient in α-linolenic acid). The better choice might be a soybean oil-based diet, containing both linoleic and α-linolenic acids. Such problems are avoided by the existence of an agreed-upon selection of PUFA diets.

As a second issue, it would be extremely useful to specify the membrane PUFA changes that should be expected with such diets and to encourage their quantitation. The current database suggests, for example, that linoleic acid-deficient diets should reduce the membrane levels of 20:4n-6 (arachidonate) (29), and diets with high ratios of linoleic acid to α-linolenic acid should diminish the levels of one or more long-chain n-3 PUFA (e.g., DHA) while raising n-6 PUFA levels (e.g., 22:5n-6) (8,31,52). By specifying expected membrane PUFA changes associated with the use of a particular dietary formulation, the investigator new to dietary PUFA research has a basis for validating his/her ability to set up the model, and others have a reference point for comparing results across laboratories and disciplines.

A third issue is the age(s) at which the animals should be studied. Many animal studies involve institution of the diets during gestation and examination of the animals at different postnatal timepoints through single or multiple generations of dietary exposure (8,18). Other studies focus on early postnatal development (34,35). Indeed, a good deal of interest currently revolves around the question of what fatty acids to feed a baby in order that its brain and retina undergo proper postnatal development (1). Breast milk contains certain PUFA that infant formulas do not (e.g., AA, DHA, eicosapentaenoic acid), and an important question is if these fatty acids should

be added to infant formulas (1,53,54). Still other studies institute diets at or after weaning and explore for membrane effects several weeks or months later (7,17,29,38,55–57); a variation examines the reversibility of dietary PUFA deficiency produced during gestation and early postnatal life by provision of a diet adequate in PUFA at or after weaning (31). While sound reasons exist for employing each of these temporal designs, good arguments also may exist for further refining the time periods during which dietary PUFA modifications are provided and their effects examined. For the neurochemist or neuropharmacologist, the basis for such refinement might be related to the selection of a particular neuron for study. For example, NE neurons first appear in rat brain around gestational day 12; the multiplication of this cell population ends by gestational day 16. Axonal proliferation then occurs, and major projections are present at birth. However, NE and tyrosine hydroxylase contents in almost all brain regions undergo their largest increases postnatally, with adult values being reached by postnatal day 40 (58,59). These facts suggest that a treatment provided before gestational day 16 might conceivably influence the population size of the NE neuronal pool, while a treatment instituted at and after day 16 might influence axonal extensions and neuronal connectivities of these neurons. A treatment provided after postnatal day 40 would not be expected to affect the number of NE neurons or their fundamental connections but might influence their functional properties *via* membrane remodeling. A similar case can be made for autonomic NE neurons (33). Developmental data are also available for other chemically specific neurons in brain (e.g., 5HT, DA); a similar argument could be made for each. Agreement on such time posts for treatment would minimize ambiguity in the interpretation and comparison of NE findings, such as when a study is somewhat vague as to the gestational age at which diets were instituted (days 13–18) (18), or when one study institutes the diets in "weanling rats" (presumably postnatal days 17–21) (39), and another almost 2 wk later (100 g rats) (38). The timing of treatment could be an important source of variability within and among such studies.

And fourth is the issue of animal model. The neurochemist/neuropharmacologist usually prefers the rat because of its size and cost and because it has proven itself to be a good model (more often than not) for humans. Indeed, most of the neuroscience literature is based on this animal. Hopefully, the rat can be used as the focus of exploration for dietary PUFA effects on the nervous system. If not, the preferred model will have to be characterized neurochemically and pharmacologically for its similarity to the more familiar rat model before results obtained with it will be widely accepted. As an example, we recently conducted studies of the influence of dietary cholesterol intake on the synthesis and turnover of 5HT in brain, to evaluate the hypothesis that lowering serum cholesterol enhances suicidal and violent thoughts in humans by reducing 5HT function in brain (60). The rat is the standard model for studying the neuropharmacology and "nutrition" of 5HT. But the rat is not an acceptable model for the study of biological effects of dietary cholesterol, since serum cholesterol levels are minimally responsive to diet in this species; the gerbil is the animal of choice (61). Very few studies are present in the literature dealing with 5HT function in gerbil brain. Hence, whatever the outcome of cholesterol–5HT studies in this species, their relevance would be unknown, since there is no basis for determining if results obtained in gerbils are neurochemically applicable to rats (and thus ultimately to humans). In conducting gerbil studies, therefore, we were compelled to include a set of basic pharmacological experiments to establish if 5HT neurons in gerbil brain respond in the expected manner to treatments known to influence these neurons in rats. This approach turned out to be particularly important: the pharmacological studies showed that gerbil and rat 5HT neurons behave alike, while the ultimate outcome of the cholesterol studies was that 5HT synthesis and turnover are unaffected in gerbil brain over an enormous range of serum cholesterol levels (and thus did not support the original hypothesis) (62).

Hopefully, with the recognition that changes in dietary PUFA intake can influence neuronal membranes and neurotransmission, as well as the definition of standard dietary paradigms, neuroscientists will become more involved in the exploration for the neurochemical effects of dietary PUFA that underlie functional effects in both the CNS and the peripheral nervous system.

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Polyunsaturated Fatty Acid Status and Neurodevelopment: A Summary and Critical Analysis of the Literature[1]

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ABSTRACT: The rationale for randomized trials designed to measure the effects of variable docosahexaenoic acid (DHA) status on neurodevelopment in human infants came from earlier studies of neurodevelopment in animals that were deficient in DHA owing to diets low in α-linolenic acid. The session on neurodevelopment looked at the results of these animal studies and discussed outcomes that appear to be analogous in human infants with variable DHA status. Presentations focused mainly on measures of development that may be attributed to more specific developmental domains (e.g., visual attention, recognition memory, problem-solving), some of which have been shown to be affected by long-chain polyunsaturated fatty acid (LCPUFA) status. This paper derives from discussions that took place during the session and reviews subsequent developments in this area. Although more difficult to interpret, global measures of infant development (e.g., the Bayley Scales of Infant Development, and Brunet-Lezine) can only suggest a relationship to specific developmental domains, but they have been applied in some randomized trials of LCPUFA and infant development. Those results are also summarized here.

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The session on polyunsaturated fatty acids (PUFA) and neurodevelopment/infant behavior began with short presentations by Neuringer, Jacobson, Willatts, and Morley. Neuringer focused on the effects of n-3 fatty acid deficiency on primate behavior, including effects on visual attention that appear to be analogous to those found in preterm infants with lower docosahexaenoic acid (DHA) status. Jacobson gave an overview of similar and of differing effects of toxic agents such as alcohol, cocaine, and polychlorinated biphenyls on visual attention of human infants. Willatts presented evidence that infant problem-solving is affected by n-3 fatty acid status. Morley demonstrated what normal variability is among infants on the Bayley Scales of Infant Development in relation to the number of infants that must be studied to determine if a variable influences global development. The paper that follows is based on the presentations and discussion in this session and summarizes work from other scientists on points crucial to the design of future studies of neurodevelopment.

Animal studies of n-3 deficiency, particularly those in nonhuman primates, have provided the rationale to study the functional effects of varying DHA status of human infants. The animal studies of development deliberately provided little α-linolenic acid, that is, they were designed to be n-3 fatty acid deficient. These n-3 fatty acid-deficient diets led to lower retinal and brain DHA, which could be interpreted as the cause of changes in behavior.

These studies were done with rhesus monkeys fed vegetable oils containing large amounts of linoleic acid and very small amounts of α-linolenic acid. Because the diets contained good amounts of n-6 fatty acids, the n-3 fatty acid-deficient animals grew normally and accumulated amounts of long-chain polyunsaturated fatty acid (LCPUFA) like n-3 fatty acid fed animals. Had these factors been affected by n-3 fatty acid deficiency, additional control groups would have been required. Instead, there was only a qualitative change in LCPUFA in retina and brain, with DHA replaced by docosapentaenoic acid (22:5n-6) synthesized from linoleic acid. Consequently, changes in behavior could be attributed to this change in composition. The underlying mechanism for changes in behavior is not known. Studies to learn the mechanism(s) by which variable amounts of neural LCPUFA influence behavior are currently being done in a number of laboratories using several approaches and are likely to become a topic for discussion at future meetings on PUFA and development.

In contrast to animal studies of n-3 fatty acid deficiency, the human studies of PUFA have asked if α-linolenic acid, at either low or high levels as present in standard feedings, provided optimal DHA for brain and retina of infants. These studies have sometimes varied α-linolenic acid in infant formula, but more frequently have included an experimental formula with some amount of DHA. The latter type of study has been used to test the hypothesis that DHA is a "conditionally essential" nutrient for rapidly growing infants, especially infants who, because of preterm birth, have been deprived of the normal DHA accumulation that occurs in the last intrauterine trimester.

One may conceptualize the difference between the animal and human infant studies of development as one of degree of

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Abbreviations: ARA, arachidonic acid; BPD, bronchopulmonary dysplasia; DHA, docosahexaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; MDI, Bayley Mental Developmental Index; SES, socioeconomic status.

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depletion of tissue DHA, especially neural DHA. The animal studies, which provided little n-3 fatty acids of any kind, are more likely to result in greater tissue DHA depletion than studies in humans, who are not deliberately fed n-3 fatty acid-deficient diets (estimates of the degree of depletion in brain have been made by some investigators and are included later in the paper). Consequently, studies in human infants addressed here and in the paper by Gibson and Makrides (1) on visual development have reported outcome measures analogous to those affected by reduced neural DHA accumulation in nonhuman primates.

These studies differ in background and design from a separate body of literature focused on the developmental differences between breast-fed and formula-fed infants (for example, Ref. 2). Studies of DHA supplementation in formula-fed infants are sometimes compared or confused with studies comparing breast milk and formula, because of the recognized differences in DHA status between these groups. This confusion is unfortunate, because effects of human milk feeding cannot be attributed to DHA alone but are likely to be related to a host of other nutritional or environmental factors. Comparisons of breast-fed and formula-fed infant development have focused almost exclusively on cognitive development (as distinct from other domains of development such as sensory, temperament, motor, or social). However, as discussed by Jacobson in this issue (3), recent studies that have controlled for all of the parental variables associated with the choice to breast-feed do not show a cognitive advantage of breast-feeding over formula-feeding using standardized tests of intelligence. Moreover, as will be pointed out in the following section, the animal literature suggests that even extreme reductions in neural DHA do not affect learning or memory directly, although they do affect other developmental domains that may indirectly influence cognitive function.

DHA DEFIENCY AND ANIMAL DEVELOPMENT

Caldwell and Churchill in 1966 (4) and Paoletti and Galli in 1971 (5) were the first to report effects of essential fatty acid deficiency (combined deficiency of both n-6 and n-3 fatty acids) on behavior, implying effects of low brain LCPUFA on neural function. These and other early animal studies found differences in performance on learning tasks that were interpreted as evidence of lower ability to learn. More recent studies of n-3 fatty acid-deficient rodents and primates (6–8) suggest that lower performance on some tasks of learning is secondary to effects on other behavioral domains (9,10). As Wainwright and Ward (9) and Neuringer and Reisbick (10) indicated, effects on other behavioral domains such as sensory, motivation/arousal, motor or social are not less interesting or important than primary effects of deficiency on learning. Although lower performance on learning tasks can occur secondary to a primary effect on some domain other than cognition, it is important to identify the primary domain affected so that attention can be focused on outcomes more likely to demonstrate true effects in studies involving observation or intervention.

Sometimes an unambiguous assignment of function to a specific neural domain (e.g., sensory, cognitive/learning, motivational, motor or social) is not possible in studies of behavior. Wainwright and Ward (9) highlighted the importance for behavioral research of construct validity, i.e., the correspondence between what is measured and the ultimate interpretation of the findings. This is not to imply that a positive or negative meaning may not be assigned to a given effect on behavior, only that the neural domain responsible for the effect on behavior may not be clear. By analogy, Wainwright and Ward (9) suggested that there is generally little concern about construct validity for biochemical measures, such as tissue DHA.

In animal studies, those that involve feeding deficient diets to the mother or through several generations generate the highest degree of concern about attributing changes in behavior to a specific neural domain. This is because the diet may affect the behavior of the mother as well as the newborn, and both effects have the potential to influence the quality of maternal care and the ultimate behavior of the offspring. These and other excellent points are raised by Wainwright and Ward (9) in their chapter "Early Nutrition and Behavior: A Conceptual Framework for Critical Analysis of Research." Because n-3 fatty acid-deficient animals have normal growth and physical health, it may not be apparent that maternal behavior could be a mediating variable. In contrast, n-6 fatty acid deficiency has obvious effects on growth and physical health, mediating variables that are routinely monitored in nutrition research and for which the need for control is understood.

Studies in nonhuman primates have been particularly critical for the human studies of LCPUFA and development. Neural DHA accumulation is higher and neural function of newborn monkey infants is more mature than in newborn human infants. For this reason, rhesus monkeys were exposed to n-3 fatty acid-deficient diets both *in utero* and after birth, effectively producing different degrees of DHA depletion in retina and brain (11). In the course of these studies, a number of behavioral effects of n-3 fatty acid deficiency in rhesus monkeys were identified.

Monkeys exposed to n-3 fatty acid-deficient diets *in utero* and postnatally had lower n-3 LCPUFA accumulation in retina and brain (11,12) and abnormal retinal function (11). Smaller a-wave amplitudes seen with n-3 fatty acid deficiency were corrected by 2 yr of age, but differences from the control group in implicit time and relative refractory periods actually became more pronounced with age (13). Moreover, these differences were not corrected even when the retina and cerebral cortex DHA were returned to normal at 10 mon of age or later by feeding n-3 LCPUFA (14). Monkeys that were n-3 fatty acid-deficient also had lower visual acuity during the period after birth when grating acuity increases most rapidly (12). Infant monkeys fed diets deficient in α-linolenic acid after birth also developed abnormal electroretinograms compared to monkeys with a high intake of α-linolenic acid (15).

Deficiency of n-3 fatty acids in monkeys also led to longer look duration in a test of visual attention relative to monkeys with higher brain DHA accumulation (16). This could have

been due to lower visual function, although look duration was not related to concurrent visual acuity (17). Look duration decreases with age, and this maturational effect has been attributed to faster processing speed (a cognitive effect) (18,19). However, there are alternative explanations for relatively shorter look duration such as increased ability to disengage attention (20) and lower reactivity (8). Any of these explanations might apply to animals developing with higher neural DHA; and each of these possible explanations suggests a positive effect of higher neural DHA on development. Whatever the neural domain affected, the difference in attention has the potential to provide insights into the effects of n-3 LCPUFA on cognitive development.

In addition to the effects of n-3 fatty acid deficiency already noted, deficient monkeys had polydipsia and polyuria but with normal renal function, growth, and physical health (21). Reisbick *et al.* (8,22) also described a higher frequency of stereotyped behavior, locomotor activity, and general behavioral reactivity in n-3 fatty acid-deficient monkeys than in a control group fed α-linolenic acid.

Not all of the neural domains investigated in studies of n-3 fatty acid-deficient primates have been affected, however. For example, Reisbick *et al.* (8) measured performance on several kinds of learning tasks in monkey infants and adults. Based on these studies, they concluded that even severe, long-term n-3 fatty acid deficiency did not cause general deficits in learning or memory.

Wainwright and Ward (9) put forth the theory that vulnerability to insults such as poor health and environmental deprivation could interact with poor n-3 LCPUFA status to create adverse effects on behavior even in the absence of a main effect of fatty acid status on behavior. In their studies of stereotyped behavior, activity and reactivity, Reisbick *et al.* (8,22) compared laboratory-reared, randomized groups to a nonrandomized group of "standard" monkeys that were breast-fed as infants, fed food containing DHA after weaning, and raised in a social group until at least 2 yr of age. At 4 yr of age, standard monkeys had lower frequencies of whole body activity, and stereotyped behavior and lower reactivity to a variety of social and nonsocial stimuli than the controls fed α-linolenic acid, who in turn had lower scores (a positive finding) than n-3 fatty acid-deficient monkeys.

One possible explanation for the lower scores on these measures by standard monkeys was that they accumulated more neuronal DHA than monkeys fed α-linolenic acid in the early months of life, because they were nursed by the mother and received a postweaning diet with DHA. At 2 yr of age (the only age data available other than at birth), however, DHA in the retina and frontal cortex of standard and soybean oil-fed monkeys did not differ (8). Alternatively, Reisbick *et al.* (22) suggested that partial social deprivation (social experience with agemates but not with mothers) heightened the behavioral response to n-3 fatty acid deficiency.

Given the large amount of n-3 and n-6 LCPUFA in neural membranes, and the relatively few behavioral outcomes that have been studied, it seems likely that many effects of low brain n-3 LCPUFA accumulation on neural function remain to be discovered.

DHA INTAKE AND DEVELOPMENT OF HUMAN INFANTS

Human infants cannot be fed diets that are deficient in any required nutrient, but studies to optimize the intake of required nutrients are permissible, indeed desirable, if a plausible case for a meaningful difference in function can be made. Human infants fed formula rather than human milk have lower red blood cell and plasma lipid DHA (23–26). The assumption was made that this might represent lower accumulation of DHA in the retina and/or brain with possible effects on infant behavior analogous to those observed in n-3 fatty acid-deficient animals.

Although declines were larger in infants fed a low-linolenic acid formula (24), they still occurred when the control groups were fed formulas containing soybean oil that contributed very large amounts of α-linolenic acid and a good ratio (~7:1) of linoleic acid to α-linolenic acid (27,28).

Recent analyses of DHA in infant brains (29,30) confirmed that infants fed DHA from human milk accumulated more DHA than infants fed formula. Infant formulas with greater than 1% of energy from α-linolenic but no DHA led to 20 to 25% less DHA in brain phospholipids than human milk. An even greater reduction of 35 to 45% compared to human milk was calculated for term infants fed formulas low in α-linolenic acid without DHA (29). One preterm infant fed α-linolenic acid-containing formula had a 60% reduction in brain phospholipid DHA compared to term, human milk-fed infants (29). These reductions can be compared to n-3-fatty acid-deficient monkeys that have approximately 75% lower DHA than n-3-fatty acid-sufficient monkeys at birth (11). Because the degree of reduction in brain DHA increased with age in both human infants and monkeys, the relative reduction in brain DHA among preterm infants may be comparable in some cases to that of monkeys deprived of n-3 fatty acids *in utero* (31).

All of the clinical trials of DHA supplementation conducted in the United States have compared a group of infants fed formula with n-3 LCPUFA to a group of infants fed at least 1% of energy from α-linolenic acid, an amount recently recommended for preterm infants (32). This was true of the first published trials in preterm infants (28,33,34) as well as three recent studies in term infants (35–37), although one of the earlier preterm studies (33) also included a group that was fed a formula with corn oil (a fat low in α-linolenic acid). Because the control groups in all but this one case were fed α-linolenic acid, observations of higher neural functions in the groups fed experimental formulas with DHA compared to formulas without DHA are evidence that DHA is a conditionally essential nutrient for preterm infants (38).

Some randomized studies of term infants performed outside the United States compared formulas with n-3 LCPUFA (with or without n-6 LCPUFA) to formulas containing less

than 1% of energy (~2.0% of total fatty acids) from α-linolenic acid. Importantly, even when formulas with DHA resulted in higher neural function, the studies cannot be used to conclude that DHA *per se* is a conditionally essential nutrient when the control formula contained low α-linolenic acid. However, such studies have contributed to evidence that there are functional effects of low neural DHA accumulation.

To be fair to investigators who have compared commercially available low α-linolenic acid formulas to experimental formulas with DHA, one should note that investigators (33,39–41) have only recently demonstrated effects of low dietary α-linolenic acid on human infant development. Because functional information in human infants prior to these reports was absent, recommendations for α-linolenic acid in artificial infant feeds were necessarily provisional. Frequently, the composition of formulas was based on human milk, in which α-linolenic acid is typically much less than 1% energy (42,43), and ignored the fact that human milk, unlike formula, provided DHA and arachidonic acid (ARA).

Effects of DHA on sensory function. Of the13 reported randomized trials that were designed to study the effects of variable DHA status on neurodevelopment, 11 trials (28,33–37, 40,41,44–46) measured sensory development, in particular, visual or retinal development. At the meeting, studies of visual and retinal development were presented in a different session from studies of other neurodevelopmental domains, despite the fact that some studies measured both types of outcomes. Gibson and Makrides (1) summarized the session on visual development. This paper will not address visual development except to note when effects on sensory development could be an explanation for differences in behavior related to lower n-3 LCPUFA status.

Are there effects of n-3 LCPUFA status on cognition/learning ability? Other than sensory effects, the neurodevelopmental outcomes measured in human infants with variable n-3 LCPUFA status have been more limited than those employed in animal studies. Despite reports of altered performance on tests often used to assess these functions, neither animal nor human studies have provided convincing evidence that the primary effect of n-3 LCPUFA status is on learning ability or memory. Performance differences are more likely to be attributable to sensory, motivational or other behavioral differences rather than to changes in cognitive ability. For example, past studies of malnutrition and development found an apparent poorer ability to learn. However, most of these effects were secondary to the effects of malnutrition on infant responsiveness and temperament, which influenced the caretaker's interactions with the infant (for example, see Ref. 47). There could be an analogy between these studies and those that varied LCPUFA status during development.

Several studies have used the Fagan Infantest (FTII) to assess visual recognition memory in infants with different DHA status (46,48,49). In this test the infant is presented with a series of photographs of human faces. For most of the test items, the infant is allowed to gaze at a face [or two identical copies of the same face] for a predetermined number of seconds of cumulative looking time, and then is shown the now familiar face paired with a novel one. Consistent preference for the novel pictures is an index of memory for the pictures that are familiar and, therefore, that attract less attention. Only one study found an effect on recognition memory (46). In that preliminary report, term infants fed human milk and LCPUFA-containing formula had normal visual recognition memory on the test, whereas infants fed a commercially available formula of undefined composition had abnormally low recognition memory. The lower scores in that group suggested that the control diet could have interacted with some other variable to influence outcome.

In addition to its designed use to assess visual recognition memory, the FTII has been used experimentally to measure the average duration of individual looks during both the familiarization and paired comparison phases of the test. An analogous test was developed for monkey infants. Both monkey (16) and human infants (48,49) with relatively higher DHA status had shorter look duration without effects on visual recognition memory. Using a classical habituation paradigm (exposure to a single visual stimulus until attention wanes), Forsyth and Willatts (50) also found shorter look duration in 9-mon-old term infants with higher DHA and ARA status. However, the reduction in look duration with DHA and ARA occurred only in a subgroup with lower birthweight, who may have accumulated less DHA *in utero*. The control formula contained only 0.35% energy from α-linolenic acid (51), and the effect might not have been observed had the control contained more α-linolenic acid.

Longer look duration may be used to infer an undesirable effect of lower n-3 LCPUFA status on brain function. Longer look duration in infancy correlates moderately well with poorer performance on later tests of cognitive ability (52–54). The usual interpretation has been that longer look duration represents slower information processing speed (a cognitive function) (18,19). More recent studies have led to the alternative suggestion that longer look duration could represent an inability to disengage or shift attention from the stimulus (20) or higher reactivity to visual stimuli (8,10). Differences in sensory function are also a possible explanation for differences in look duration in the animal and human studies, because groups with higher DHA status were shown to have higher visual acuity either concurrently or earlier in development. Neither concurrent visual acuity in monkey infants (17) nor earlier visual acuity in preterm infants (55) was correlated with look duration, however, making this explanation somewhat less likely.

More advanced infant problem-solving ability and language development have also been used as indices of higher cognitive function, and some early tests of both have been related to higher performance on tests of development in childhood. Willatts *et al.* (51) reported higher means-end problem-solving scores in 10-mon-old term infants fed a formula with DHA and ARA. Because the control formula provided only 0.35% energy from α-linolenic acid, these data, in conjunction with studies of brain composition (29,30), bolster the

case for positive effects of higher brain DHA accumulation on infant development. Again, however, they cannot be used to conclude that DHA is a conditionally essential nutrient for term infants.

Janowsky *et al.* (56) reported lower language scores at 14 mon in term infants fed formula with DHA throughout the first year of life in comparison to infants fed human milk, formula without n-3 or n-6 LCPUFA, or formula with both DHA and ARA. However, at 3.25 yr of age, cognitive and language scores of these same infants were unaffected by their early diet (57). It is not entirely clear how the early language data from this study should be interpreted. On one hand, the data could be used to suggest that feeding only LCPUFA of the n-3 fatty acid family created an imbalance of n-3 and n-6 LCPUFA. On the other hand, an inverse relationship has been reported between productive vocabulary at 16 mon of age and nonverbal memory performance, with the difference emerging over longer delays between 1 and 12 mon (58).

Effects of n-3 LCPUFA on global tests of mental and physical development. Several reports of higher global development among infants fed formulas with n-3 (or n-3 and n-6) LCPUFA have appeared, although the numbers of infants per group have been relatively small. Two scales of global development have been employed, the Brunet-Lezine Test (59) and the Bayley Scales of Infant Development (60). Both have elements of the Gesell Developmental Schedules (61), the first system developed to assess behavioral development in infants. Global tests of development were designed to assess current abilities, not to predict long-term outcomes, which are susceptible to many intervening variables. Some studies have shown modest relationships between infant performance on global tests and performance on tests later in childhood, but the relationships generally are weaker than more specific measures such as look duration and recognition memory in infancy.

Agostoni *et al.* (62) compared psychomotor development of term infants fed a formula with 0.3% DHA and 0.44% ARA to those fed formula containing 0.35% energy from α-linolenic acid. At 4 mon, infants fed LCPUFA had higher psychomotor development scores. Effects of diet were not found when the groups were compared again at 12 and 24 mon of age, although blood LCPUFA at those ages did correlate with development (63). Damli *et al.* (44) compared preterm infants fed formulas with LCPUFA (0.2% DHA and 0.3% ARA) to a formula without LCPUFA, but with an unspecified amount of α-linolenic acid. They found higher performance scores on the 6-mon Bayley Mental Developmental Index in the group of infants who were fed LCPUFA-supplemented formula. It was not clear if infants were assessed after 6 mon of age, and, therefore, if these effects persisted. Janowsky *et al.* (56) also measured Bayley Scales of Infant Development scores of term infants at 12 mon and found no effect of supplementing formula with DHA on performance at that age.

Preterm infants fed formula with DHA compared to formula with at least 1% energy from α-linolenic acid but no DHA had higher scores on the 12-mon Bayley Mental Developmental Index (MDI) in one of two randomized trials conducted in the same center (64). Lucas (65) pointed out that a higher mean developmental score in one trial could represent a Type I error, a possibility that cannot be ruled out. However, differences in design between the trials might also have been influential. In particular, both control and experimental groups in the study that resulted in higher 12-mon Bayley MDI scores with DHA supplementation received formulas with higher concentrations of most nutrients (designed for preterm infants) until they were 2 mon corrected age (28). In contrast, infants in the study that found no effect of dietary DHA on the Bayley MDI were fed a lower nutrient (term) formula beginning approximately 1 mon before expected term delivery (34).

The developmental scores for the control groups in both trials were similar despite the differences in nutrient densities of the formulas. As well, the scores of both groups were similar to the historical scores for preterm infants in the same center. This suggested that the higher Bayley MDI in DHA-supplemented infants in the second trial could have been the result of an interaction between higher nutritional status and higher DHA status (38). Marginal nutritional status may be an unreported mediating variable in many developmental studies, especially those involving preterm infants, who have been reported to have low indices of many nutrients that can continue for months after discharge from the hospital. Unfortunately, published articles rarely provide information about the nutrient content of the diet fed in studies of infant development, much less provide measurements of nutritional status.

Bronchopulmonary dysplasia (BPD), characterized by periods of hyperoxemia and hypoxemia, may adversely influence development of the central nervous system. The diet groups in the second randomized trial, in which n-3 LCPUFA led to higher scores on the Bayley MDI, were subsequently divided into infants with and without BPD. The already higher mean score in the experimental group increased even more and was equivalent to breast-fed term infants from the same study population when infants without BPD were excluded. On the other hand, DHA-supplemented infants with BPD and infants with and without BPD who consumed the control formula had scores that were similar to each other and lower than DHA-supplemented infants without BPD (66). These results suggested that something about the condition of BPD prevented infants from experiencing the benefit of n-3 LCPUFA—on this test of global mental development—in the presence of higher nutrient intakes. The infants in these studies were not followed after 12 mon of age, so whether any effects of diet on behavior persisted is unknown.

DISCUSSION

As reviewed here, some but not all randomized trials have found effects of an experimental formula with DHA on some aspects of infant behavior. The tests employed in most of these studies have been used to infer effects on intellectual development. However, studies in n-3 fatty acid-deficient monkeys

suggest that lower brain accumulation of DHA may influence neural domains such as sensation, motivation or temperament, but not cognition (8). The most consistent effect identified to date in human and animal studies has been in one specific measure, that of look duration in tests of visual attention.

Although data on brain composition from infants fed a variety of infant diets are very limited, those that are available support the premise that variations in n-3 fatty acid intake (especially dietary DHA) affect DHA accumulation in brain (29,30). Preterm infants appear to accumulate less DHA relative to term infants, and infants fed little α-linolenic acid accumulate less brain DHA relative to infants fed at least 1% of energy from α-linolenic acid (29,30).

There are now a few examples in which effects of higher DHA status on early performance in a specific neural domain are not found later; e.g., early effects on visual acuity may not be found later in infancy (1). Although such results could be taken as evidence of functional recovery with time, it cannot be accepted that the effects of higher early DHA status are transient without further testing. For most tests of infant development, their sensitivity to developmental influences is highly age dependent. The dilemmas are (i) that there are not straightforward tests to sample the same or analogous abilities continuously from infancy through childhood, and (ii) that effects on one function at an early age may affect the development of other related functions at later ages. Before it can be concluded that a neural domain does not show long-term functional effects, it will be necessary to determine test(s) that sample that domain at the later age and show that it is unaffected by early DHA status. For the most part, there is an absence of evidence (studies have not been done) rather than evidence of absence of an effect on later development (studies that show transient effects on a specific domain of neural development).

Factors other than early birth and n-3 fatty acid intake may also influence the accumulation of DHA in developing brain. Some variables that have been suggested are intrauterine growth retardation (67), maternal alcohol use (68,69), maternal tobacco use (68,69), zinc status (70), *trans* fatty acid intake (71–73), and preeclampsia/ hypertension (74). As yet, no study has controlled for all of these possible variables, and differences in their occurrence among populations could be responsible for some variability in clinical studies that have been reported.

In addition to these variables, Wainwright and Ward (9) have suggested effects of socioeconomic status (SES) and/or health that could interact with DHA status to influence behavior. For example, poor DHA status may have little or no effect on development of healthy term babies or babies going to high SES homes but contribute to developmental risk in sick preterm or low-SES infants (9). The degree of formal education received by low-SES parents affected look duration in the same way and to the same degree as DHA-supplemented formula (55), perhaps an example of an interaction between SES and PUFA. Had all parents in the study been highly educated, DHA-supplemented formula might have had little additional effect on look duration.

In summary, only a limited number of behavioral studies in animals and humans address the question of neural DHA accumulation and developmental measures other than vision. It is encouraging that the human studies found several effects of n-3 LCPUFA status that appear analogous to those found in nonhuman primates. Such parallels add validity to the idea that lower neural DHA is responsible for the differences in behavior found in human infants fed diets with compared to without DHA. However, the optimal concentration of neural DHA and the optimal ratio of n-3 and n-6 LCPUFA in neural membranes are still not known. Moreover, they are not likely to be discovered until basic structure/function studies in animals identify the mechanism(s) by which LCPUFA act on the developing central nervous system and clinical studies more clearly identify variables that influence neural DHA accumulation in specific populations.

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Polyunsaturated Fatty Acids and Infant Visual Development: A Critical Appraisal of Randomized Clinical Trials

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ABSTRACT: At the Consensus and Controversies Conference held in Barcelona in November 1996, one of the sessions focused on an evaluation of the effects of dietary polyunsaturated fatty acids (PUFA) on infant visual development. The intervention trials in preterm and term infants were reviewed and discussed in detail. Results of these trials, particularly those in term infants, were inconsistent; much discussion occurred concerning the causes of these diverse results. We attempt to reflect, rather than report exactly, the discussion relating to these issues and address the clinical trials according to recently published guidelines for conduct and reporting of randomized clinical trials (RCT). Compared with these recent guidelines, the published papers of RCT involving PUFA and visual function are often incomplete, making it difficult to assess if we can have a high degree of confidence in the reported effects (or lack of effects). Despite this, valuable data relating to the effect of diet on the visual development of infants were obtained. Our evaluation of the trials to date suggests that the definitive answer to the degree to which dietary long-chain PUFA is likely to influence visual development may only be resolved with impeccably conducted RCT.

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The AOCS conference entitled "PUFA in Infant Nutrition: Consensus and Controversies" held in Barcelona in 1996 included a session entitled PUFA and Infant Visual Function Development. The purpose of this session was to review the evidence relating to a role for n-3 polyunsaturated fatty acids (PUFA) in infant visual development. While it is well established that n-3 PUFA are important and even essential for visual development (1,2), the debate is now focused on the possibility that an adequate level of the precursor PUFA, α-linolenic acid (18:3n-3, ALA) is sufficient or the possibility that docosahexaenoic acid (22:6n-3) is also required in the infant diet. Speakers included Eileen Birch, who reviewed the visual maturation process; Maria Makrides, who reported on clinical trials involving acuity as an outcome measure; Martha Neuringer, who reviewed electroretinogram studies; and Bob Gibson, who made general comments about the use of randomized clinical trials (RCT) in nutrition (Tables 1 and 2). Discussion was extensive, facilitated by many questions from the floor. Most of the vocal participants were eager for an explanation of the fact that some trials report effects of n-3 long-chain LCPUFA on visual development while others do not.

In the field of PUFA research, we have become used to conducting and reporting relatively small, carefully designed studies that often have specific biochemical outcomes. Only recently have studies been reported that were designed to determine functional outcomes, particularly in the area of visual development. These newer outcome measures bring a new level of complexity to study design and conduct because the differences expected are often small and may be influenced by a range of factors other than diet, and special testing skills and equipment are required. Given these facts, not surprisingly, variation appears in the reported visual responses of infants to changes in dietary LCPUFA. In other words, the fact that some investigators report an improvement in acuity while others conducting a similar trial can report no effect may have a lot more to do with the complexity of the development of the visual process in infants than any shortcomings in the design and conduct of the study. Clinical trials with infants, particularly preterm infants, are extremely difficult to conduct. Recognition of this fact is pivotal to our understanding of the field. The following discussion is designed to help the general reader understand some of the complex issues relating to the conduct of clinical trials with particular reference to issues relating to detecting effects of dietary n-3 PUFA on visual development in infancy.

Although many reports exist of differences between breast- and formula-fed infants, the only way to test if an ingredient of breast milk causes a reported difference is to conduct a RCT. In the case of LCPUFA, a RCT cannot be conducted in breast-fed infants with a zero LCPUFA treatment since LCPUFA are always present in breast milk. Thus, most RCT reported to date were conducted in formula-fed infants. However, testing the effect of a range of LCPUFA doses in breast-fed infants by supplementation of breast-feeding mothers is possible (3,4).

Rationale for studies. The underlying hypothesis of all studies involving n-3 PUFA and neural/developmental out-

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Abbreviations: ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ERG, electroretinogram; LA, linoleic acid; LC PUFA, (long-chain) polyunsaturated fatty acids; RCT, randomized clinical trial; VEP, visual-evoked potential.

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TABLE 1
Visual Outcomes of Randomized Clinical Trials (published in full) of PUFA Supplementation in Preterm Infants

Reference	Diet	Test	Age	Results
5,18,30	HM (supplemented with	Rod ERG	36 wk PCA	HM and FO > LA/ALA 24:0.5
	FO formula)		57 wk PCA	No difference with diet
	LA/ALA 24:0.5	Cone ERG	36, 57 wk PCA	No difference with diet
	LA/ALA 21:2.7	Steady-state VEP	36, 57 wk PCA	HM and FO > LA/ALA 24:0.5
	LA/ALA 20:1.4 + FO	Acuity cards	57 wk PCA	HM and FO > LA/ALA 24:0.5
6	SF, LA/ALA 33:4.8	Acuity cards	48, 57 wk PCA	FO > SF
	+ FO		68, 79, 93 wk PCA	no difference with diet
7	SF, LA/ALA 21.2:2.4	Acuity cards	2 mon CA	TO > SF, if no BPD
	+ TO		4, 6, 9, 12 mon CA	No difference with diet
8	HM	ERG latencies	52 wk PCA	No difference with diet
	SF, LA/ALA 18:0.3	ERG amplitudes	52 wk PCA	No difference with diet
	+ egg PL	Flash VEP latency	52 wk PCA	N4: HM and +eggPL < SF

Abbreviations: PUFA, polyunsaturated fatty acid; HM, human milk; SF, standard formula; LA, linoleic acid; ALA, α-linolenic acid; PL, phospholipid; FO, fish oil; TO, tuna oil; VEP, visual-evoked potential; ERG, electroretinogram; PCA, postconceptional age; CA, corrected age; BPD, bronchopulmonary dysplasia.

comes is that the demonstrated effects on development of visual acuity may reflect an underlying, more widespread difference in neural maturation that might affect other (perhaps many) aspects of brain function. This hypothesis, although often stated, has not been adequately tested by measuring a range of developmental outcomes. Visual acuity is the only functional outcome that has received repeated attention in trials of LCPUFA supplementation, largely because earlier work in monkeys demonstrated an effect of dietary ALA and because it is the only visual ability for which there are standardized, validated, and readily available tests for infants. This does not mean that other visual, nonvisual sensory, cognitive, or behavioral outcomes might be equally or more sensitive to effects of early LCPUFA intake.

Treatments. The RCT of n-3 PUFA and vision reported thus far vary in their dietary treatments (5–16). Differences are present both in the level and type of PUFA supplementation and in the background or placebo formulas (17). Only two trials have formally addressed the issue of 18 carbon PUFA levels and examined the effect of linoleic acid (18:2n-6) to ALA ratios on visual performance (13,18). In terms of n-3 LCPUFA, the source used varied and has included high-eico-sapentaenoic acid (20:5n-3) fish oil, low-eicosapentaenoic acid fish oil, egg phospholipids, and unicellular oils (17). Some recent trials included sources of arachidonic acid (20:4n-6) as well as n-3 LCPUFA. The spread of treatment fatty acids and doses encouraged workers to speculate about dose-dependent effects. For example, studies using relatively

TABLE 2
Visual Outcomes of Randomized Clinical Trials (published in full) of PUFA Supplementation in Term Infants[a]

Reference	Diet	Test	Age	Results
10	HM	Transient VEP	16, 30 wk	HM and FO, EPO > SF
(*n* = 55)	SF, LA/ALA 17:1.5			
	+ FO, EPO			
11	HM ≥ 3 mon	Acuity cards	2 mon	HM and +egg PL > SF
(*n* = 58)	SF, LA/ALA 22:2		4, 6, 9, 12 mon	No difference with diet
	+ egg PL			
31	HM ≥ 3 mon	Sweep VEP	2, 4, 6, 9, 12 mon	No difference with diet
(*n* = 120–200)	SF, LA/ALA 22:2	Acuity cards	2, 4, 6, 9, 12 mon	No difference with diet
	+ egg PL			
	+ TO			
13	HM	VEP latency	4 mon	No difference with diet
(*n* = 63)	LA/ALA			
	18:0.4			
	17:1			
	17:1.7			
	16:3.2			
12	HM	Sweep VEP	4 mon	HM different to formula
(*n* = 39)	SF, LA/ALA 12.7:1.2			No difference between
	+ FO			formula groups
	+ FO, BO			
16	HM	Sweep VEP	6,17,52 wk	HM, +DHA, +DHA + AA > SF
(*n* = 79)	SF, LA/ALA 15:1.5		26 wk	No difference with diet
	+DHA single cell oil	Acuity cards	6,17,26,52 wk	No difference with diet
	+DHA + AA single cell oil			

[a]BO, borage oil; see Table 1 for other abbreviations.

TABLE 3
Summary of Full-Published Reports of Randomized Clinical Trials Involving PUFA

Study	Method of allocation	Placebo	Blinded outcome assessment	Losses to follow-up	Power calculation	Notes
Preterm infants						
5,18,30	Random, method not stated	Yes	Not reported	40%	Not reported	?Coinciding of blood sample and visual assessment
6	Random, method not stated	Yes	Partial	28%	Yes	13% (10/79) infants were replaced post-randomization
7	Random, method not stated	Yes	Yes	46%	Yes	Infants were replaced (more controls than supplemented) postrandomization
8	Random, method not stated	Yes	Not reported	Not clear	Not reported	Inappropriate visual assessment
Term infants						
10	Random, method not stated	Yes	Yes	11%	Yes	Uneven randomized group due to unavailability of test formula
11	Random, method not stated	Yes	Yes	38%	Yes	
31	Three-site trial with separate randomization at each center method of allocation not stated	Yes	Yes	28%	Not reported	Each of the three sites had different assessment protocols.
13	Random and masked assignment	Yes	Not clear	29%	Yes	Inappropriate visual assessment
12	Random and masked assignment	Yes	Yes	2%	Yes	Formula fed infants were initially breast-fed. Formula feeding began ≈25 day postpartum
16	Random and masked assignment	Yes	Yes	24%	Yes	

high levels of n-3 LCPUFA supplementation (>0.5% total fatty acids) have more often found beneficial effects than those providing lower levels. In addition, Heird *et al.* (19) proposed that visual effects of dietary supplementation with n-3 LCPUFA were seen only in studies where the placebo diet contained less than 2% ALA. However, this hypothesis is not consistent with the current literature. Presently, two trials exist with <2% ALA in the placebo diet which demonstrate no effect of LCPUFA treatment (12,15) and four trials with >2% ALA in the placebo formula which demonstrate beneficial effects of n-3 LCPUFA supplementation on the measures of visual function (6,7,11,16). The issue of dietary treatment was reviewed in detail elsewhere (17,19).

Another issue related to the source of LCPUFA is the type of lipid (triglycerides or phospholipids) in which the LCPUFA are esterified. The exact source of dietary LCPUFA (triglycerides, phospholipids) may be irrelevant to the recipient infant provided that docosahexaenoic acid is present, since there is much reconstruction of dietary lipids postdigestion. Because the varied treatments used in many of the studies generally result in similar LCPUFA status in the infant, we believe that the source of LCPUFA may not be a cause of the disparate results. Other issues relating particularly to the design and conduct of RCT as well as the small size of the anticipated differences in visual development may be more important.

Methodology. By far the most common measure used to assess visual function is acuity, assessed either by electrophysiological or behavioral methods. The specific methods for visual acuity and retinal function assessment were extensively documented and reviewed elsewhere (20). Most trials used standardized and widely accepted visual assessment methods to assess the effect of n-3 PUFA on visual outcome. However, two trials used inappropriate methods (8,13) (Table 3). Faldella *et al.* (8) used an electroretinogram method to assess retinal function that did not involve the pupil being dilated or the use of a contact lens electrode, two test conditions considered necessary by the international standard for clinical visual electrophysiology (20). In a similar vein, Jensen *et al.* (13) measured visual-evoked potential in response to a single high-contrast checkerboard pattern (30-min arc), which provides information about waveform amplitude and latency but cannot be used to evaluate acuity. Therefore, lack of effect in these trials cannot be interpreted as treatment having no influence on visual development, as the specific outcomes sensitive to LCPUFA were not assessed.

The size of the effect. If we assume, for the sake of argument, that all the reported trials were unaffected by any limitations in study design and conduct and that all papers report true effects, effects on visual development (where seen) were generally modest and close to the limit of clinical significance. Because the anticipated effect of dietary LCPUFA on

some outcome measures is likely to be small, not surprisingly some trials report effects while others do not. Some of the apparent inconsistent effects reported in the PUFA field, especially in term infants, may derive from this fact. Having said that, one must not dismiss effects simply because they are small, particularly when global developmental effects are monitored. Effects of small size may be significant when considered on a population basis. Thus, a shift of 1 or 2% in test scores in a whole population can translate to a large increase in those individuals falling below the threshold of normal.

Some studies have aimed to increase the power of the study by enrolling large numbers of infants at multiple sites. Crucial to obtaining a valid result is the need to have absolute consistency between operators (or testers) at all the study sites. The results from each of the operators should be monitored and the separate results examined statistically for variation between sites and then presented in any report of the overall findings. Statistical procedures may be necessary to combine data that show site differences, or variation in testing procedures may result in small diet-induced effects being masked. Operator–operator error may be due to different sites or simply due to more than one operator carrying out an assessment at one site; nevertheless, it may contribute to a larger variance than estimated at the start of the study. The reader should therefore do simple power calculations on data that purport to demonstrate no effect, as operator error could have reduced the power of the study to a degree that a type II error was introduced.

Post hoc analysis of data can easily determine if either insufficient numbers in the groups existed or excessive noise (or variance) was present in the data for an effect to be seen (type II error). However, no test is available for detecting if confounding influences (parental smoking, socio-economic variables) or unintentional bias as a result of nonadherence to the randomization code had a major influence on the final result. For this reason, the remainder of this paper concentrates on study design and conduct issues that can possibly infuence the outcome of trials (21–26).

Clinical trial issues: (i) Bias in RCT. The word "bias" often raises connotations of deliberate intent, as this is the general usage in a social sense. This is not the meaning of the word in regard to RCT. Essentially, it refers to any influences that have the potential to distort group comparisons other than the assigned treatment. Most commonly, such influences or biases occur as a result of trial design or conduct (27). The sources of bias can stem from the initial randomization (selection bias), the loss of study participants (attrition bias), and the way participants are assessed during the trial (performance and detection bias).

(ii) Method of allocation. The purpose of a randomized study is to ensure that any event that arises has an equal (or random) chance of arising in both the treatment and the placebo groups unless it is influenced by the treatment (treatment effect). This can be ensured only if the groups of the trial are assembled in a truly random way and not in a way that is at all influenced (even accidentally) by the investigators. Random assignment is vital because unless everything except the treatment effects is distributed equally among the groups, the true effect of the treatment cannot be accurately determined. This will be due to two inherent problems: first, an unequal variance or confounding in the data sets exists such that an effect can be obscured or created and second, the use of basic statistical procedures will be invalidated.

Many ways of allocating subjects into a trial are possible, and some forms of allocation are better than others. For this reason, great emphasis has been placed on the method of allocation in recent publications that aims to improve the quality and reporting for randomized clinical trials (22,27). For example, to use an open list of random numbers of assignment or other methods of allocation such as odd and even medical record numbers or dates of birth is insufficient. These allocation procedures are transparent before assignment, and now evidence suggests that transparent allocation is associated with selection bias (28,29). This bias is best avoided by using a method that prevents foreknowledge of treatment assignment and is often referred to as allocation concealment (27). For example, when allocating infants into a trial of placebo or LCPUFA-supplemented formula, the person responsible for recruiting participants should determine the eligibility of the infant, speak to the family, and gain consent before contacting an independent person in the research team to obtain the next formula allocation or opening a sealed, opaque envelope containing the next allocation. In such a process the person recruiting participants can in no way influence the allocation.

Ideal allocation practice also necessitates no deviation from the predetermined randomization schedule, and following assignment, it should not be possible to alter the assignment or the decision about eligibility (27). In the n-3 PUFA field, examples exist of deviations from ideal practice in the trials reported to date. In an RCT reported by us in 1995, a greater number of infants was recruited into the control group compared with the supplemented formula group (10). This occurred because we were supplied with an inadequate amount of the LCPUFA supplement, and we continued recruiting into the control group until the appropriate number of infants was enrolled, resulting in only 13 infants in the n-3 LCPUFA-supplemented formula-fed group and 19 infants in the placebo formula-fed group (10). Other examples exist of studies that have involved deviation from the predetermined assignment schedule by replacement of infants lost to follow-up that may have resulted in an unintended selection bias, hence affecting the outcome of the trial (6,7). While authors clearly do not consciously select better infants for one group than another, any deviation from the randomization schedule means that the process is no longer random, raising the possibility of an imbalance in the groups that can override or enhance treatment effects.

(iii) *Blinded outcome assessment.* As this is one of the areas in which investigators are most aware of the possibility of unintentional biases, many of the authors may in fact have conducted blinded outcome assessments but failed to report them (Table 3).

(iv) Losses to follow-up. Many of the clinical trials in the areas of n-3 PUFA and infant visual function have had large dropout rates (Table 3). Indeed, it is extremely difficult to conduct relatively long-term trials in the real world of mothers and their babies, and intense emphasis and energy are required to assess as many infants as practically possible. Loss of participants from the trial postrandomization may be due to withdrawal of consent, failure to attend clinic appointments, or exclusions made by the investigative team. These losses may result in systematic differences between groups, especially if greater numbers are lost from one group relative to another. The systematic differences between groups that result because of loss to follow-up are referred to as attrition bias. The new guidelines for reporting of RCT emphasize that it is vital that authors attempt to better describe the characteristics of all the subjects enrolled, those lost to follow-up as well as those of the subjects completing the trial in each treatment group. In that way, any major aberrations in characteristics between groups that may result in attrition bias will be evident.

In conclusion, valuable data relating to the effect of diet on the visual development of infants has been obtained from the published papers of RCT involving PUFA. However, details about the conduct of the trials are often incomplete, making it difficult to assess if we can have a high degree of confidence in reported effects or lack thereof. Power calculations suggest that many of the studies were of sufficient size to detect differences in visual acuity due to dietary treatment. Variations in reported results could possibly be due to unintentional bias as a result of accidental inclusion of confounders, deviations from the randomization code, or loss to follow-up. A definitive answer to the degree to which dietary LCPUFA are likely to influence visual development may only be resolved with RCT that are impeccably conducted and that are of sufficient size to avoid potential perturbations from unreported confounders. Evaluation of a more comprehensive range of outcomes initiated to more completely assess the global implications for infant development would be valuable.

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Statistical Considerations in Infant Nutrition Trials

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ABSTRACT: Infant nutrition trials usually require developmental follow-up, often to 18 mon, and sometimes beyond reading ability at age 7 yr. They are therefore logistically complex and costly, and should be conducted to a high statistical standard. With examples, we focus on: good practice in nutrition trials and the goal of a common protocol; how to set plausible trial targets and to work out trial size accordingly; statistical observations on assessing visual maturation; and methods of randomization, including the method known as minimization, which can be adapted to select more appropriately a comparison cohort of breast-fed infants. We end with discussion of analysis and reporting standards, including the give-aways (or tell-tale signs) to be on the look out for. A Cochrane Collaboration for systematic review of randomized trials of infant nutrition is proposed.

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CONSULTATION OF "GOOD PRACTICE" DOCUMENTS

Randomized controlled trials (RCT) in infant nutrition should be conducted to a high statistical standard. Investigators may therefore find it instructive to consult two excellent "good practice" documents: Guidelines for Standard Operating Procedures for Statistical Practice in Clinical Research (1) and Biostatistical Methodology in Clinical Trials in Applications for Marketing Authorizations (2) (Scheme 1). The first has 12 practical sections ranging from clinical development plans and clinical trial protocol through to randomization and blinding, interim analysis plans, and quality assurance. Infant nutrition trials usually entail developmental follow-up, often to 18 mon and sometimes beyond reading ability at age 7 yr. They are therefore logistically complex, and costly. Longer-term follow-up is unlikely to be funded unless predicated upon evidence of early benefit for, say, short-term anthropometry. Choosing wisely the infant nutrition trials in which to invest both intellectually and financially needs careful attention to basic scientific rationale and to rigorously presented short-term results.

Statisticians in the Pharmaceutical Industry (PSI)

GUIDELINES FOR STANDARD OPERATING PROCEDURES FOR STATISTICAL PRACTICE IN CLINICAL RESEARCH

1. Clinical development plans
2. Clinical trial protocol
3. Statistical analysis plans
4. Evaluability of data for analysis
5. Randomization and blinding
6. Data management
7. Interim analysis plans
8. Statistical reports
9. Archives and documentation
10. Data overviews
11. Quality assurance and control
12. Interactions with Contract Research Organization

European document

BIOSTATISTICAL METHODOLOGY IN CLINICAL TRIALS IN APPLICATIONS FOR MARKETING AUTHORIZATIONS . . .

SCHEME 1

COMMON PROTOCOL AS A GOAL

A goal for investigators of polyunsaturated fatty acids (PUFA) in infant nutrition should be a common protocol to which all can refer.

At the very least, in any protocol, sample size should be clearly specified and justified in terms of trial objectives. The methods used to arrive at trial size should be referenced and documented, as should estimates of any quantities used in trial size calculations. I shall give some examples of how to do this in a later section. Plans for sample size reviews, to alter the planned number of patients—up (for example, because variability is greater than had been anticipated) or down (for example, because of poor accrual)—should be conducted blinded and noncomparatively: that is, without breaking the code for treatment assignments.

Multiplicity should be addressed in the protocol. By multiplicity I mean any or all of: multiple trial objectives, multiple measures of the same endpoint, or multiple endpoints. Priorities should be set, or an appropriate hierarchical analysis

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Abbreviations: AA, arachidonic acid; ALEC, Artificial Lung Expanding Compound; BREATHE, British Randomized Evaluation of ALEC Therapy; CIK, Cambridge, Ipswich, and Kings Lynn; CONSORT, Consolidation of Standards for Reporting of Trials; DHA, docosahexaenoic acid; FM, formula; LCPUFA, long-chain polyunsaturated fatty acids; N&S, Norwich and Sheffield; NEC, necrotizing enterocolitis; PUFA, polyunsaturated fatty acids; RCT, randomized controlled trial; sd, standard deviation; se, standard error.

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strategy should be set out explicitly, which safeguards against false positive inferences. More generally, generic statistical analysis plans should outline the methods of analysis for all aspects of the trial data. Before actual analysis, these plans should be developed into a comprehensive description and set of statistical programs.

Rules to determine the evaluability of patients should be laid out in the protocol, especially in the case of protocol violations, withdrawal of patients from study by the investigator, or patient drop-outs. An off-study form might be completed in such cases to explain the circumstances.

DETERMINING TRIAL SIZE: PRELIMINARIES

How do we determine *a priori* a rational target difference for each major outcome variable on which infants randomized to different diets/supplements/formulas will be assessed in the course of follow-up?

The effect size (or estimated difference) in animal studies or in related randomized trials on other formulas may suggest the size of differential that is reasonable and prudent to expect with a new formulation in infants. Epidemiological studies, especially those which have reported covariate-adjusted comparisons of outcome for breast-fed vs. formula-fed infants, may suggest an upper bound for plausible effect size.

Target differentials may be set relative to the gender or gestational age effect on outcome. How much better do female infants perform than males? Could the new formulation achieve as much? Is it reasonable to target the new formulation to achieve more than the gender differential, or more than the effect on outcome of one additional week of gestational age?

Do we need to consider a differential effect of the new formula? For example, is its effect likely to be greater in preterm than in term infants, or less in infants who receive the new formulation only as supplement to mother's own milk rather than as sole nutrition? An example follows.

Also, consideration needs to be given to whether the new formulation has a persistent or transient effect. If its effect is thought to be transient, when in the course of follow-up do investigators expect that the maximum effect is likely to occur, and why so? They should ensure that assessments are timed for the period of maximum effect and, if scientifically important, document its transience.

EXPLAINING TARGET DIFFERENTIALS BY REFERENCE TO OUR MULTICENTER LOW-BIRTHWEIGHT FEEDING TRIAL

Design outline. Our five-center low-birthweight (<1850 g) feeding trial was designed in the early 1980s as follows. Three centers [Cambridge, Ipswich, Kings Lynn (CIK)] had donor milk banks, but in the other two centers [Norwich, Sheffield (N&S)] the control diet was term formula rather than donor milk. Pilot studies around Cambridge showed that two-thirds of mothers of low-birthweight infants chose to express breast milk: the infants of these mothers were to be randomized in the so-called supplement trial—that is, they would receive the trial diet (control = banked milk or term formula according to center; or special pre-term formula) only as supplement to mother's own milk if mother was not providing enough. Other low-birthweight infants, those whose mothers elected not to express breast milk, were to be randomized in the primary trial—that is, they would receive the trial diet as sole nutrition.

Major outcome variables. Outcome variables were ordered in time and prioritized as follows. First, we were interested in the effects of the preterm formula on short-term anthropometry: days to regaining birthweight, steady-state weight gain in g/kg/d, length gain, head circumference, and skinfold thickness. Steady-state weight gain was the major short-term outcome. The last three were to check that babies did not just put on weight but also grew in length and head circumference and did not become excessively fat. The first was important because it entailed a careful definition of the day of regaining birthweight: from that date until discharge was the period over which steady-state weight gain was assessed using linear regression of the natural logarithm of weight on day. A secondary economic variable was also reported: days to discharge from special care, irrespective of survival status—a harsh outcome to contemplate.

Second, we were interested in the effect of the special preterm diet on developmental outcome, as assessed by Bayley scores at 18 mon. A longer horizon stretching from reading ability at age 7 and beyond was not formally part of our trial size considerations in 1982, when the reality was that unless the preterm formula had a significant effect on short-term anthropometry, it was unlikely that industry or the Medical Research Council would underwrite continued randomization to achieve the trial size that would be necessary to powerfully discern (by the benchmark of statistical significance) moderate effects on developmental scores, if such effects pertained.

Research strategy. Our research strategy was therefore a four-group sequential design allowing up to three formal interim analyses when recruitment was 1/4, 1/2, and 3/4 complete. This design was selected to allow early reporting of major effects on steady-state weight gain, but randomization continued to ensure that sufficient infants were randomized to discern moderate effects on Bayley scores at 18 mon. In effect, we assumed that publication of results on short-term anthropometry would encourage funders to underwrite the continued randomization and more costly, actual follow-up to 18 mon and Bayley assessments.

This plan differed from the usual reason for a group sequential design, which is to curtail randomization to the inferior treatment if important effects are soon seen. With a four-group sequential design—each group of size s and half the infants per group having been randomized to the preterm formula—analysis at a more extreme significance level than the 5% level is made after s, $2s$, $3s$, and $4s$ infants have been recruited: up to four analyses. But if, at any of the three planned interim analyses, the design-specific significance

level is met, then randomization may cease and the significant differential between preterm and control diet can be reported with confidence that false positive inference, so-called Type 2 error, has been appropriately controlled. Type 1 error occurs when statistical significance is observed but there is truly no difference between treatments. Type 2 error is when there is truly an important difference between treatments but statistical significance is not achieved.

How were target differentials determined? Going back a stage, how were rational target differentials determined for steady-state weight gain and for Bayley developmental scores? Professor Alan Lucas will forgive me, I hope, for paraphrasing the then process as: he provided the differentials and I provided the rationale!

(i) Steady-state weight gain. From prospective pilot studies and also from analysis of data from case-notes of low-birthweight babies, we estimated the standard deviation (sd) for steady-state weight gain as 6 g/kg/d and the mean as 16 g/kg/d. Professor Lucas was confident that the effect would be large. A large target was set (3.8 g/kg/d), about two-thirds of a standard deviation in the primary trial; that is, twice as large as would nowadays be considered to constitute a moderate effect.

The rationale that I imposed on this exuberance was that a more modest target (set at two-thirds of the target in the primary trial = 2/3 × 3.8 g/kg/d = 2.5 g/kg/d) should be used for the supplement trials in which babies would receive the trial diets only as supplement to mother's own milk, and hence could receive anything from 0 to 100% of their nutrition from the randomized diet. In the event, and regrettably, data which we had not obtained from a formal pilot study before the start of the main trials, infants in the supplement trials received about half their nutrition from mother's own milk, and so—on crude proportionality—the second target should perhaps have been smaller still.

(ii) Bayley developmental scores. Bayley developmental scores, like IQ scores, have a published mean of 100 and standard deviation of 15.

For the 18-mon assessments, it was prudent to set a more modest, but still confident-that-early-dietary-effects-would-persist, 5-point differential in the primary trials, equivalent to one-third of the then-published standard deviation. For the supplement trials, the same shrinkage to two-thirds effect was applied as for short-term anthropometry, giving a target 3.3-point difference between preterm diet and control.

Simple trial size sums. Using the simple trial size sum illustrated in Scheme 2, we calculate that for the primary trials to have 80% power to discern a 5-point differential by the benchmark of statistical significance at the 5% level, 140 infants whose mothers elected not to breast feed should be randomized to the control group and 140 to preterm diet. Thus, for primary trials,

$$A1 = 2 \times 15 \times 15/(5 \times 5) = 18 \quad [1]$$

and, for 80% power and 5% significance level,

$$\text{number per dietary group} = 8 \times A1 = 8 \times 18 = 144 \quad [2]$$

Trial size (ratio 1:1)

Step 1. Rational target difference ... *science, not arithmetic*
Step 2. Literature, historical or pilot data . . . standard deviation (SD)
Step 3. Back-of-envelope sum, as follows:

Compute:
A = 2 × SD × SD/(target difference × target difference)

For 80% power[a] and 5% significance level:
number per treatment group = 8 × A

For 50% power[a] and 5% significance level:
number per treatment group = 4 × A
[= *half the number required for 80% power*]
$= (1.96 + 0.84)^2 = (Z_{1-\alpha/2} + Z_{1-\beta})^2$.

[a]For 80% power, multiplier 8 (actually, 2.8 × 2.8 = 7.84)
For 50% power, 4 = approximately (1.96 × 1.96) with $Z_{1-\beta} = 0$.

SCHEME 2

How did these requirements measure up to the numbers of infants (4 groups of 32 each) to be randomized in the CIK (4 × 32 = 128 babies) and N&S (also 4 × 32 = 128 babies) four-group sequential primary trials? The answer is: not well for the primary trials separately (less than 50% power each, see Scheme 2), but comfortably for the CIK + N&S primary trials combined. The combined primary trial thus addressed powerfully a generalized question: whether the special preterm formula improved upon the control diet (be it term formula or banked human milk) as sole early nutrition in respect of Bayley developmental scores at 18 mon.

For each of the supplement trials we needed to randomize 330 per dietary group for 80% power and 5% significance level per trial. The numbers to be randomized in the CIK (288) and N&S (also 288) supplement trials afforded less than 50% power per trial but approached 80% power for the combined supplement trials to answer the generalized question: as measured by Bayley developmental scores at 18 mon, did the special preterm formula improve upon control diet (be it term formula or banked human milk) as supplement to mother's own milk up to discharge from special care?

Our low-birthweight feeding trials therefore had adequate power to answer the same generalized question at 18 mon separately for infants randomized in the pair of primary trials and for those randomized to receive the trial diet as supplement to mother's own milk.

Best laid plans of mice and men . . . . But, the best laid plans of mice and men gang aft agley. In practice, the sd of Bayley scores in this population of children was around 18, nearer 20 than 15, in other words. When the above calculations are repeated with a sd of 18 rather than 15, the number to be randomized per dietary group in the combined primary trials becomes 210; the combined primary trials have only just over 50% power when the sd is as high as 18.

And similarly for the combined supplement trials, the number to be randomized per dietary group becomes 520.

In consequence, our five-center low-birthweight feeding

trials' program had adequate power in practice, rather than in concept, to answer only a single global question: does the special preterm formula confer advantage in terms of Bayley developmental score at 18 mon over the control diet (be it term formula or banked human milk) whether the infants received the trial diet as sole or only as part of early nutrition?

The original trial-specific target differentials translated to a "pooled-across-all-four-trials" 3.8 differential $[(128 \times 5 + 288 \times 3.3)/(128 + 288) = 3.8]$ between preterm and control diets, where 128 = number randomized to preterm in both primary trials; 5 = primary trial target differential (preterm formula vs. nursery standard) 228 = number randomized to preterm in both supplement trials; and 3.3 = supplement trial target differential (preterm formula vs. nursery standard). With sd of 18, for 80% power and 5% significance level, for the combined trials

$$A3 = 2 \times 18 \times 18/(3.8 \times 3.8) = 45 \quad [3]$$

$$\text{number per dietary group} = 8 \times A3 = 360 \quad [4]$$

at 18 mon, which the program achieved!

Sample size review because of change in variability. This example illustrates one context—change in anticipated variability—in which sample size review becomes necessary, but it is also an example in which nothing, practically, could be done about it, because by the time the first 50 or so infants had had their 18-mon developmental assessment, randomization had practically ceased.

AS WELL AS sd, TIME PER ASSESSMENT AND LIKELY ACCRUAL MATTER

Before turning to another, and successful, example of trial size determination I want to draw attention to Table 1 which shows four hypothetical visual acuity protocols A to D. Protocol D has the smallest sd (= 1), but it takes 4 h to test a single baby. For 80% power to discern—at the 5% significance level—a visual acuity difference of 0.5, 130 babies would need to be randomized and 520 observer hours committed for asessment of their visual acuity.

Protocol A has the second-largest sd in assessments of visual acuity (sd = 2) but it requires only 1 h to test one baby. Thus, although four times as many infants have to be randomized, the observer hours are the same as for Protocol D.

TABLE 1
Choice of Acuity Protocol[a]

Visual acuity protocol	SD	Time to test one baby (h)	Number of infants to be randomized	Observer hours
A	2	1.0	500	500
B	4	1.0	2000	2000
C	3	0.5	1150	575
D	1	4.0	130	520

[a]Assume target acuity difference = 0.50.

As well as sd of assessments, practicalities, such as time taken per assessment and number of babies available for recruitment per month, matter. If only 20 babies could be recruited per month it would take over 8 yr to complete accrual if protocol B were selected!

ELICITING PRIOR BELIEFS

Clinical trials should be designed with at least 80% power to detect *a priori* plausible treatment differences and probably should not start if they are unlikely to achieve 50% power. Also, if the only difference that is *a priori* plausible is so small that it would not affect clinical practice, the proposed trial should be vetoed and research conducted on a more promising theme in order to waste neither patient nor physician effort.

What differences are *a priori* plausible? Answers can be attained by eliciting prior beliefs, using a method which I call trial roulette.

TRIAL ROULETTE: HOW TO ELICIT, AND HOW TO FORM PRIOR BELIEFS

British Randomized Evaluation of Artificial Lung Expanding Compound (ALEC) Therapy (BREATHE) was a randomized evaluation of surfactant prophylaxis to reduce the mortality of babies born at 25–29 wk of gestation. BREATHE addressed the question: does ALEC therapy at birth reduce neonatal mortality of very premature infants? Elicitation of prior beliefs proceeded as follows.

A collaborators' meeting was held in Cambridge in June 1984. Neonatal units had forwarded their neonatal statistics for the previous 2 yr, which showed that 36% of babies born at 25–29 wk of gestation died within 28 d.

With 36% control mortality as their reference, participants were asked to place 20 bets in the betting streets illustrated in Figure 1 to indicate their prior belief in the likely mortality of ALEC-treated infants of 25–29 wk of gestation. More bets would be placed in the avenues or betting streets in which they thought it more plausible that the neonatal mortality of ALEC babies would lie. A spread of belief would be expected from most participants, indicating their uncertainty.

ALEC mortality	15%	20%	25%	30%	35%	40%	Recorded by
Centered on: 28%	4%	15%	22%	31%	25%	3%	Peers ($n = 12$)
Centered on: 22%	28%	30%	25%	10%	7%	0%	Cambridge team ($n = 3$)
Centered on **27%**	9%	18%	23%	27% ***	21% *****	2%	Pooled prior belief ($n = 15$)

FIG. 1. Prior beliefs about ALEC mortality from placing 20 bets per person. Trial roulette on the plausibility of Artificial Lung Expanding Compound (ALEC) mortality. Control mortality: 36%. Range of equivalence [*********] from 30 to 37% ALEC mortality.

Also, it is usual to elicit the so-called "range of equivalence" within which participants would be indifferent between use of ALEC and control: for example, a range of equivalence of 30 to 37% for neonatal mortality might have been cited. This would mean, for example, that if ALEC resulted in low neonatal mortality for which the upper 95% confidence limit excluded the range of equivalence, then ALEC therapy should certainly be adopted into neonatal practice forthwith.

Figure 1 also shows the total bets placed by 12 non-Cambridge peers, by the Cambridge team of three, which included me, and all bets pooled. What data and other sources were available in June 1984 on which to base opinions? Firstly, there was the rationale for artificial surfactant therapy: making up for a deficiency of natural surfactant without introducing foreign protein. Secondly, colleagues will have been aware of two small randomized trials in Oxford of ALEC which were then unpublished, and they may have inferred—rightly—"no significant difference" and concluded—wrongly—that "no significant difference" meant there was no difference. Remember that what is reported from RCT depends upon the power of the trial as well as upon the true superiority of one treatment over another. The Oxford trials were wholly inadequate in power, and hence almost completely uninformative.

Colleagues were also aware of a 1981 paper by Morley *et al*. (3) which reported zero deaths out of 22 ALEC-treated babies under 33 wk of gestation but 24% mortality in 33 control babies whose consultant was not Professor Colin Morley. This paper was received critically, not least because of confounding of consultant with therapy.

My first exercise in statistical caution in respect of Morley *et al*. (3) was to halve the difference "to adjust" for publication bias: the likelihood that the investigators had been keeping informal track of the results and rushed into print when the differential was fortuitously high. The lack of a planned stopping rule is suggested by the number of children studied being 55 rather than divisible by 2 and either 50 or 60, say. And indeed, the same design continued with recruitment of a further 76 babies prior to the start of randomization and the differential shrank from 24 to 14%.

My second exercise of statistical caution was for selection bias: I halved the differential again because the infants were not randomized to ALEC therapy vs. control and so, to me, the nonrandomized data from Cambridge were consistent with a one-third drop in mortality such as from 21% (in the under-33-wk gestational age-group) to 14%.

The foregoing description is how I formed my prior belief that a one-third reduction in neonatal mortality was plausible with ALEC. Also available at the time were randomized results by Halliday *et al*. (4) for a subgroup of 30 babies under 29 wk gestation that showed a dramatic fall in neonatal mortality from 43% in 14 controls to 25% in surfactant-treated infants. These Belfast data were consistent with interim, not fully checked data for 25–29-wk-old babies in the ongoing Cambridge-Nottingham trial (21% ALEC-treated vs. 38% control neonatal mortality, each out of 47 infants) to which only the Cambridge team was privy.

The pooled prior belief centered on 27% ALEC-treated mortality, which the two-stage trial (5) was designed with nearly 80% power to discern, given the results in hand. Ultimately, 328 babies were randomized in BREATHE, 164 to ALEC, of whom 35 (21%) died in the neonatal period compared to 59 controls (36%).

Figure 2 provides some betting streets to use to elicit prior beliefs about the impact of some long-chain polyunsaturated fatty acids (LCPUFA) on outcomes such as visual acuity (Teller acuity) at 7 mon, head circumference at 3 mon, Bayley Mental Developmental Index at 18 mon and Wechsler Intelligence Scale for Children (Wisc-R) at 7 yr (The Psychological Corp., San Antonio, TX). Notice that on each graph is documented the mean for formula-fed controls, and the standard deviation of measurements.

VISUAL MATURATION: SOME STATISTICAL OBSERVATIONS

Before leaving the topic of trial size, I want to make a series of statistical observations about visual maturation and their implications for assessment times and for analysis of visual acuity.

Since visual maturation is linked to postconceptional age rather than to postnatal age, care has to be taken that assessments are made at the intended postconceptional age and appointments booked appropriately. Visual maturation is rapid: for example, stereoacuity apparently matures to within 0.5 log of adult level by 6 to 8 mon of age. It is therefore desirable to document that bias in assessment times has been avoided, for example by reporting mean and sd of (intended – actual assessment time) per dietary group. Clearly, if the control group were seen on average at a younger age, then their visual maturation could be less on that account alone.

It is also necessary to avoid observer bias in assessing visual maturation: in particular, the assessor should be masked as to the pattern size and grating being used at all times during her/his assessment of the infant's visual maturation.

Time per test is important (Table 1). It is worth remembering that stereoacuity has been assessed on 2000 three-year-olds in the United Kingdom: it can be done.

Finally, exclusion of infants from analysis is not infrequent, for example, because of strabismus or noncompliance. The former should mean that there is a protocol for detecting strabismus that is employed for every child. The second reason for exclusion is more problematic: for example, if noncompliant children are those who see less well, such exclusions could bias the comparison between control and experimental diet by excluding from analysis precisely the controls which a successful dietary intervention might have helped!

Scheme 3 was compiled to discover something about the statistical properties of the Teller Acuity Card (TAC) assessment. Within each study, the sd is substantially lower in

Table 1: Mean Teller acuity (TAC) at 7 mon on formula is 10.0, standard deviation (sd) = 3. Place your bets (20) on change in mean with LCPUFA

–2.0	–1.8	–1.6	–1.4	–1.2	–1.0	–0.8	–0.6	–0.4	–0.2	NIL	+0.2	+0.4	+0.6	+0.8	+1.0	+1.2	+1.4	+1.6	+1.8	+2.0

Range of equivalence = ...

Table 2: Mean head circumference at 3 mon on formula is 40 cm, sd = 2 cm. Place your bets (20) on change in mean with LCPUFA

–2.0	–1.8	–1.6	–1.4	–1.2	–1.0	–0.8	–0.6	–0.4	–0.2	NIL	+0.2	+0.4	+0.6	+0.8	+1.0	+1.2	+1.4	+1.6	+1.8	+2.0

Range of equivalence = ...

Table 3: Mean Bayley Mental Development Index at 18 mon on formula is 100, sd = 16. Place your bets (20) on change in mean with LCPUFA

–20	–18	–16	–14	–12	–10	–8	–6	–4	–2	NIL	+2	+4	+6	+8	+10	+12	+14	+16	+18	+20

Range of equivalence = ...

FIG. 2. Betting streets for use to record prior beliefs about long-chain polyunsaturated fatty acids (LCPUFA).

breast-fed infants, which may be a clue to bias in their selection with, for example, replacements being drafted in for breast-fed babies with anomalous results. Typically, mean Teller acuity (cy/cm) is 2.6 times sd, an indication for logarithmic transformation of the data before analysis and when computing trial size. Notice that 3-mon adjusted visual acuity is less for preterm than for healthy term infants with between-study differentials of the order of 0.25 to 1.0 (one-eighth to one-half sd); within-trial differentials between breast- and formula (FM)-fed infants are of the same order. Thus, for 80% power to discern a modest diet-related differential of 0.25 [mean TAC (cy/cm) at 3 mon of 3.50 vs. 3.75] when sd = 1.5 requires 1200 infants to be randomized. To discern a moderate differential of 0.5, if that were *a priori* plausible, would require 300 infants to be randomized (see Scheme 2 for how to compute trial size).

Teller Acuity Card (TAC) visual acuity: statistical properties?

TAC test at 3 mon: healthy term infants
Breast-fed ~ mean TAC (cy/cm) = 3.54, SD = 1.2, n = 45
FM + PUFA ~ mean TAC (cy/cm) = 3.52, SD = 1.8, n = 32
FM . . . . . . ~ mean /tac (cy/cm) = 4.28, SD = 1.7, n = 21

TAC at 3 mon adjusted age: preterm infants
Breast-fed ~ mean TAC (cy/cm) = 4.08, SD = 1.5, n = 19
FM + PUFA ~ mean TAC (cy/cm) = 4.52, SD = 2.8, n = 18
FM . . . . . . ~ mean TAC (cy/cm) = 4.50, SD = 2.6, n = 19

(i) SD is low in breast-fed babies: *selection bias*?
(ii) Typically, mean TAC is 2.6 times SD, an indication for logarithmic transformation?
(iii) Is visual acuity less for preterm than for healthy term infants? The differential is of the order of 0.25 to 1.0.
(iv) Differential of 0.25, e.g., 3.50 vs. 3.75, SD = 1.5 leads to: for 80% power, $2n$ = 1200 infants randomized.

SCHEME 3

Critical remarks on infant habituation protocols from a statistical standpoint are inevitable. The setup is that independent observers are behind opaque screens to right and left of baby and up to 10 trials are made with the habituation stimulus. Trial j ends when baby fixates away from habituation stimulus for 2 s or more: this defines the duration of trial j.

Baseline duration of fixation is defined as (trial 1 + trial 2) duration/2 = B. Habituation criterion is reached if [trial j + trial (j + 1)] duration < B, such as in consecutive trials 3 and

4, or 4 and 5, or . . . , or 9 and 10. Baby is excluded from analysis if she or he fails to reach habituation criterion within 10 trials. Such exclusions may constitute a serious analysis bias by removing infants who are slow at recognition or are not easily bored!

Four novelty preference tests are then presented. But, inexplicably, the test order is predetermined and systematic,

*H on left, Novelty on right

+Novelty on left, H on right

*H on left, Novelty on right

+Novelty on left, H on right.

and presumably is known to the observers who assess the infant's preference. If such observers also know to which dietary group the infant has been randomized, the potential for observer bias—conscious or otherwise—would be extremely high. Moreover, bright children may habituate to the alternation! There can be no excuse for not randomizing the order in which the two * and two + sequences appear, as randomization of the order of appearance is security against observer bias.

RANDOMIZATION

Simple randomization, the equivalent of tossing a coin, is not recommended because unequal numbers per treatment group readily result, and the imbalance can be severe in small studies or for individual centers within a multicenter trial, or at interim analyses.

Balanced randomization using permuted blocks. Balanced randomization can be achieved using tables of random permutations of fixed length (for block length 8, say, ignore numbers 9 to 16 in printed permutations of length 16). Block length 8 means that after every eighth patient has been randomized—that is, after patients 8, 16, 24, 32, . . .—an equal number of patients will have been assigned between treatments, to control, and to LCPUFA, for example. In assuming a randomization ratio of 1:1 and block length 8, the maximum imbalance at any time would be 4, which is half the block length.

If treatments cannot be made double-blind, there is a serious drawback to using permuted blocks of fixed length, namely, that a proportion of assignments is knowable in advance, as illustrated below for block length 4. Knowable-in-advance assignments are shown in boldface and constitute one-third of assignments. For longer block lengths, the proportion is lower, and so there is a trade-off between maximum imbalance and the percentage of assignments that can be determined.

cc**LL** . . . . LL**cc**

cLc**L** . . . . LcL**c**

cLL**c** . . . . Lcc**L**

When treatments are not double-blind, the solution is to use permuted blocks of variable length (6 or 8 or 12, say) with the length of the first, second, third block and so on being chosen at random so that the investigator never knows when the balance will fall next.

When there is one major prognostic factor, or perhaps two, such as gestational age or gender, investigators may wish to ensure that treatments are evenly assigned to patients who differ in respect of this prognostic factor. Thus, randomization in BREATHE was stratified by center and by gestational age within each center (25–26 wk or 27–29 wk) to ensure that equal numbers of very premature babies were assigned to ALEC and to control within each center (5).

Minimization. Minimization is a method of randomization which achieves balanced treatment allocation in respect of marginal frequencies for several covariates, for example, gestational age, gender, maternal education. Thus, preterm babies would be assigned equally between control and LCPUFA, as would term babies. Male babies would be assigned roughly 50:50 between control and LCPUFA, as would female infants. Assignment would be made similarly for three levels of maternal education, but the method would not guarantee equal numbers per formula group for each cross-classification, such as: preterm female infant born to mother who completed higher education.

In multicenter studies, the choice will often be between balanced randomization within center or balanced randomization across centers (for trial as a whole, that is) or minimization. A central randomization office would usually be needed for the second and third options even though formula supplies were locally held and numbered serially such as for Barcelona in Scheme 4. The Barcelona investigators would

Randomization

Central randomization office:

Baby	Center (covariates)	Minization assignment	Local serial
1.	Flinders (female, preterm, maternal educ. 1)	A	. . .
2.	Cambridge (female, term, maternal educ. 2)	A	. . .
3.	Barcelona (male, preterm, maternal educ. 1)	B	#2 . .

.

Local supplies: BARCELONA . . . *record randomization date and to whom allocated, for example.*

1 A
2 B . . . 961102 to Baby Fernando, trial number 003: supplies B 02
3 A
4 B
5 B
6 B
7 B
8 B
etc.

*Randomization ratio: 1:1 or 2:1 or . . . , e.g., twice as many babies randomized to experimental formula.

*Randomization leaks: unblinded because . . . ; substitutions?

SCHEME 4

TABLE 2
Minimization Assignments So Far

Formula	A	B
Totals	2	0
Male	0	0
Preterm	1	0
Maternal education, level 1	1	0
Barcelona	0	0

not know that local supplies 1, 3, 11, and 12 were the A formula; that information would, however, be known by the central randomization office.

Scheme 4 shows that the first baby randomized in the trial was from Flinders, a female preterm baby whose mother achieved level 1 education. Minimization is being used at the central randomization office to achieve marginal balance in respect of center, gender, gestational age, and maternal educational level. The first baby is assigned by simple randomization to formula A. The second baby to enter the trial is from Cambridge, also female but a term infant whose mother attained education level 2. To maintain balance for female infants, it would be preferable if this second child were allocated formula B. Simple randomization is used with probability 0.8 for the assignment which minimizes the imbalance, but with probability 0.2 the imbalance is exaggerated. As it happens, the Cambridge baby was assigned to formula A. The third infant notified to the central randomization office is Baby Fernando, a preterm male from Barcelona whose mother is at educational level 1. The central randomization office checks each margin which applies to this baby as follows (Table 2). It would be preferable to assign this baby to formula B to reduce the imbalance (see Totals: 2 vs. 0), and this assignment is made using simple randomization with probability 0.8. Baby 3 is indeed assigned to formula B. The central randomization office now has to instruct Barcelona which local supplies should be assigned to Baby Fernando. The office does this by selecting a serial number at random from the first 10 which contain supplies of formula B. As luck would have it, Baby Fernando is to receive local supplies #2, but there is only a 1 in 9 chance that the next Barcelona baby who has been assigned to formula B will receive #4 as his or her supplies. Re-randomization in use of local supplies is to confound deductive disclosure such as would occur if supplies were used in sequence, Thus, used in sequence, the assignments #2, #4, #1—that is, going back to #1—would allow the local team to deduce that two babies receiving local supplies #4 and #1 were in fact receiving different diets! Re-randomization makes such deductions impossible and is a precaution that should be taken to confuse the intelligence of participants.

The randomization ratio may be different from 1:1. For example, there may be good reasons to wish twice as many babies to be assigned to the experimental formula. Some randomization give-aways (tell-tale signs) are listed in Scheme 5. Scheme 6 shows what Consolidation of Standards for Reporting of Trials (CONSORT) (6) expects investigators to detail about how assignments were made in RCT and about masking. The CONSORT flowchart, which is to be a feature of all RCT published in leading medical journals from 1 January 1997 on, is reproduced as Figure 3.

Randomization give-aways

Single-center studies
*Number randomized is not divisible by number of diets.
(more generally: does not accord with randomization ratio)
At best: untidy
At worst: clue to unplanned trial size, opportunistic stopping, exclusions from analysis

*Imbalance in numbers assigned per formula exceeds the maximum imbalance for reported permuted block lengths.

*Accrual *(number randomized per month)* does not accord with historical data on number eligible per month.

Multicenter studies
As above on a per-center basis, unless randomization was balanced only across centers.

SCHEME 5

CONSORT on Assignment

Describe:
*Unit of randomization (e.g., individual, cluster—such as twins or litters, geographic)
*Method used to generate the allocation schedule
*Method of allocation concealment, and timing of assignment
*Method to separate the generator from the executor of assignment

CONSORT on Masking (blinding)

Describe:
*Mechanism (e.g., capsules, tablets, packets of formula)
*Similarity of formula characteristics (e.g., appearance, taste, weight of packets/contents, ease of deductive biochemical or other analysis: docosahexaenoic acid in serum?)
*Allocation scheme control [location(s) of code during trial and when broken—individual breaches and across-trial]
*Evidence for successful masking of mother/baby; doctor who assigned diet to baby; outcome assessors; data analysts.

SCHEME 6

COHORT MINIMIZATION TO SELECT BREAST-FED INFANTS FOR PARALLEL STUDY

Before moving on to analysis, I'd like to suggest a novel use of minimization to select the sort of parallel cohort of breast-fed infants which features as a nonrandomized control group in many nutrition studies. Whether it is generally useful to study such nonrandomized cohorts is debated elsewhere.

If the aim is to select breast-fed infants for study so that they have the same center distribution, and same gestational age distribution, same maternal education distribution as the

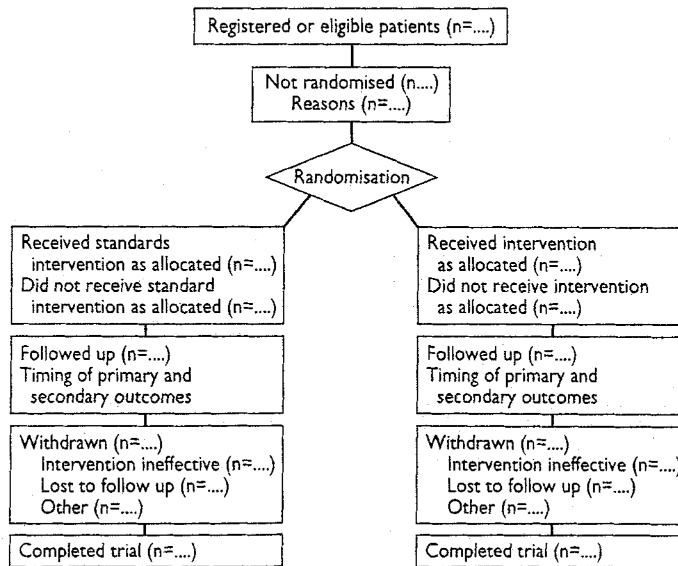

FIG. 3. Flow chart to be used in describing patient progress through randomized trials. This flow chart is intended to produce better reporting of randomized control trials and is designated as the Consolidation of Standards for Reporting of Trials (CONSORT) statement. Flow chart reprinted with permission from *JAMA,* 1996, *276* (8), 637–639. Copyright 1996, American Medical Association.

infants randomized to formula (FM vs. FM + LCPUFA), then for each breast-fed infant who is potentially eligible for study, compute its "cohort score."

Cohort score is shown in Scheme 7 for a 30–32 wk preterm infant born in Cambridge to a university-educated mother. In respect of the covariates of interest (gestational age, center, and maternal education) the cohort score basically works out whether recruitment of this baby would tend to equalize, or to imbalance, the number of Cambridge babies randomized per formula group as compared to the average of cohorted plus randomized babies from Cambridge, and likewise for the other two covariates that apply to this potentially eligible breast-fed baby. The cohort score sums the discrepancies over the three covariates of interest for this potential recruit.

If the baby's cohort score ≥ 0, then the infant is recruited into the cohort with probability 0.8 because its recruitment will make for a better balance between randomized and cohorted babies. But, if its cohort score < 0, baby is cohorted with probability 0.1 only because its recruitment exaggerates the existing imbalance.

Such design considerations can thus aid selection of a more appropriate breast-fed cohort. There is also the assurance that since all potential recruits are notified and detailed to the central randomization office, there is minimal opportunity for untoward selection bias on investigators' part. Recall that previously low sd for Teller acuity was a clue to such untoward selection bias.

Cohort minimization

Selection of breast-fed infants for study so that they have same center distribution, same gestational age distribution, and same maternal education distribution as the infants randomized to FM or FM + PUFA.

Centers = Adelaide, Barcelona, Cambridge
Gestation = 27–29 wk preterm, 30–32 wk preterm, or term
Education = school to age 16, high school, or university

Next eligible breast-fed baby: 30–32 wk preterm infant born in Cambridge to university-educated mother. Its cohort score:

#RCT(Cambridge)/2 − [#RCT(Cambridge) + #Breast(Cambridge)]3 +
#RCT(30–32 wk)/2 − [#RCT(30–32 wk) + #Breast(30–32 wk)]/3 +
#RCT(university)/2 − [#RCT(university) + #Breast(university]/3
That is, Cohort score = sum of Observed − (expected marginal)

If Cohort Score ≥ 0, baby is cohorted with probability of 0.8.
If Cohort Score < 0, baby is cohorted with probability of 0.1.

If All (expected marginals) = 0, cohort with probability of 0.5.

SCHEME 7

ANALYSIS AND REPORTING STANDARDS

Cochrane Collaboration and systematic reviews. Consideration should be given to establishing a Cochrane Collaborative Center to conduct systematic reviews of RCT of infant nutrition according to the standards expected of Cochrane Collaborations and to update them regularly.

Statistical overview, or meta-analysis, of a series of clinical trials requires from each: integrity of design, analysis and reporting. The goal of a common protocol available to all investigators would assist in ensuring adequacy of trial design, including plausible target differentials, sufficient trial size to ensure not less than 50% power and ideally at least 80% power, randomization and masking to CONSORT standards,

and attention to the timing of visual acuity measurements and assessment protocol.

Integrity of analysis concerns the avoidance of untoward stopping and careful consideration of potential biases, for example in the handling of protocol deviations. Particular care is needed in the conduct and reporting of regression analyses. Some analysis give-aways include the following: too little variation; *n* not divisible by number of formulas; NS without 95% confidence interval; inconsistency between outcome measures or across observation times; selective emphasis in results/discussion on comparisons which were not of prior interest; *P*-values rather than clinically meaningful data summaries/results; effect sizes which are *a priori* implausible; subgroup analyses without formal tests of interaction; and interactions which influenced neither randomization nor trial size considerations.

Factorial treatment structure or dose response studies. The interplay between design and analysis is pre-eminent when randomized treatments have a factorial structure such as: group 1, FM; group 2, FM + DHA (0.35%); group 3, FM + arachidonic acid (AA) (0.70%); and group 4, FM + DHA (0.35%) + AA (0.70%); this structure allows the main effect of DHA (0.35%) to be estimated efficiently by comparison of groups 2 and 4 vs. 1 and 3 and of AA (0.70%) by comparison of groups 3 and 4 vs. 1 and 2.

If it is considered *a priori* likely, for example, that AA (0.70%) may potentiate the effect of DHA (0.35%), then this interaction can be assessed by comparing the effect of DHA (0.35%) in the absence of AA (0.70%) (group 2 minus group 1 differential) with the effect of DHA (0.35%) in the presence of AA (0.70%) (group 4 minus group 3 differential). Factorial treatment structure is the only way to address the question of potentiation, but answering it powerfully requires that trial size sums be done in respect of plausible magnitudes for the interaction, that is, for the differential effect of DHA (0.35%) in the presence vs. absence of AA (0.70%). Statistical collaboration is advisable. However, a factorial treatment structure has much to commend it, specifically when no such interaction is anticipated, because the four-way randomization we have been discussing effectively allows two trial questions to be answered for the price of one.

Another design feature related to analysis occurs when treatments are selected so that dose response can be estimated as in comparison of 7-mon visual acuity between infants randomized to formulas differing only in α-linolenic acid content (e.g., 0.4, 0.8, 1.6, 3.2% of fatty acids). The objective at analysis is to model the dose-response relationship. Is it significantly nonlinear to an important degree?

Reporting standards: adjusted vs. unadjusted analyses. Whenever regression methods are used there should be a statistical strategy for doing so. This requires consideration both of the coding of covariates [one coding would be simply an indicator variable for preterm (vs. term) babies; a more informative coding might be number of weeks before or after 40 wk of gestation at which the babies were born, namely, (gestational age − 40 wk); the reader can think of others] and the prioritizing of covariates. Some covariates relate to trial design: randomized treatment allocation, center, randomization stratum such as birthweight <1200 g or 1200–1849 g. They should be fitted for that reason. Other covariates are widely recognized to be prognostic and could be expected to be in the regression model, whether they are formally statistically significant in these data or not, such as gender, maternal education, ... Yet others may have been recorded speculatively, and the statistical strategy should be designed to guard against false positive signals in trawling through this set of speculative covariates. This applies *a forteriori* to trawling for interactions. Finally, there may be some specific objective of analysis such as to explore dose-response, whether in a randomized or nonrandomized context. Consider our multicenter feeding trials: does the percentage of its nutrition that an infant receives from the trial diet explain developmental outcome at 18 mon for babies randomized in the supplement trials? Another specific objective of analysis may be to check out an interaction that affected trial size considerations or a lesser one that influenced randomization, say, as a stratification or minimization factor. The trial size considerations for our multicenter feeding trials anticipated a quantitative interaction by assuming a smaller effect size but benefit from preterm formula nonetheless as in the supplement vs. primary trials. We did not expect a so-called qualitative interaction, wherein the direction of effect switches from benefit to harm.

In preparing tables and figures for publication, investigators should ensure that readers can follow how covariates have been recoded and that they are provided with descriptive statistics for the recoded covariates. Standard errors for regression effects should be cited, and also the residual standard deviation. The tables or text should also allow readers to follow the investigators' statistical thinking by documenting the regression chi-square (or equivalent other statistic) and degrees of freedom for salient intervening models by which the end simplification was arrived at.

Readers should expect to see justification of linearity, for example by indicator variables having been fitted for quartiles of the data and the regression coefficients for these showing a suitably linear progression, or because a quadratic term had been fitted to check for nonlinearity, or otherwise.

Readers should be wary of specious (that is: wrong!) statistical reasoning such as "covariate does not differ significantly between treatment groups, therefore no need to adjust for it." Differences between properly randomized groups in the distribution of a highly prognostic covariate may influence the comparison between treatments and therefore need to be adjusted for, despite the distributional difference not being statistically significant. Contrariwise, a highly significant distributional difference in an irrelevant covariate, that is, one which does not affect outcome, while it might lead to checks that there had not been some breach of the randomization procedure, would be unlikely to alter estimation of the treatment effect.

Reporting standards: an example. Table 3 is taken from a much-quoted *Lancet* paper (7) on breast milk and subsequent IQ in children born preterm. Only 11% of infants who re-

TABLE 3
Breast Milk and Subsequent IQ in Children Born Preterm

Factors relating to IQ	Increase in IQ	95% Confidence interval
Received mother's milk	8.3	4.9 to 11.7
Social class[a]	−3.5 per class[b]	−5.5 to −1.5
Mother's education[a]	2.0 per group[c]	0.5 to 3.5
Female gender	4.2	1.0 to 7.4
Days of ventilation[a]	−2.6 per wk	−3.7 to −1.5

[a]Full descriptive data given in Lucas and Morley (7).
[b]Classes 1 and 2; 3 nonmanual; 3 manual; 4 and 5.
[c]5-point scale from 1 = no qualifications to 5 = degree or higher.

ceived no mother's milk were from social class 1 or 2 compared to 30% of the 210 infants whose mothers intended to breast-feed. Of these 210, 17 mothers failed to express any breast milk. Of the mothers who were unsuccessful, 24% were from social class 1 or 2 compared to 31% of mothers who successfully expressed breast milk, a difference which was not statistically significant. Nonetheless, social class is a major correlate of IQ and so should feature anyway in regression analyses. The investigators chose to exclude the 17 from analysis of factors relating to IQ, perhaps because their overall IQ was closer to that of the 90 whose mothers opted not to express breast milk, and despite their social class 1 or 2 advantage. Leaving that decision aside, the published paper clearly explains the covariate coding that was adopted and cites 95% confidence intervals, which go roughly from 2 standard errors (se) below to 2 se above the estimated regression coefficient. Less clear is the distribution of covariate codes for the 90 vs. 193 infants who feature in the analysis. And linearity has not been clearly demonstrated. However, the regression model presented here is that which the investigators had used in a previous publication, and there is much to be said for a consistent literature.

Further analyses were summarized in the text as follows without the actual results being shown: "When % diet as mother's milk was regressed against IQ, while adjusting for the above confounders, there was a significant linear relation ($P < 0.05$)—a finding that was greatest for the verbal scale ($P < 0.01$) with a 9-point advantage [95% CI from 6 to 12]."

Notice that we are left to work out for ourselves that the 9-point advantage was associated with 100% mother's milk; the linearity of the relationship has not been justified; and the adjusted advantage for overall IQ has not been cited. Can we deduce it?

We may apply some statistical thinking to the deduction task as follows: (i) unadjusted se for verbal IQ is 8% higher than for overall IQ [see Table II in (7)]; (ii) assume that the same holds for the adjusted comparisons; (iii) verbal IQ advantage = 9, significant at 1% level, which implies [by statistical reasoning]: $2.58 \times$ se (verbal) = 9, that is, se (verbal) = 9/2.58 = 3.5, which implies (using i and ii) that se (overall) = 3.5/1.08 = 3.2; (iv) overall IQ advantage, we were told, was significant at 5% level, hence $1.96 \times$ se (overall) = overall IQ advantage = $1.96 \times 3.2 = 6.3$ (associated with 100% mother's milk). Recall that median %diet as mother's milk was around 50% in the low-birthweight feeding trial, and so the typical adjusted overall IQ advantage might have been of the order of 3. Future investigators, of LCPUFA for example, should bear in mind the typical IQ rather than the greatest differential (which happened to be in verbal IQ).

Analysis and other give-aways in published trials. Analysis give-aways listed earlier include NS = not statistically significant, but without appropriate qualification such as by quoting se or 95% confidence interval. I interpret NS as a non-statement in such contexts.

Beware, also, of selective emphasis in Results or Discussion on comparisons that were not of prior interest. Subgroup analyses without formal tests of interaction, which are the statistical equivalent of the drunk man who leans on a lamp post for support rather than illumination, are often paraded in desperate defense of selective emphasis. Beware also flaunted interactions which are *post hoc* in that they influenced neither trial design through stratified randomization nor trial size considerations. Also be concerned about inconsistency of inference between outcome measures or across observation times unless transience of effects was postulated *a priori*.

Another give-away is claimed effect sizes in trial size calculations which have not been authenticated and are not *a priori* plausible.

Too little variation is another give-away, which alerts the reader to possible exclusions from analysis; and, if the number randomized is not divisible by the number of treatments when permuted block randomization was used, there should be some explanation given thereof in the text.

Worst of all are abstracts and text which cite only *P*-values without giving a single clinically meaningful data summary.

SUMMARY

Recommended reading are: Guidelines for Standard Operating Procedures for Statistical Practice in Clinical Research (1), Biostatistical Methodology in Clinical Trials in Applications for Marketing Authorizations (2), and the CONSORT recommendations (6) on randomization, masking, and flow-diagram on accrual and follow-up (Scheme 6, Fig. 3).

Scheme 8 is an *aide memoire* to data sources and considerations to be taken into account in determining rational tar-

Rational target difference for each major outcome variable?

Animal studies
Related randomized studies on other formulas
Epidemiological studies, e.g., breast-fed vs. formula

Order of magnitude: relative to gender effect or effect per extra week of gestation?

Differential effect: greater in preterm than in term infants; less in infants who receive formula only as supplement to mother's own milk?

Transient or persistent effect: maximum impact when (and why so)?

SCHEME 8

get differences for each major outcome variable. Prior opinion of participants in the Clinical Trials Workshop was elicited about the likely change in Bayley developmental scores at 18 mon for infants randomized to LCPUFA-enriched formula vs. control formula. The mean *a priori* plausible differential was 3.3. By assuming sd of 16 for the revised Bayley test, this means (see Scheme 2) that $A4 = 2 \times 16 \times 16/(3.3 \times 3.3) = 47$ and, for 80% power and 5% significance level, number to be randomized per formula $= 8 \times 47 = 376$ and hence some 800 infants in total.

Figure 4 shows the corresponding sum when the outcome variable is binary (success/failure, necrotizing enterocolitis (NEC)/no NEC) rather than continuous or measured (weight gain or Bayley developmental score), for which use Scheme 2. If a meta-analysis of several RCT, or a single study, wanted to have 80% power to detect a doubling in the incidence of NEC with control vs. experimental diet, how many infants would need to be studied? The answer depends upon the nursery incidence of NEC which in Cambridge, for example, is nearer 1% than 5%. To detect a doubling in incidence from 1% to 2% requires: $A5 = 99 \times 1 + 98 \times 2/[(2 - 1) \times (2 - 1)] = 295$. The number per treatment group $= 8 \times 295 = 2360$, and hence requires some 5000 infants in total.

If other serious infections were added to NEC, the nursery incidence in Cambridge would rise to about 5%, but detecting a doubling in incidence of "NEC and other serious infections" from 5 to 10% requires randomization of only some 1000 infants in total.

If, however, the randomized trials are sited in nurseries which have an incidence of "NEC and other serious infections" of around 15%, then detecting a doubling in incidence from 15% to 30% requires only 250 randomized infants. It would therefore be scientifically efficient to focus attention on such nurseries because an answer can be obtained more quickly.

Compute:

$$A = \frac{\text{success} \times \text{failure rate on control diet} + \text{success} \times \text{failure rate on experimental diet}}{\text{(target difference in failure rates} \times \text{target difference in failure rates)}}$$

For 80% power and 5% significance level:
number per treatment group = 8 × A

For 50% power and 5% significance level:
number per treatment group = 4 × A

(= half the number required for 80% power)

Table: Necrotizing enterocolitis (NEC) example

Nursery incidence of NEC (%)	For 80% power to discern doubling in nursery incidence of NEC on experimental diet, number of infants randomized (ratio 1:1) needs to be
15	250
5	1,000
1	5,000
0.5	10,000

FIG. 4. Trial size when comparing failure rates.

TABLE 4
Common Protocol: Goal

Goals	Comments
Sample size	Clearly specified and justified in terms of trial objectives; methods used should be referenced or documented, as should estimates of any quantities used in calculations.
Sample size reviews	Reviews, for purpose of altering planned number of patients, should be conducted blindly and noncomparatively.
Multiplicity	Should be addressed: any or all of multiple trial objectives, measures, or endpoints.
Rules to determine evaluability of patients	Especially needed in case of protocol violations, withdrawals, or dropouts (off-study form?).
Generic statistical analysis plans	Outline methods of analysis.
Comprehensive description	Before analysis, plans should be developed into comprehensive description and suite of analysis routines; see Statisticians in the Pharmaceutical Industry (1).

Table 4 serves as a reminder of some topics which a common protocol for infant nutrition studies should seek to agree on, such as rules to determine evaluability of patients, generic statistical plans, and how multiplicity should be addressed (see also 8,9).

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Long-Chain Polyunsaturated Fatty Acids and Eicosanoids in Infants–Physiological and Pathophysiological Aspects and Open Questions

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ABSTRACT: Eicosanoids are highly active lipid mediators in physiologic and pathologic processes, with their effects ranging from cytoprotection and vasoactivity to modulation of inflammatory and proliferative reactions. Generation of eicosanoids can be affected by changes in the pools of their precursors, the long-chain polyunsaturated fatty acids (LCPUFA). Thus, dietary interventions such as supplementation of infant formula with specific n-3 and n-6 LCPUFA will alter formation as well as activity of the eicosanoids produced. This report summarizes the results and discussion of the workshop on "Eicosanoids and Polyunsaturated Fatty Acids in Infants." The intention of the workshop organizers was to give an overview of the role of eicosanoids in physiological and pathophysiological processes in infants, to discuss the implications that an increased n-3 and n-6 LCPUFA intake may have on eicosanoid generation, and to point out open questions and controversies for future research.

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The term eicosanoids was coined to describe collectively the oxygenated derivatives of long-chain polyunsaturated fatty acids (LCPUFA) containing 20 carbon atoms that can be formed either enzymatically or by nonenzymatic (per)oxidation. Arachidonic acid is the predominant precursor fatty acid, owing to its high abundance in phospholipids. Other fatty acids serve as precursors for enzymatically oxygenated fatty acids with 20 carbon atoms, such as dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-6). PUFA with 18 or 22 carbon atoms, such as linoleic acid (18:3n-6) or docosahexaenoic acid (22:6n-3), are also oxygenated by some but not all of the enzymes of the arachidonic acid cascade. Because the role of these metabolites in physiologic processes is not understood, the workshop focused on the metabolites with 20 carbon atoms.

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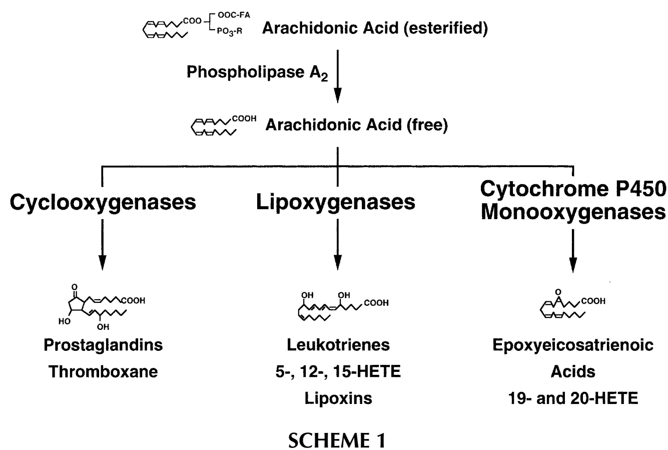
Abbreviations: CNS, central nervous system; HETE: hydroxyeicosatetraenoic acid; LCPUFA: long-chain polyunsaturated fatty acid; LT: leukotriene; PG: prostaglandin; Tx, thromboxane.

THE ARACHIDONIC ACID CASCADE

Arachidonic acid can be oxygenated by three different enzymatic systems: cyclooxygenases, lipoxygenases, and cytochrome P450 monooxygenases (Scheme 1, where FA = fatty acid; for review, see Refs. 1–3). The expression of these enzymes can be rather specific for a certain cell lineage, as, for example, expression of the 5-lipoxygenase in cells of myeloid origin.

After release of arachidonic acid from its storage site in cellular phospholipids or after its transcellular exchange, metabolism by cyclooxygenases leads to the formation of prostaglandins (PG) and thromboxane A_2 (TxA_2), whereas 5-, 12-, or 15-lipoxygenase activity results in the formation of the respective monohydroxylated fatty acids (mono-HETE). Production of leukotrienes (LT) is initiated by 5-lipoxygenase, whereas subsequent oxidation by the 5-lipoxygenase and 12- or 15-lipoxygenases results in the formation of trihydroxylated compounds, the lipoxins. Metabolism of arachidonic acid by cytochrome P450 monooxygenases gives rise to epoxides, the epoxyeicosatrienoic acids, and ω-hydroxylated derivatives of the precursor fatty acids or other eicosanoids. Cross-talk between these different enzymatic systems leads to the formation of additional eicosanoids, e.g., 15-epilipoxins, which are generated by the subsequent metabolism of as-



SCHEME 1

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pirin-inhibited cyclooxygenase-2 and 5-lipoxygenase (4).

Eicosanoids are highly active lipid modulators of a multitude of physiologic and pathologic processes (1,2,5). Among their most important properties are cytoprotective effects, modulation of renal function, mediation of allergic and inflammatory reactions, induction and inhibition of thrombotic processes, and regulation of smooth muscle cell tone leading, for example, to vaso- and bronchoconstriction or -relaxation and to the postnatal closure of the *ductus arteriosus Botalli*. *In vitro* observations further indicate that eicosanoids may have a multitude of additional biochemical and physiologic effects; the most important ones are outlined in Table 1. Apart from being intercellular mediators, arachidonic acid and eicosanoids also have been identified as novel intracellular second messengers in inflammatory and mitogenic signaling (6). However, since we are just beginning to understand this role of eicosanoids, it was beyond the intention of this workshop to discuss possible effects of PUFA on intracellular signaling by eicosanoids.

EICOSANOID SYNTHESIS FROM ALTERNATIVE PUFA

In addition to arachidonic acid, dihomo-γ-linolenic acid (20:3n-6) and eicosapentaenoic acid (20:5n-3) also serve as precursors for eicosanoids. Dr. Howard R. Knapp, University of Iowa, gave an overview of the *in vivo* generation and detection of eicosanoids derived from these alternative fatty acid precursors. He pointed out that the metabolism of these PUFA to PGE_3 or LTB_5, which has been observed *in vitro*, does not necessarily correspond to an abundant generation of these compounds *in vivo*. Dihomo-γ-linolenic acid is principally converted to the 1-series PG and the 3-series LT. However, *in vivo* formation of these eicosanoids is barely detectable, with the exception of PGE_1 in seminal fluid. In contrast, eicosapentaenoic acid is readily metabolized to PG of the 3-series such as PGI_3 or TxA_3 and to LT of the 5-series such as the peptidoleukotrienes LTC_5 and LTD_5. This is of physiologic importance, since some eicosapentaenoic acid-derived eicosanoids have different activities from those of the respective arachidonic acid-derived compounds (7,8). Thus, TxA_3 has a much lower potency to activate platelets or to induce vasoconstriction than TxA_2, and LTB_5 is much weaker in stimulating neutrophil activation than LTB_4, although a reduction of its generation by n-3 LCPUFA has not yet been proven *in vivo* (9). In contrast, PGI_3 inhibits platelet activation and aggregation to the same degree as does PGI_2 and has equal vasodilatory activity. Furthermore, the proallergic and proinflammatory effects of the peptidoleukotrienes LTC_5/LTD_5 are also equal to the effects of LTC_4/LTD_4. Despite *in vitro* observations suggesting that PGE_3 may be as active as PGE_2, its physiologic relevance remains to be determined, since formation of PGE_3 has not yet been detected in abundant amounts *in vivo* after supplementation with n-3 PUFA.

In addition to conversion to PG and LT, dihomo-γ-linolenic acid, eicosapentaenoic acid, and other LCPUFA can also be oxygenated *in vitro* to various monohydroxylated or epoxide derivatives by lipoxygenase and cytochrome P450 monooxygenase activities, respectively. However, the physiologic effects of these compounds *in vivo* are just being elucidated.

TABLE 1
Physiological and Biochemical Effects of the Most Physiologically Important Eicosanoids[a]

Eicosanoid	Effects	
PGE_2	Vasculature	Vasodilation (arterial), vasoconstriction (venous)
	Airways	Bronchodilation
	Pain	Hyperalgesia
	Stomach	Cytoprotection
	Kidney	Reduction of water or NaCl reabsorption, increase in renin secretion
	Neutrophils	Reduction of activation
	Lymphocytes	Reduction of apoptosis, reduction of cytokine production
	Bone	Increase in osteoblast activity
	Reproductive system	Uterus contraction, induction of ovulation
	Body temperature regulation	
$PGF_{2\alpha}$	Vasculature	Vasoconstriction
	Reproductive system	Uterus contraction, induction of ovulation
PGI_2	Vasculature	Vasodilation, reduction of platelet aggregation and activation
	Airways	Bronchodilation
TxA_2	Vasculature	Vasoconstriction, platelet aggregation and activation
	Airways	Bronchoconstriction
LTB_4	Neutrophils	Chemotaxis, superoxide anion generation, degranulation, increased expression of adhesion molecules, aggregation
	Monocytes	Increase in cytokine production
	Lymphocytes	Induction of T-suppressor cells, reduction of apoptosis in premature cells, increase in cytokine production
LTC_4/LTD_4	Vasculature	Vasoconstriction, vascular leakage, plasma extravasation
	Airways	Bronchoconstriction, mucus secretion

[a]For references see reviews (1,13,51) for prostanoids and (16,52,53) for leukotrienes. PG, prostaglandin; Tx, thromboxane; LT, leukotriene.

TABLE 2
Eicosanoids and Diseases in Infants[a]

Diseases	Eicosanoids	Δ	Suggested mechanism	Reference
Bleeding disorders	TxA_2	⇓	Platelet aggregation, vasodilation	54
Patent ductus arteriosus	PGE_2, PGI_2	⇑	Inhibition of occlusion	10
Necrotizing enterocolitis	PGE_2, TxA_2,LTB_4	⇑	Cytokine formation, PMNL activation	55
Juvenile arthritis	LTB_4	⇑	Inflammation	52
Neonatal hypoxia with pulmonary hypertension	LTC_4, LTD_4	⇑	Vasoconstriction	56
Bronchopulmonary dysplasia	TxA_2, LTB_4	⇑	Inflammatory response	57,58
Cystic fibrosis	LTC_4, LTD_4, LTB_4	⇑	Mucus secretion, inflammation	52
Allergic responses	LTC_4, LTD_4	⇑	Vascular leakage, edema formation	52
Renal failure	PGE_2, PGI_2, LTB_4	⇑	Natriuresis, diuresis, inflammation	59
Celiac disease	PGE_2, TxA_2, LTB_4	⇑	Mucosal injury, increased motility	52

[a]PMNL, polymorphonuclear leukocytes; for other abbreviations see Table 1.

EICOSANOIDS AND DISEASES IN INFANTS

Most of the physiologic and pathophysiologic effects of eicosanoids have been established *in vitro* and in animal models and have been confirmed in adults. Less is known about the actions of eicosanoids in infants. Many basic physiologic effects of eicosanoids may be similar in infants and adults, such as activation or inhibition of platelet aggregation, vasorelaxation or vasoconstriction, stimulation of inflammatory and allergic reactions or cytoprotection. Dr. Ricardo Uauy, University of Santiago de Chile, discussed the potential role of eicosanoids in the manifestation and/or progression of several diseases in infants. Some of these are listed in Table 2. Clear proof that the suggested pathophysiological mechanisms are active *in vivo* and underlie the disease process exists for only a few diseases, such as the effects of cyclooxygenase inhibition on the occlusion of patent ductus arteriosus (10). In infants with congenital heart diseases that depend hemodynamically on an aortopulmonary shunt, the ductus can be kept open by continuous infusion of a PGE_1 mimetic (10). Furthermore, decreased urinary excretion of 6-keto-$PGF_{1\alpha}$, the stable metabolite of PGI_2, and of PGE metabolites has been observed in the first days of life (11). Whether the decrease in vasodilatory PG formation in the newborn period underlies additional changes of physiologic processes during the first days of life remains to be examined. However, in most examples listed, only an association between eicosanoid levels in biological fluids or tissue samples and a certain disease has been made, without demonstration of a clear causal relationship.

Effects of supplementation with dihomo-γ-linolenic acid or n-3 LCPUFA on eicosanoid formation related to the course of diseases have not yet been studied in infants. Given the observations in adults, it may be expected that n-3 LCPUFA could modulate immunologic, inflammatory, vascular, and thrombotic responses in infants. Indeed, marine oil supplementation in preterm infants led to a significant increase in bleeding time vs. infants fed nonsupplemented control formula (12). However, the bleeding times remained in the normal range (below 5 min) and were not associated with an increase in bleeding complications.

SPECIAL ASPECTS OF DIETARY MODULATION OF EICOSANOID GENERATION IN INFANTS

Whereas in many physiological or pathological processes modification of eicosanoid synthesis by n-3 and n-6 LCPUFA may lead to the same responses that have already been identified in adults, some effects which have not yet been sufficiently investigated may be of special importance in infants and merit further consideration. Thus, possible effects on the maturation and activity of the immune system and the modulation of growth responses may be of special importance.

Maturation and activity of the immune system. Eicosanoids, especially PGE_2 and LTB_4, have been implicated as modulators of proliferation, differentiation, and cytokine synthesis in cells involved in the immune response (13–16). PGE_2 and LTB_4 have been found to reduce apoptosis of premature thymocytes (13,15), whereas apoptosis is increased by TxA_2 (13,15). Furthermore, LTB_4 induces formation of $CD8^+$ T-suppressor cells while inhibiting proliferation of $CD4^+$ T-helper cells (16). PGE_2 also inhibits proliferation of T-helper cells (15,16). Both eicosanoids may also affect B-cell function by increasing immunoglobulin synthesis and modulating B-cell differentiation and growth (14,16). In addition, cytokine synthesis in lymphocytes and monocytes is greatly inhibited by PGE_2, whereas LTB_4 in most studies had a stimulatory effect (Table 3; Refs. 15,16). Although most of these observations have been made in lymphocytes or prelymphocytes of different animal species and although they do not yet definitively link the action of either PGE_2 or LTB_4 to a specific immune response, they support the view that the generation of eicosanoids can modulate the maturation and activity of immunocompetent cells.

These observations raise the question whether supplementation with n-3 LCPUFA may alter the maturation and activity of an infant's immune system. In particular, the observed decrease in overall PGE formation [reduced generation of PGE_2 and PGE_1 without abundant synthesis of PGE_3 (17)] after supplementation with n-3 LCPUFA may affect proliferation and differentiation of lymphocytes and cytokine production. In accordance with such an effect, Dr. Jane Carver, University of South Florida, reported preliminary data indicating that sup-

TABLE 3
Modulation of Immune Responses by PGE_2 and LTB_4[a]

Response		PGE_2	LTB_4
Apoptosis of prelymphocytes		+	+
Proliferation of T-helper cells		—	—
Proliferation of T-suppressor cells		?	+
Proliferation of B-cells		—	+
Immunoglobulin synthesis of B-cells		±	+
Migration of T-cells		+	+
Cytokine synthesis			
T-helper cells	IL-3	—	?
	GM-CSF	—	?
	IFN-γ	—	+
T-suppressor cells	IFN-γ	?	—
Monocytes	IL-1	—	+
	TNF-α	—	+

[a]+, stimulation; —, inhibition; ?, effect not yet determined; IL, interleukin; GM-CSF, granulocyte/macrophage colony stimulating factor; IFN-γ, interferon-γ; TNF-α, tumor necrosis factor α. For other abbreviations see Table 1. For references see 13–16.

plementation with n-3 PUFA in rat pups increased thymocyte differentiation to a $CD4^+CD8^-$ cell type while reducing $CD4^+CD8^+$ prelymphocytes. Since LTB_4 has been found to induce $CD8^+$ suppressor cells, these observations also point to the necessity to determine whether the activity of LTB_4 differs after n-3 LCPUFA increases. Thus, supplementation with eicosapentaenoic acid ameliorated the clinical state in children with cystic fibrosis (18), and this was correlated with an upregulation of LTB_4 receptor activity (19). In contrast, a reduction in the *in vivo* production of LTB_4 has not been observed after supplementing adults suffering from allergic rhinitis with moderate doses of n-3 LCPUFA (9).

Observations and experiences in populations with a traditionally high fish intake seem to argue against a strong immunosuppressive effect of dietary n-3 LCPUFA, since no increase in immunodeficiencies or decreases in the success of vaccination programs have been reported so far in these populations. Supplementation with n-3 LCPUFA has been observed to slow the progression of adult diseases that potentially involve autoimmunity, such as rheumatoid arthritis (20) and immunoglobulin A nephropathy (21). In contrast, n-3 LCPUFA had no effect on graft rejection after renal transplantation (22,23) and did not delay the decrease in renal function in patients with lupus nephritis (24), but they had a clearly beneficial effect on autoimmune-induced kidney diseases in rodent models (25,26). Whether these positive effects of n-3 LCPUFA are related to a decrease in the immune response or to a decrease in the accompanying inflammatory reaction, both of which may result from the well-documented reduction in cytokine formation by n-3 LCPUFA, is not known (27). Therefore, controlled trials should be performed in infants to clearly determine whether infant formulas that decrease n-6 LCPUFA or increase n-3 LCPUFA levels alter the immune response.

Modulation of the growth response. The effects of eicosanoids and LCPUFA on growth are not yet fully established. Taken together, epidemiological as well as animal studies indicate that increased consumption of n-3 LCPUFA is associated with a decrease in pathologic and hyperproliferative growth responses such as in tumor progression or chronic proliferative disorders (28,29). In studies of adult humans and of other mammals no adverse growth-inhibiting effects of n-3 LCPUFA on physiologic (i.e., regenerative) growth, such as of hematopoietic stem cells or intestinal epithelial cells, have been reported. In addition, supplementation of pregnant women with n-3 LCPUFA had no effect on growth and weight of the newborns, although gestational length increased slightly but significantly (30). Furthermore, lower levels of arachidonic acid in red blood cell phospholipids in term infants fed formulas containing different amounts of n-3 and n-6 LCPUFA were not associated with reduced growth at 3 (31) or 12 (32) mon after birth. In contrast, feeding term infants a formula containing either high or low levels of α-linolenic acid while linoleic acid content was kept constant resulted in a significant reduction in plasma levels of phospholipid arachdonic acid and in body weight during the first 4 mon of life in the infants receiving the high α-linolenic acid formula (33). Similar observations were made in preterm infants: Reduced growth was observed after supplementation with a fish oil rich in the n-3 LCPUFA eicosapentaenoic acid (34), and lower arachidonic acid status in preterms was associated with lower first-year growth even among infants who were fed formula without n-3 LCPUFA (35). In several other studies in premature infants, depletion of n-6 LCPUFA has been associated with reduced growth (36–38). Although these data from feeding studies are still controversial, a number of these studies suggest that levels of n-6 LCPUFA, especially of arachidonic acid, may correlate with growth. This assumption is further supported by *in vitro* observations demonstrating that cellular arachidonic acid metabolism stimulates and promotes cell growth (39,40).

On a cellular and molecular level, various eicosanoids have been found either to stimulate or to inhibit the mitogenic

response. Thus, PGE_2, TxA_2, $PGF_{2\alpha}$, or LT in the main increased cell growth (41), whereas PGI_2, PGA_2, and in certain cell types also PGE_2 inhibited cell growth. In a recent *in vitro* study, we further demonstrated that the mitogenic response in mouse 3T3 fibroblasts was induced by the cellular metabolism of arachidonic acid to PGE_2, whereas n-3 LCPUFA antagonized mitogenic stimulation with arachidonic acid by reducing PGE_2 formation (42). Dr. Bruce A. Watkins from Purdue University indicated that PGE_2 may have a biphasic effect on bone formation, increasing osteoblast activity and bone formation at low concentrations but reducing bone formation at high concentrations by increasing osteoclast activity as well. n-3 LCPUFA decreased PGE_2 levels and increased bone formation in animals.

These observations indicate that a delicate balance between pro- and antiproliferative effects of eicosanoids is likely to exist and that the superimposed effects of n-3 LCPUFA will likely add further complexity. Observations in various organs and tissues in populations with a traditionally high intake of n-3 LCPUFA detected neither growth retardation of term infants at birth nor growth delay of infants and children, suggesting that other factors also must come into play such as the balance of n-3 and n-6 LCPUFA achieved *in utero* and beyond. Further research is required to determine which amounts of n-3 and n-6 LCPUFA have to be supplied to pregnant women and infants for optimal physiologic balance between these two classes of PUFA.

EICOSANOIDS IN THE CENTRAL NERVOUS SYSTEM

The role eicosanoids have in the maturation of the central nervous system (CNS) and related functions such as cognitive processes, visual acuity, or behavioral responses is difficult to establish. However, observations in animals and in a few clinical trials in infants indicate that eicosanoids may be important signaling molecules in the CNS. That PGE_2 regulates body temperature (43) and modulates nociception of pain, mainly by inducing hyperalgesia, is well documented. Prostaglandins and hydroperoxy derivatives of arachidonic acid have been suggested to influence synaptic plasticity through their effects on long-term potentiation and depression of neuronal function (44). PGE_1 has also been suggested to increase behavioral activation and to exert antidepressant activity (45). Furthermore, PGD_2 (46) and to a lesser degree lipoxins and LT (47) have been found to promote sleep in rodents, an activity which is counteracted by PGE_2 (48). In support of a potential role of PUFA in regulating sleep, it has been observed that slow-wave sleep is reduced in children maintained on a totally fat-free parenteral nutrition for months as compared to children whose parenteral nutrition was supplemented with essential fatty acids (49).

Taken together, these observations indicate that eicosanoids modulate important processes in the maturation of the CNS as well as in behavioral responses. Further work is required to characterize more clearly the role of eicosanoids in the CNS and to delineate the effects of LCPUFA supplementation on these processes.

OPEN QUESTIONS

Apart from clearly determining the effects of n-3 and n-6 LCPUFA supplementation on the maturation and the activity of the immune system and on physiological growth patterns, several other issues related to these items await further research and clarification in infants.

One central issue is the determination of the amount of n-3 and n-6 LCPUFA which can be supplied to infants without provoking severe adverse effects due to a shift in eicosanoid generation from these LCPUFA. This implies that individual needs may differ, as various diseases and disorders may benefit from different eicosanoid levels. In some situations (e.g., in thrombotic conditions or in inflammation) a higher ratio of n-3 over n-6 fatty acids may be more beneficial than merely supplying LCPUFA for a balanced nutrient intake. In contrast, individuals with bleeding disorders may profit from a formula with a reduced n-3 LCPUFA content. These considerations may be extended to inflammatory disorders, pulmonary hypertension, or patent ductus arteriosus. Basic to such concepts is, of course, the determination of *in vivo* eicosanoid synthesis in infants and the effects of LCPUFA supplementation. Dr. Margaret Craig-Schmidt, Auburn University, reported that neonatal piglets fed infant formula enriched with 20:4n-6 had higher formation of PGI_2 and TxA_2 in lung tissue than piglets fed no arachidonic acid, whereas dietary 22:6n-3 decreased the levels of both eicosanoids (50). These observations indicate the importance of investigating the effects of single n-3 and n-6 LCPUFA in animal models. Preparations now available allow the addition of a single LCPUFA to experimental formulas, as well as combinations of LCPUFA. In the appropriate animal model, studying these effects on eicosanoid formation can be useful in helping design strategies for formula supplementation.

Finally, the sources and the form of the LCPUFA supplied may be of importance for eicosanoid formation. Thus, it may be necessary to determine whether n-3 LCPUFA extracted from single-cell oils have effects on eicosanoid formation that are identical to those of n-3 LCPUFA from marine oils. Furthermore, the lipid class containing the LCPUFA may be of importance. It has to be ruled out whether the different forms in which LCPUFA can be supplied, either the free ethyl esters which allow a highly concentrated supply of the respective fatty acids, LCPUFA esterified in phospholipids, or LCPUFA supplied as triglycerides, have different biological activities and, if so, the form of supplementation which is the most suitable must be determined.

CONCLUSION

The workshop on eicosanoids in infants and their modulation by LCPUFA supplementation made evident that eicosanoids are important physiologic and pathophysiologic mediators in

infants. Various diseases in infants are asssociated with a change in eicosanoid formation. Further research is needed to better define the effect of LCPUFA supplementation, especially that of n-3 LCPUFA on eicosanoid formation and its impact on the modulation of physiologic processes including growth and immune responses as well as their effects on pathologic conditions.

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Biological Effects and Safety Issues Related to Long-Chain Polyunsaturated Fatty Acids in Infants[1]

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ABSTRACT: The purpose of this workshop at the American Oil Chemists' Society Symposium, "PUFA in Infant Nutrition: Consensus and Controversies," was to enumerate the safety issues raised by the prospect of supplementing infant formulas with long-chain polyunsaturated fatty acids (LC-PUFA), to evaluate the evidence that these concerns are problematical, or theoretically problematical, and to identify the safety issues most in need of resolution. This was approached by reviewing briefly the known biological effects of LC-PUFA and how these effects might give rise to concerns about safety of LC-PUFA as components of infant formulas. Some of these issues were then discussed in more detail by invited participants, all of whom had submitted abstracts concerning the issue discussed. The pertinent aspects of all issues discussed during the workshop are summarized. In addition, since the symposium was held over 2 yr ago, an addendum summarizing additional data reported since the symposium that either support or refute issues discussed during the workshop also is included.

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Considerable evidence exists that a dietary source of long-chain polyunsaturated fatty acids (LC-PUFA) during infancy may be beneficial with respect to subsequent visual and neural development. This evidence is based on comparisons of relatively short-term visual and neurodevelopmental outcomes of breast-fed vs. formula-fed infants as well as infants fed formulas with LC-PUFA vs. without LC-PUFA. These data, as well as the methods used to obtain them, were evaluated in other sessions of this conference and are reviewed in the summaries of the respective sessions.

At the outset, it is important to note that the described benefits of LC-PUFA are subtle. Formulas without LC-PUFA have been fed to infants for decades and have not resulted in epidemics of either poor vision or neurodevelopmental delays. This highlights the importance of questions that have been raised concerning the immediate and long-term safety of LC-PUFA intake during infancy. For example, it is logical to ask, as several have, if addition of bioactive substances such as LC-PUFA to infant formulas for their perhaps beneficial but, nonetheless, subtle effects on visual and neural development might result in undesirable effects on other systems that, on balance, negate any beneficial effects on visual and/or neural development. Within this context, even the largely theoretical concerns that have been raised represent problems that must be resolved before widespread supplementation of infant formulas with LC-PUFA can be unconditionally recommended.

BIOLOGICAL EFFECTS OF LC-PUFA AND SAFETY ISSUES RAISED BY THESE EFFECTS

Addition of LC-PUFA to infant formulas is known to influence the fatty acid pattern of both plasma and erythrocyte membrane lipids, and it is assumed that the fatty acid pattern of tissue membranes is similarly influenced. The fatty acid pattern of membrane lipids, in turn, is thought to affect the characteristics of the membrane. In fact, the beneficial effects of LC-PUFA on visual and central nervous system development are usually attributed to this general effect. LC-PUFA also are known to affect eicosanoid metabolism; for example, increased availability of the major n-3 precursor of eicosanoids [i.e., eicosapentaenoic acid (EPA)] relative to the major n-6 precursor [i.e., arachidonic acid (AA)] results in decreased synthesis of eicosanoids derived from AA and, perhaps, increased synthesis of those derived from n-3 precursors (see summary of the session devoted to eicosanoid metabolism, Ref. 1). These two general effects of LC-PUFA have been known for years. More recently, both n-3 and n-6 LC-PUFA have been shown to influence the rate of transcription of a number of genes including genes controlling hepatic lipogenic and lipolytic enzymes (2).

Any or all of these effects, of course, could result in both beneficial and undesirable effects. For example, the better visual and central nervous system development of breast-fed vs. formula-fed infants and of infants fed formulas supplemented with docosahexaenoic acid (DHA) vs. unsupplemented formulas have been attributed to a greater content of retinal and central nervous system DHA secondary to dietary intake of DHA. However, supplementation of formulas with DHA, particularly supplementation with sources of DHA that also have a high content of EPA (e.g., fish oil), is associated with a lower content of AA in plasma and erythrocyte phospholipids and this, in turn, has been associated with lower rates of growth (3).

This effect on growth could be mediated by the expected effects of n-3 LC-PUFA intake on eicosanoid metabolism, i.e., a

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Abbreviations: AA, arachidonic acid; ALA, α-linolenic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LC-PUFA, long-chain polyunsaturated fatty acids; NEC, necrotizing enterocolitis.

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decrease in eicosanoids derived from AA. Alternatively, it could be mediated by changes in hormone sensitivity and/or binding of hormones to receptors secondary to changes in membrane fatty acid pattern and, hence, changes in membrane characteristics. Altered transcription of genes controlling enzymes involved in metabolism of energy substrates also could affect substrate metabolism and, hence, growth. Unfortunately, data to distinguish among these theoretical mechanisms for lower growth and/or to implicate any or all mechanisms are not available.

Changes in eicosanoid metabolism secondary to LC-PUFA intake might also affect a number of other systems, e.g., hemostasis, and immune function. Uauy *et al.* (4) reported that infants receiving formula supplemented with high-EPA fish oil have longer bleeding times than infants receiving an unsupplemented formula; however, the mean bleeding times of both groups were within normal limits. Inflammatory responses also are affected by LC-PUFA; in general, n-6 LC-PUFA are proinflammatory and n-3 LC-PUFA are antiinflammatory. While an antiinflammatory effect obviously may be beneficial in some circumstances (e.g., autoimmune disorders), such an effect may be undesirable in others (e.g., infection).

In addition to alterations in hormone sensitivity and/or binding to receptors, changes in the fatty acid pattern of membranes might enhance the likelihood of oxidative damage. For example, increasing the unsaturation index of membranes, as is assumed to occur with an increased intake of LC-PUFA, might well increase the likelihood of peroxidation. This, in turn, could contribute to development of a variety of conditions that are thought to be caused and/or aggravated by peroxidative damage; in the infant, such conditions include bronchopulmonary dysplasia and necrotizing enterocolitis (NEC).

EVIDENCE FOR SAFETY CONCERNS

Unfortunately, available studies provide little definitive information concerning the extent to which the safety concerns incident to the known biological effects of LC-PUFA are warranted. Exceptions include the aforementioned difference in bleeding times of infants receiving a formula supplemented with fish oil vs. an unsupplemented formula and the effects of the same type of supplement on growth.

Several participants cited a presentation at a recent symposium in which data from a recent study of Carlson *et al.* (5) were reanalyzed to show that preterm infants who received formula supplemented with low-EPA fish oil had a higher combined incidence of NEC plus sepsis than control infants—an outcome that was not analyzed by the investigators. In this study, 94 infants were randomized to either an unsupplemented formula ($n = 45$) or the same formula with 0.2% of total fatty acids as DHA and 0.06% as EPA ($n = 49$); linoleic (LA) and α-linolenic acid (ALA) contents of both formulas were 21.2 and 2.4% of total fatty acids, respectively. Three infants assigned to the control formula (6.7%) and nine infants assigned to the supplemented formula (18.4%) developed NEC. Two assigned to the control formula (4.4%) and five assigned to the supplemented formula (10.2%) developed sepsis. The difference in incidence of neither condition between infants fed control vs. supplemented formulas was statistically significant. Further, power analysis suggested that 100 subjects per group would have been required to detect statistically significant differences of the magnitude observed in rates of either NEC or sepsis between supplemented and unsupplemented infants. Equally important, the incidence of neither condition in the supplemented group differed significantly from the incidence of that condition within the total nursery population. However, the incidence of the two conditions combined, which was not evaluated by Carlson *et al.* (5), apparently was significantly greater in the supplemented group.

Overall, the incidence of sepsis in both groups was lower than the roughly 20% incidence observed in the late 1980s among 1,765 infants (birthweight < 1500 g) delivered at the seven centers participating in the National Institute of Child Health and Human Development Neonatal Intensive Care Network (6). The 6.7% incidence of NEC observed in the unsupplemented group was similar to that reported among the same 1,765 infants (6). The 18.4% incidence of NEC observed in the supplemented group was high, although not higher than the incidence of this condition at some of the centers making up the National Institute of Child Health and Human Development Neonatal Network (6). Nonetheless, the n-3 LC-PUFA supplement theoretically could result in an increased incidence of NEC by decreasing eicosanoids derived from AA and/or by increasing eicosanoids derived from EPA, thereby decreasing the enteric cytoprotective effects of prostaglandins derived from AA and, perhaps, the ability to mount an effective inflammatory response against invading pathogens. Theoretically, the supplement also could have increased unsaturation of enterocyte lipid membranes, thereby increasing the likelihood of peroxidative damage which also could contribute to development of NEC. However, more information than provided in the report of Carlson *et al.* (5) is necessary to conclude that the difference in incidence of NEC and/or the difference in the combined incidence of NEC and sepsis between the two groups was statistically significant.

Discussion of this anecdotal report, as occurred during the session, is included to illustrate the need for studies with sufficient power to determine if formulas supplemented with LC-PUFA increase the incidence not only of NEC and sepsis but also the myriad other conditions that are common in preterm infants and, theoretically, might be increased by LC-PUFA. Unfortunately, studies of the effects of formulas with a variety of combinations, amounts, and sources of LC-PUFA are likely to be necessary.

In contrast to the lack of data concerning the safety of 3 LC-PUFA in human infants, some data are available from studies conducted in animals. Such studies are particularly useful because relatively large doses of LC-PUFA can be given, thereby identifying potential problems that subsequently can be evaluated in human infants receiving more reasonable LC-PUFA intakes.

Data from a study in rats that examined the effects of maternal diet during pregnancy and lactation on auditory brain stem response, excitoxic neural injury, hypoxic ischemic injury, and immune response of the offspring were presented at the symposium by Carver *et al.* (7). In this study, dams were fed a control

chow diet, a diet supplemented with corn oil (22% of energy), or a diet supplemented with fish oil (22% of energy). The expected effects of the diets on the fatty acid patterns of the dams' plasma and milk as well as the pups' plasma were observed. The latency component of the auditory brain stem response of the offspring of dams fed the fish oil diet was longer at 16, 19, and 29 d of age suggesting a slower rate of conduction. However, cerebral edema 4 d following injection of methyl aspartate into the left ventricle of the cerebrum (to produce excitoxic damage) was less in the offspring of dams fed the fish oil diet, suggesting a protective effect. Hypoxic ischemic injury produced on day 3 of life by ligation of the right carotid artery followed by 2 h in an 8% oxygen environment delayed developmental milestones in all groups, but the delays were more marked in the fish oil group. Contrary to theoretical expectations, mortality following injection of Group B streptococci was lower, not higher, in offspring of dams assigned to the fish oil diet. The percentage of immature CD4+/CD8+ thymocytes also was lower in the offspring of dams fed the fish oil diet while the percentage of mature CD4/+CD8+ thymocytes was higher. Studies addressing at least some of these issues in infants are badly needed.

AMOUNTS OF LC-PUFA THAT SHOULD BE ADDED TO INFANT FORMULAS

Many believe that some of the theoretical problems discussed above can be avoided by supplementing formulas with both AA and DHA. Most also believe that high intakes of EPA should be avoided. Unfortunately, few published data exist either to support or to refute these beliefs. Representatives from several formula manufacturers cited preliminary data from just-completed or soon-to-be-completed multicenter studies showing no difference in either growth or morbidity between infants assigned to an unsupplemented formula and those assigned to the same formula supplemented with DHA and AA. However, the amounts and sources of DHA and AA differed considerably among the studies, highlighting concerns regarding the amounts and sources of fatty acids that should be added to formulas.

Regulatory agencies usually condone addition of components of human milk to formula in the same amounts that are present in human milk. However, there are nutrients for which this guideline clearly is not appropriate. For example, more vitamin D than is present in human milk appears to be desirable. In addition, iron and zinc are more bioavailable from human milk than formula. Thus, modern infant formulas contain more vitamin D, iron, and zinc than human milk.

Although advocated by many, it is not clear that the amounts of the various LC-PUFA in human milk are the appropriate guidelines for the amounts that should be present in formulas. First, these fatty acids may be conditionally essential for some infants, but they are not essential nutrients in the traditional sense. Further, the amount of LA and ALA, the precursors for endogenous synthesis of AA and DHA, respectively, as well as the amounts of all longer-chain, more unsaturated n-3 and n-6 fatty acids in human milk are quite variable (8); coefficients of variation are at least 50% for most. This variation in part reflects the maternal plasma fatty acid pattern which, in turn, reflects the fatty acid content of the mother's diet rather than regulation of the fatty acid content of human milk fat at the level of the mammary gland. In contrast, the ratio of AA and DHA in human milk appears to be somewhat less variable than the absolute amount of either (the coefficient of variation of the ratio is roughly half that of either AA or DHA). Thus, it has been argued that the ratio of these two fatty acids is more important than the absolute amount of either.

Another potential problem in adopting either the amounts of AA and DHA or the ratio of these two fatty acids in human milk as a guideline for the amounts of each that should be in formula is that human milk contains LA and ALA as well as LC-PUFA other than AA and DHA. Since infants can synthesize DHA and AA (9–12), the amounts of these fatty acids that are produced endogenously are likely to be influenced by the amounts of the precursors as well as the amounts of other fatty acids in human milk that, once ingested and absorbed, can be converted to AA and DHA and/or compete with other fatty acids for conversion. According to data presented at the symposium by Sauerwald *et al.* (13), intake of DHA and AA decreases endogenous synthesis of both fatty acids. In this study, enrichments of plasma lipid DHA and AA following administration of uniformly ^{13}C-labeled ALA and LA were considerably lower in breast-fed infants and infants fed formulas supplemented with AA and DHA than in infants fed an unsupplemented formula.

Thus, even though formulas are supplemented with the same amounts of AA and DHA and/or the same ratio of AA/DHA present in human milk, there is no *a priori* reason to expect that such supplementation will result in the same plasma and tissue patterns of these fatty acids observed in breast-fed infants. For this to occur, one would predict that the amounts of the precursors of n-6 and n-3 LC-PUFA, i.e., LA and ALA, as well as the content of all n-6 and n-3 polyunsaturated fatty acids in formula must be the same as in human milk.

An alternative and more reasonable strategy is to supplement formula with a mixture of fatty acids that results in a plasma lipid pattern of fatty acids similar to that of breast-fed infants. However, the plasma lipid fatty acid pattern of breast-fed infants, although not as variable as the fatty acid pattern of human milk, also is quite variable. Moreover, application of this strategy clearly will require considerable time and effort.

Four posters presented at the symposium included plasma and/or erythrocyte phospholipid fatty acid contents of preterm infants fed formulas supplemented with varying amounts of AA and DHA. Two of these as well as an additional poster also included data from infants fed formulas supplemented with only DHA—an algal triglyceride in one study and a low-EPA fish oil in the other two. All studies included a group of preterm infants fed human milk. Combinations of AA and DHA (% of total fatty acids), respectively, of 0.50:0.35, 0.6:0.4, 0.72:0.36, and 0.75:0.6 all resulted in plasma and/or erythrocyte contents of AA and DHA similar to those observed in infants fed preterm human milk. The amount of DHA was similar in all studies of only DHA supplementation (0.35, 0.36, and 0.37% of total fatty acids; the latter, the low-

EPA fish oil, also included 0.07% of total fatty acid as EPA). The algal triglyceride DHA supplements resulted in plasma or erythrocyte phospholipid contents of DHA similar to those observed in infants fed preterm human milk. The low-EPA fish oil supplement resulted in higher contents of both DHA and EPA than observed in infants fed preterm human milk. In all studies, the AA content of plasma or erythrocyte phospholipid was lower than observed in infants fed preterm human milk.

Thus, despite the considerations discussed above, it appears that supplementing preterm infant formulas with ratios of AA/DHA from 1.4–2.0 (with AA ranging from 0.5–0.75% of total fatty acids) results in plasma and erythrocyte phospholipid contents of AA and DHA similar to those observed in infants fed preterm human milk. Although all studies were small, were performed by investigators from three continents, and included measurements of the fatty acid contents of different lipid fractions using different methods, the data are reassuring.

AVAILABLE SOURCES OF LC-PUFA

In addition to the issues discussed above concerning the amounts of LC-PUFA that should be added to formulas, there are issues concerning the source(s) of supplementation to be used. Currently, three general sources are available. The first, fish oil, contains large amounts of n-3 LC-PUFA but minimal amounts of n-6 LC-PUFA; further, some fish oils contain at least 1.5 times as much EPA as DHA, and the high EPA content has been implicated as the cause of adverse effects on growth. Some fish oils contain considerably less EPA but one such oil also was shown to have an adverse effect on growth of preterm infants (5), although a smaller effect than high-EPA fish oil. All fish oils, theoretically, are subject to contamination with heavy metals and, perhaps, other components; however, it should be relatively easy to ensure that such contamination is minimal.

Other sources of LC-PUFA include egg yolk lipid, or phospholipid, and triglycerides, usually derived from algae or other single-cell organisms. Egg yolk lipid, of course, contains large amounts of cholesterol; for example, the amount necessary to supply even the lowest amounts of AA and DHA in human milk will result in a formula with considerably more cholesterol than is usually present in human milk. For this reason, egg yolk phospholipid is a more logical choice. Although egg yolk phospholipid contains AA and DHA, the proportions of the two are not necessarily the same as the proportions of these fatty acids in human milk. However, the proportions of AA and DHA in egg yolk phospholipid can be modified by alterations in the diet of the hens. There is some concern, although no evidence, that egg yolk lipid or phospholipid may contain allergens. Of more concern is the possible difference in intestinal digestion/absorption and/or metabolism of phospholipid vs. triglyceride sources of LC-PUFA.

Both phospholipid and triglyceride sources of LC-PUFA contain fatty acids other than the LC-PUFA, including unusual fatty acids. These sources also may contain toxins, pigments, and/or other contaminants from fermentation or extraction processes utilized in their manufacture but, again, this theoretical concern should be easily allayed. The algal triglycerides contain positional distributions of LC-PUFA different from those of human milk triglycerides; whereas AA and DHA in human milk triglycerides are present primarily in the *sn*-1 and *sn*-2 positions, they are present in all three positions in the algal oils. Further, while human milk triglycerides rarely contain more than one molecule of AA and/or DHA, some algal triglycerides contain two, even three, molecules of AA and/or DHA. Whether this is a problem—or, perhaps, a desirable attribute—remains to be determined.

COMPARISON OF PHOSPHOLIPID AND TRIGLYCERIDE SOURCES OF LC-PUFA

Carnielli *et al.* presented data at the symposium (14) from a study in preterm infants showing that absorption of DHA was better at 1 mon of age in infants who received DHA as a component of phospholipid than in infants who received this fatty acid as a component of triglyceride (i.e., 91.6 ± 1.5% of intake in infants who received the phospholipid source vs. 80.4 ± 2.1% of intake in infants who received the triglyceride source). Absorption of AA from the phospholipid vs. triglyceride source also was higher (i.e., 84.7 ± 1.7% vs. 80.4 ± 2.3% of intake), but this difference was not statistically significant. Absorption of DHA and AA by infants fed human milk was 78.4 ± 4.4% and 81.1 ± 3.6% of intake, respectively. In this study, the intake of DHA and AA from the phospholipid source was lower than the intake of these fatty acids from the triglyceride source, i.e., 13.6 ± 0.1 and 19.4 ± 0.2 mg/kg/d of DHA and AA, respectively, in infants who received the phospholipid source vs. 37.8 ± 0.7 and 49.7 ± 1.0 mg/kg/d of DHA and AA, respectively, in infants who received the triglyceride source. This raises the question of whether the apparently better absorption of DHA from the phospholipid source is secondary to the known physiological differences in digestion and absorption of phospholipid vs. triglyceride or simply reflects the usual greater percentage absorption of lower intakes. Despite apparently poorer absorption of DHA and AA from the triglyceride source, the amounts of the two fatty acids absorbed were higher in infants who received the triglyceride vs. the phospholipid source of these fatty acids, i.e., 30.6 ± 1.1 vs. 12 ± 0.3 mg/kg/d of DHA and 40.0 ± 1.4 vs. 16.4 ± 0.4 mg/kg/d of AA in infants who received the triglyceride and phospholipid sources, respectively.

Questions arose concerning the effect of calcium supplementation of human milk on absorption of both fatty acids from human milk as well as the effect of the calcium content of formulas on absorption of both as components of phospholipid vs. triglyceride. No information concerning the specific questions was available. However, recent data of Schanler *et al.* (15) show that addition of a commercially available human milk fortifier (protein, calcium salts, and other mineral salts) to human milk markedly decreases total fat absorption.

Despite evidence that human infants absorb DHA better from phospholipid than from triglyceride sources, Goustard *et al.* presented data at the symposium (16) showing that the DHA content of retinal phosphatidylethanolamine was similar in piglets fed formulas enriched with egg yolk (phospholipid),

salmon oil (high-EPA triglyceride) or low-EPA fish oil (triglyceride), i.e., 40–42% of total fatty acids, the same as in sow-fed controls, vs. only 35% of total fatty acids in animals fed a formula with no LC-PUFA. However, interpretation of these findings is complicated by the fact that the egg yolk (phospholipid) supplement contained more DHA than either triglyceride supplement [0.4% of total fatty acid vs. 0.2% of total fatty acids in the salmon oil supplement (plus 0.3% 20:5n-3 and 0.1% 22:5n-6) and 0.3% in the low-EPA fish oil supplement].

EFFECT OF POSITIONAL DISTRIBUTION OF DHA

Data presented at the symposium by Christensen *et al.* (17) provide some insight into the biological significance of the positional distribution of DHA. In this study, newborn rats were fed dietary supplements of either specifically structured or randomized oils. Both oils had the same total content of DHA but, in the structured oil, DHA was present exclusively in the *sn*-2 portion whereas, in the randomized oil, it was equally distributed among *sn*-1, -2, and -3 positions. At 12 wk of age, the liver and brain DHA contents of both supplemented groups were higher than the content of the control group which was suckled and then fed chow. Liver DHA content was higher in animals supplemented with the randomized vs. structured oil. Brain content of DHA, on the other hand, was highest in the group supplemented with the structured oil. In both tissues, the increase in DHA was accompanied by a decrease in n-6 LC-PUFA. Fatty acid profiles of the retina did not differ among the three groups. Despite the apparent differences in metabolism and distribution of DHA as a result of positional distribution within the oils, there were no differences among groups in learning ability and only minor differences in electroretinograms and auditory brain responses.

LC-PUFA AND INSULIN SENSITIVITY

Data from extensive studies in both animals and human volunteers showing statistically significant positive correlations between the content of various LC-PUFA in skeletal muscle membrane and insulin sensitivity (18) were reviewed by Baur. Specifically, there are statistically significant positive correlations between insulin sensitivity and the content of AA, DHA, the sum of all n-3 LC-PUFA and the sum of all n-3 and n-6 LC-PUFA in skeletal muscle phospholipid. In adults, these relationships are thought to be important with respect to development and/or progression of diseases linked to insulin resistance—i.e., obesity, coronary heart disease, hypertension. Although it has not been conclusively established that diet during infancy and childhood is an important antecedent for development of these adult diseases, there is concern that this may be the case. Thus, these relationships may be equally, perhaps even more, important in children.

Altered insulin sensitivity secondary to the fatty acid pattern of skeletal muscle membranes also may affect the rate as well as the composition of weight gain during infancy. For example, greater insulin sensitivity should be associated with a lower plasma insulin concentration and, hence, less marked effects of insulin on skeletal muscle and adipose tissue. This, as well as eicosanoid-mediated effects on growth, is a rational explanation of the lower rate of weight gain observed by Carlson *et al.* (3) in low-birthweight infants supplemented with a high-EPA fish oil and the lower weight-for-length Z-scores observed in a subsequent study by the same group in low-birthweight infants supplemented with a low-EPA fish oil (5). Greater insulin sensitivity could also explain the lower rates of weight gain observed by Jensen *et al.* in both low-birthweight (18) and term infants (20) fed formulas with a high (i.e., 3.2% of total fatty acids) vs. a low (i.e., 0.4–1.0% of total fatty acids) content of ALA.

Baur also reviewed her group's more recent studies in children (21). In these studies, the fatty acid pattern of muscle membranes obtained at elective operation was related to diet as well as to fasting glucose concentrations. Infants who were breast-fed vs. formula-fed at the time of operation had higher muscle phospholipid contents of DHA as well as total n-3 and n-6 LC-PUFA. Plasma insulin concentrations of the two groups did not differ, but the breast-fed infants had lower fasting plasma glucose concentrations, suggesting greater insulin sensitivity (or less insulin resistance). Moreover, among the total study population, there was a wide range of skeletal muscle phospholipid contents of DHA and other LC-PUFA and, as observed in adults, there was an inverse correlation between fasting plasma glucose concentration and the percentage of DHA ($r = -0.47$) as well as the sum of n-3 and n-6 LC-PUFA ($r = -0.38$) in skeletal muscle phospholipid. Baur concluded that diet and probably genetics determine the fatty acid composition of the muscle membranes of children as well as adults and that this pattern affects insulin sensitivity and hence regulation of glucose metabolism.

Whether insulin sensitivity and/or resistance secondary to LC-PUFA intake and the resulting effect on the fatty acid content of skeletal muscle membrane are responsible for or contribute to the lower rates of weight gain observed in infants fed formula supplemented with n-3 LC-PUFA or containing a high content of the precursor of these fatty acids remains to be established. In the initial study of Carlson *et al.* (3), there was a positive correlation between weight at 1 yr of age and the plasma phosphatidylcholine content of AA early in infancy, i.e., those with higher AA contents early in life weighed more at 1 yr of age. Jensen *et al.* (20) also observed a positive correlation between weight of term infants at 120 d of age and the plasma phospholipid content of AA as well as the plasma phospholipid content of other n-6 LC-PUFA at the same age. The AA content of cord plasma lipid also has been related to the size of infants at birth (22).

As discussed elsewhere, this apparent relationship between AA status and growth might also be explained by an imbalance in eicosanoids derived from AA vs. EPA. Recent studies showing that growth of infants supplemented with both AA and DHA is not lower than that of infants fed unsupplemented formulas tend to support this possibility. However, a role of insulin sensitivity cannot be discounted. If LC-PUFA increase insulin sensitivity, infants supplemented with these fatty acids should de-

posit less fat. Hence, the impression that the lower rate of growth of infants fed formulas supplemented with n-3 LC-PUFA or formulas with a high content of ALA is "bad" may need to be reevaluated. In other words, this current safety concern may prove to be a reason for adding LC-PUFA to formulas. Clearly, much more data are needed concerning this issue.

RESEARCH NEEDS

As discussed throughout this summary, much more data are needed concerning all of the safety issues discussed above. While a number of posters presented data from studies with formulas containing a variety of LC-PUFA supplements, few of these studies included outcome variables related specifically to the safety issues of concern. Moreover, most were reasonably short-term studies and included relatively few subjects. Virtually no data are available concerning the effects of feeding formulas supplemented with LC-PUFA for the entire first year of life, as is likely to occur if such formulas become generally available. The extent to which formulas supplemented with LC-PUFA are "safe" for all groups of preterm infants is even more problematical. In these infants, LC-PUFA theoretically could interfere with host defense mechanisms, leading to an even greater incidence of infection. These fatty acids also could exacerbate effects of oxidant stress secondary to increased unsaturation of lipid membranes and, if oxidant stress is an etiological factor in development of such conditions as NEC, bronchopulmonary dysplasia and retrolental fibroplasia, as some suggest, supplemented formulas could result in an even higher incidence of these conditions. Effects on hemostasis theoretically, could also result in an increased incidence of intraventricular hemorrhage and/or other bleeding disorders; however, intraventricular hemorrhage usually occurs during the immediate neonatal period, frequently before infants are fed. As noted above, longer bleeding times, although still within normal limits, were observed in preterm infants supplemented with fish oil (4).

Although large, possibly multicenter, studies will be required, data concerning the extent to which these theoretical safety issues are real should be relatively easy to obtain and, until such data are available, questions concerning the safety of LC-PUFA are likely to persist. Data concerning the purity of various sources of LC-PUFA should be even easier to obtain and some such data, in fact, are available. On the other hand, the data required to answer questions concerning the bioavailability and metabolic consequences of LC-PUFA from various sources will be more difficult to obtain, and these data are needed to determine both the optimal amounts of n-3 and n-6 LC-PUFA that should be added to formula and the optimal sources of these fatty acids. However, with respect to amounts of LC-PUFA with which formulas should be supplemented, it is somewhat reassuring that a variety of levels of supplementation with both DHA and AA appears to result in plasma and/or erythrocyte lipid contents of these fatty acids similar to those of breast-fed infants.

Manufacturers of LC-PUFA supplements and infant formulas are urged to address at least some of the outstanding safety issues at the same time issues concerning the efficacy of LC-PUFA supplementation of infant formulas are being addressed. However, manufacturers should not be expected to assume responsibility for resolving all the issues that have been raised. Many of these issues also should be of sufficient concern to governmental health and research agencies to warrant greater investment in studies to help resolve them. Until some of the outstanding questions concerning safety are resolved, there is likely to be resistance to adding LC-PUFA to formula. This might be unfortunate if these fatty acids, indeed, are important for optimal visual and central nervous system development.

ADDENDUM

Some of the data presented in this session of the symposium in November 1996 (and reviewed above) have since appeared in peer-reviewed publications. These include the study of Carnielli *et al.* (23) showing more efficient intestinal absorption of DHA from a phospholipid vs. a triglyceride source and the study of Baur *et al.* (24) concerning differences in insulin sensitivity of breast-fed vs. formula-fed infants. Other peer-reviewed publications and abstracts addressing biological effects and safety issues related to LC-PUFA in infants also have appeared since November 1996 and some of the more pertinent of these are discussed below.

Recent data from both animals and infants suggest that balanced intakes of AA and DHA do not result in altered eicosanoid metabolism. Huang and Craig-Schmidt (25) found that the tissue contents of eicosanoids derived from n-6 LC-PUFA were lower, and those derived from n-3 LC-PUFA were higher, in piglets fed a diet supplemented with only DHA, whereas eicosanoids derived from n-6 and n-3 LC-PUFA were not deranged in piglets fed diets supplemented with both AA and DHA. Stier *et al.* (26) found no difference in mean urinary excretion of stable metabolites of prostaglandin E_2, thromboxane A_2, and prostacyclin among groups of preterm infants fed a standard formula, a formula supplemented with AA and DHA (~0.35 and 0.20% of total fatty acids, respectively) or human milk. However, the urinary prostanoid excretion of all groups was quite variable.

Additional data also are available concerning the amounts of AA and DHA with which formulas should be supplemented. Clandinin *et al.* (27) reported the fatty acid contents of erythrocyte membrane phospholipid of preterm infants who were assigned randomly to be fed an unsupplemented formula or a formula supplemented with one of three combinations of AA and DHA for the first 6 wk of life, i.e., 0.32 and 0.24, 0.49 and 0.35%, or 1.1 and 0.76% of total fatty acids, respectively, as AA and DHA. A clear dose response between intake and the AA and DHA contents of erythrocyte membrane phospholipid was observed. Based on these data, AA and DHA intakes of 0.6 and 0.4% of total fatty acids, respectively, appeared adequate and, perhaps, optimal with respect to achieving an erythrocyte phospholipid pattern of fatty acids similar to that of breast-fed infants.

A considerable amount of data concerning the safety of preterm infant formulas supplemented with AA and DHA

produced by single-cell organisms also has become available since November 1996. In addition, the manufacturer of the supplements and infant formula manufacturers have reported additional data concerning both the safety and toxicology of the supplements in animals (28,29). The latter show no untoward effects of doses of AA and DHA from single-cell source orders of magnitude higher than likely to be added to infant formula. I am not aware of similar published data concerning other LC-PUFA sources.

Unfortunately, the major sets of data concerning the biological effects and safety issues related to LC-PUFA in infants have been published only as Abstracts (30,31). These data are from two randomized, double-blind, controlled clinical trials of LC-PUFA-supplemented *vs.* unsupplemented formulas in preterm infants. The specific details of the two studies differed somewhat, but both included a relatively large number of infants as well as a reference group fed human milk and both included follow-up at least through 57 wk postmenstrual age.

The two studies plus two smaller published studies (27,32) provide data from roughly 175 preterm infants fed formulas containing 0.32–1.1% of total fatty acids as AA and from 0.24–0.76% of total fatty acids as DHA, both from a single-cell source and produced by the same manufacturer. The combined data show that formulas containing approximately 0.6% of total fatty acids as AA and 0.3–0.4% of total fatty acids as DHA result in plasma and erythrocyte lipid levels of these fatty acids similar to those observed in either preterm or term infants fed human milk. More important, these data suggest that formulas supplemented with AA and DHA from single-cell organisms are safe. In no study was the incidence of any adverse outcome or all adverse outcomes combined greater in the supplemented group than in the control group. Further, in no study was growth of infants receiving a supplemented formula less than that of infants receiving the control formula (a standard preterm formula fed to preterm infants for a number of years). In fact, one study suggests that infants fed a formula supplemented with both DHA and AA grow better than infants fed either the control formula or a formula supplemented with only DHA.

Despite the lack of specific data concerning the effects of preterm formulas supplemented with LC-PUFA on eicosanoid metabolism, peroxidation, etc., these recent data suggest that these concerns may be more hypothetical than real. Certainly, the incidence of diseases which might be related to these biological effects of LC-PUFA (e.g., NEC, bronchopulmonary dysplasia, and retrolental fibroplasia sepsis) did not differ between supplemented and unsupplemented groups enrolled in these studies. A caveat, of course, is that this conclusion applies only to formulas containing the sources of AA and DHA used in the studies evaluated. Also, since data concerning the safety of formulas supplemented with only DHA are limited, this conclusion may not apply to such formulas.

Much less information is available concerning the safety of preterm formulas supplemented with other sources of AA and DHA. One exception is a recent study of Carlson *et al.* (33) in which preterm infants (birthweight, 725–1375 g) were randomly assigned to be fed a control formula without LC-PUFA until 12 mon corrected age, this formula until discharge and, then, a formula containing egg yolk phospholipid (75% phosphatidylcholine; 0.4% of total fatty acids as AA and 0.13% as DHA) until 12 mon corrected age or the latter formulas through 12 mon corrected age. Only one infant fed the formula supplemented with egg yolk phospholipid during hospitalization developed NEC (2.9%) whereas 15 fed the control formula during hospitalization developed this condition. The incidence of sepsis, bronchopulmonary dysplasia and retrolental fibroplasia did not differ between groups.

The extent to which conclusions concerning the safety of preterm formula containing AA and DHA are applicable to all preterm and low-birth-weight infants remains to be addressed. While all studies cited above included a few infants who weighed less than 1000 g at birth, none included an appreciable number of these infants who, theoretically, are more likely to require an exogenous source of LC-PUFA and also more likely to be vulnerable to the potential adverse effects of these fatty acids. Thus, lack of such data, although understandable, is unfortunate.

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Long-Chain Polyunsaturated Fatty Acids in Diets for Infants: Choices for Recommending and Regulating Bodies and for Manufacturers of Dietary Products

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ABSTRACT: While the scientific evaluation of the physiologic effects, potential benefits, and risks of dietary preformed long-chain polyunsaturated fatty acids (LCPUFA) for infants have been discussed elsewhere, this manuscript addresses some of the resulting consequences and challenges of interpreting the available knowledge from the perspective of recommending and regulating bodies, and of manufacturers of dietary products and their ingredient materials. Traditionally human milk composition has served as a reference point for infant formula design. With respect to the use of LCPUFA in infant formula, much more emphasis than ever before invested for any other group of micronutrients has been put on clinical studies investigating the potential advantages and disadvantages of LCPUFA in infant foods, including growth, safety aspects, and a variety of other outcome measures.

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CURRENT NATIONAL AND INTERNATIONAL RECOMMENDATIONS AND REGULATIONS ON THE LONG-CHAIN POLYUNSATURATED FATTY ACID (LCPUFA) SUPPLY WITH INFANT DIETS

It is common knowledge that the nutritional and metabolic requirements of children differ from that of adults, and age-specific dietary guidelines are particularly important in infancy (1) However, current recommendations of national and international bodies on the LCPUFA supply with infant diets, and the respective legal regulations, show considerable variation.

With respect to national recommendations, no formal comment of a scientific society in the United States on this question has been made. The U.S. Food and Drug Administration (FDA) has not approved any commercial preterm or term infant formula with added LCPUFA for sale in the United States. The FDA commissioned the Life Sciences Research Office to convene an expert panel in order to review the need for nutrients supplied with formulas for healthy, full-term infants, and the report has recently become available (2,3). This North American expert panel followed the general concepts of previous European recommendations (4) and regulations (5,6) with regard to increasing the minimum required fat content in infant formulas to 40% of energy, adding α-linolenic acid (U.S. recommendation 1.75–5% of total fatty acids), limiting the linoleic/α-linolenic acid ratio (U.S. recommendation 6–16:1), as well as avoiding a high exposure to *trans* fatty acids and to myristic acid. However, the committee "did not recommend the addition of arachidonic acid (AA) or docosahexaenoic acid (DHA) to infant formulas at this time." The committee considered the evidence available at present insufficient to warrant a recommendation for adding LCPUFA to formulas, but advised that the potential short- and long-term effects of LCPUFA should be further characterized and "the question of requiring the addition of specific LCPUFAs to infant formulas be reassessed within five years" (2,3).

Similarly, the Canadian Paediatric Society, the Dieticians of Canada, and the government department Health Canada concluded in a recent joint statement on the nutrition of healthy term infants: "Although it is reasonable to presume that infants not fed breast milk might benefit from dietary sources of AA and DHA, until the safety and efficacy of the sources of these fatty acids and of the formulas supplemented with them have been established, the routine addition of these nutrients to formulas designed for full-term infants is not recommended" (7).

National regulations in Asian countries on PUFA in infant formulas are mostly restricted to linoleic acid content, e.g., in Japan (linoleic acid > 0.3 g/100 kcal), the People's Republic of China (linoleic acid in special grade formula > 3 g/100 g, first-grade > 2 g/100 g, passed > 1.5 g/100 g), and Taiwan (linoleic acid > 0.3 g/100 kcal). Thailand regulates not only linoleic acid content (> 0.3 g/100 kcal) but also acknowledges the addition of LCPUFA up to an upper limit of no more than 1% of dietary energy. Several term formulas with added LCPUFA are commercially available in Japan, Thailand, and other Asian countries as well as Australia.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; ESPGHAN, European Society for Paediatric Gastroenterology, Hepatology and Nutrition; FAO, Food and Agriculture Organization of the United Nations; FDA, United States Food and Drug Administration; LCPUFA, long-chain polyunsaturated fatty acids; PUFA, polyunsaturated fatty acids; WHO, World Health Organization; WIC, United States Special Supplemental Food Program for Women, Infants and Children.

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In the United Kingdom the British Nutrition Foundation issued a report providing a recommendation of the authors for the PUFA supply per day and kg body weight (8). The numbers given for preterm as well as for term infants are 280–1130 mg linoleic acid, 50 mg α-linolenic acid, 20 mg docosahexaenoic acid (DHA) and "some" arachidonic acid (AA).

International recommendations were provided by a joint expert consultation of the Food and Agriculture Organization (FAO) of the United Nations and the World Health Organization (WHO) (9). It was stated that infant formulas for term infants should contain preformed DHA and AA. Recommendations for Europe were issued by the Committee on Nutrition of the European Society for Paediatric Gastroenterology, Hepatology and Nutrition (ESPGHAN) (4). For preterm and term infant formulas as well as follow-on formulas, the committee recommended a linoleic acid content of 0.5–1.2 g/100 kcal and a linoleic acid/α-linolenic acid ratio in the range of 5–15. With respect to LCPUFA it was stated: "The Committee feels that enrichment with metabolites of both linoleic and α-linolenic acids approximating levels typical for human milk lipids (n-6 LCPUFA 1%, n-3 LCPUFA 0.5% of total fatty acids) is desirable for formulas for low birthweight infants. LCPUFA supplementation to infant formulas, i.e., for infants born at term, might be of advantage but further data on this question are required prior to a definite recommendation." It was also recommended that n-3 and n-6 LCPUFA contents of formula should not exceed 1 and 2%, respectively, of total formula fatty acids.

The Commission of the European Union issued regulations on infant formula composition, including permission for addition of LCPUFA up to 1 and 2%, respectively, of total fat content as n-3 and n-6 LCPUFA (6).

In Europe, several term and particularly preterm infant formulas are commercially available (since the early 1990s) as a consequence of the recommendations and regulations issued (Tables 1 and 2). In addition, two phenylalanine-free dietary products for the treatment of infants with phenylketonuria with added LCPUFA were introduced (10,11). Interestingly, even though numerous infants were fed with such LCPUFA-enriched formulas, no observations of LCPUFA-related adverse effects have been reported with the enriched formulas available on the market.

IS HUMAN MILK COMPOSITION A SUITABLE MODEL FOR FATTY ACID CONTENT OF INFANT FORMULAS?

In view of the differences of recommendations to include or not include LCPUFA, and of the practice of infant formula manufacturers, the question arises to what extent guidance on infant formula design may be derived from human milk composition. From a historical perspective, the aim of making infant formula similar to human milk appears logical and valid. Unmodified animal milks were widely used for infant feeding well into the nineteenth century, when results of biochemical investigations on human milk composition sparked first efforts to modify the nutrient content of cows' milk preparations (12). During the twentieth century, improved understanding of human milk composition and infant physiology led to a dramatic development from only slightly modified animal milks to modern sophisticated infant formulas. Many infant formulas approached a composition that was much more similar to human milk, with respect to energy density, protein quantity and composition, relative contents of saturated and unsaturated fats, carnitine, and taurine.

In 1977 the Commitee on Nutrition of the ESPGHAN (then still named ESPGAN) summarized some of the underlying basic concepts by stating that "Breast feeding is superior to any other feeding system for infants . . ." while infant formula was considered a supplement to or substitute for breast feeding (13). The compositional and quality requirements for infant formula are higher than for any other food product because it is "intended to cover, by itself, all the nutritional needs during the first 4–6 months . . ." It was further concluded that infant formula "should resemble human milk as far as is possible" (13).

The conclusion that human milk composition should be the model for infant formula, however, has a number of limitations. The nutrient supply with breast feeding is rather variable, since milk composition varies within and between individuals, with duration of lactation, during a day, within one nursing and with changes of maternal diet (14,15), even though recent stable isotope studies indicate that the effects of short-term changes of dietary PUFA intake on milk composition are metabolically buffered (16). For example, average total LCPUFA content of human milk falls from about 3% of total fatty acids in the first week of lactation to about 1.8% at 1 mon (17). Moreover, breast-milk volumes consumed by the exclusively breast-fed infant vary markedly, even in well-nourished populations between some 550 to 1100 mL per day. Hence, it is rather difficult to define reference nutrient intakes of breast-fed babies. Furthermore, the bioavailability and metabolism of nutrients may differ considerably between breast- and formula-fed infants, and they can be influenced by a complex interaction of several components in human milk. The absorption and metabolism of LCPUFA from human milk lipids cannot be assumed to be identical to different forms of LCPUFA added to formula. As another example, the linoleic/α-linolenic acid ratio in human milk may not necessarily be the best guidance for the ratio in formula, not only because it is markedly influenced by maternal diet but also because the linoleic/α-linolenic acid ratio may have a fundamentally different importance in formula- than in breast-fed infants. In babies fed conventional vegetable oil-based infant formula without LCPUFA, an unbalanced ratio of the two precursor fatty acids may have untoward effects on the synthesis of AA or DHA (18). In contrast, this is probably of little relevance in breast-fed infants who receive sufficient amounts of preformed LCPUFA with human milk lipids to cover their needs for growth of membrane-rich tissues (19). It is also well known that contents of some human milk ingredients are markedly influenced by maternal intakes, and in some cases suboptimal supplies to breast-fed infants may

occur, for example of the vitamins K, D, B_{12}, the trace elements iodine, zinc, fluorine, and iron (14,15,20). In conclusion, there is no scientific basis to presume that average fatty acid and LCPUFA contents in human milk found under given dietary and living conditions in a population would represent the ideal substrate supply either of the breast-fed or the formula-fed infant.

The concept that infant formula should resemble human milk as closely as possible is further challenged by the identification of a several bioactive components in breast milk (15). With the developments of biotechnology, it appears technically feasible in the foreseeable future to also add several of these bioactive compounds found in human milk to infant formulas. In addition to LCPUFA, nonpeptide and peptide hormones, lactoferrin, oligosaccharides, polyamines, a variety of different nucleotides and nucleosides, and enzymes such as bile salt-stimulated lipase might theoretically be incorporated into formulas, particularly into products with special indications for defined subgroups of infants. However, at present the addition of a number of these compounds may not be economically affordable. In fact, the introduction of some modifications of standard infant formula that may be technically

TABLE 1
Commercial Low Birthweight Infant Formulas with Metabolites of Linoleic and α-Linolenic Acid in Europe, Based on Manufacturers' Data[a]

Manufacturer	Product	Major metabolites (% of fatty acids)	Raw materials used
Alete (Munich, Germany)	Aletemil preterm formula	0.3 GLA, 0.1 AA, 0.3 DHA	Marine oil, black currant seed oil, egg lipids
Cow & Gate (Trowbridge, United Kingdom)	Nutriprem LBW	0.4 GLA, 0.4 AA, 0.35 DHA	Borage oil, marine oil, egg lipids
Friesche Flag (Leeuwarden, Netherlands)	Frisopre	0.35 GLA, 0.2 EPA, 0.3 DHA	Borage oil, marine oil
Farley (Nottingham, England)	OsterPrem with LCPUFA	0.9 GLA, 0.1 EPA, 0.5 DHA	Borage oil, marine oil
Gallia (Paris, France)	PreGallia au DHA	0.1 EPA, 0.4 DHA	Marine oil
Humana (Herford, Germany)	Humana 0, Humana 0 HA	0.2 GLA, 0.2 DHA	Borage oil, egg lipids
Laboratorios Ordesa (Barcelona, Spain)	Bleviprem	0.3 AA, 0.15 DHA	Egg lipids
Milupa (Friedrichsdorf, Germany)	Prematil	0.2 GLA, 0.4 AA, 0.2 DHA	Evening primrose oil, egg lipids
Nestlé (Vevey, Switzerland)	Beba preterm formula, Pre Nidal + LCPUFA	0.3 GLA, 0.1 AA, 0.3 DHA	Marine oil, black currant seed oil, egg lipids
Nutricia (Zoetermeer, Netherlands)	Nenatal	0.6 AA, 0.4 DHA	Single-cell oils
Nutricia (Zoetermeer, Netherlands)	Premilon (for preterm infants after hospital discharge)	0.45 AA, 0.3 DHA	Single-cell oils
SMA Nutrition (Taplow, United Kingdom)	SMA Low Birthweight	0.6 AA, 0.4 DHA	Single-cell oils

[a]GLA = γ-linolenic acid, AA = arachidonic acid, EPA = eicosapentaenoic acid, DHA = docosahexaenoic acid. Modified from Reference 25.

TABLE 2
Commercial Infant Formulas for Healthy Term Infants with Metabolites of Linoleic and α-Linoleic Acid in Europe and New Zealand, Based on Manufacturers' Data[a]

Manufacturer	Product	Major metabolites (% of fatty acids)	Raw materials used
Alete	Aletemil preterm formula	0.3 AA, 0.15 DHA	Egg lipids
Humana	Humana ES	0.2 DHA	Marine oils
Laboratorios Ordesa	Blemil 1 plus	0.3 AA, 0.15 DHA	Egg lipids
Milupa	PreAptamil	0.2 GLA, 0.4 AA, 0.2 DHA	Evening primrose oil, egg lipids
Nestlé	Beba Start HA	0.3 AA, 0.15 DHA	Egg lipids
Nutricia Ltd. (Auckland, New Zealand)	Karicare	0.4 AA, 0.2 DHA	Single-cell oil, tuna oil
Wander (Bern, Switzerland)	Adapta 90 Inicio	0.2 EPA, 0.2 DHA	Marine oil

[a]See Table 1 footnote for company source locations and abbreviations.

possible could absorb so much resources that other modifications, which might confer as much or even greater benefits for the recipient infant, would not be affordable any more. Hence, one needs both to set priorities and to select some of the possible multitude of formula modifications, based on a thorough evaluation of possible benefits and disadvantages.

EVALUATION OF FORMULA MODIFICATIONS

An important question is how the incorporation of new concepts or new ingredients into an infant formula and its nutritional and clinical effects should be assessed. An expert committee in the United Kingdom recently provided guidelines for such evaluations (21). The committee concluded that nutritional assessment should be performed if modifications that aim at a functional or other clinical benefit are applied to infant formula. If modifications are introduced for other reasons than these two benefits, for example for economic, technological or commercial reasons, these products should at least be subjected to studies of acceptability. In these cases it should also be assessed whether the modification might have nutritional implications, and that the new product is no less satisfactory than the product that went before. The committee further concluded that results of clinical studies should be interpreted in the light of outcomes of healthy infants exclusively breast-fed for 4–6 mon. If adequate data on breast-fed populations are not available, the inclusion of breast-fed groups in clinical studies should be considered as the reference. Some further recommendations of the committee on the design and conduct of clinical studies evaluating formula modifications, as provided by the Working Group on Nutritional Assessment of Infant Formulas, are: (i) At the outset of a nutritional study there should be a clear hypothesis of functional or clinical benefit with defined selection criteria and outcome measures. (ii) Appropriate preclinical studies should be performed for previously untested components of infant formula. (iii) A pilot study should be considered to provide the information necessary to design an adequate study. (iv) Nutritional, including metabolic, outcome measures should be justified as relevant to the modification under test. (v) The need for continuing follow-up to 2 yr or beyond, and the consequent ethical and practical implications, should be considered in all studies. (vi) Studies to assess infant formulas should comply with the principles of Good Clinical Practice and Good Laboratory Practice. (vii) The possibility of unpredicted adverse outcomes should be addressed by adequate clinical monitoring of the participants and by independent scrutiny of the accumulating data. (viii) Results from clinical studies of infant formula, including those partly completed which have been abandoned, should be published.

SPECIFIC ISSUES RELATED TO LCPUFA INCORPORATION INTO INFANT FORMULAS

Following are several specific questions and concerns with regard to LCPUFA incorporation into infant formula.

(i) *What evidence of benefit, and of benefit/cost ratio, is required for incorporation of LCPUFA into infant formula?* A far more detailed evaluation of LCPUFA effects, and of the relationship between benefit and risk as well as cost, has been performed by manufacturers, scientific bodies, and regulatory agencies than was previously applied with respect to decision making on the addition of other nutrients to infant formula, e.g., taurine. This detailed evaluation that is more similar to the approach of evaluating pharmaceutical products is generally welcomed. Nonetheless, no consensus has been reached on the criteria and evidence base required to make decisions regarding formula composition. This lack of consensus is underlined by the differences in strategies that have been applied by various manufacturers and regulatory bodies in different parts of the world. Some experts feel that any change of product design would only be justified by conclusive scientific evidence on biologically relevant long-term benefits, such as improved neurodevelopmental outcome at school age or even in adulthood. In contrast, others raise concern that such conclusive long-term evaluation would not be available for almost any of the components of current infant diets. Based on the lack of such long-term outcome data and the limited feasibility to perform intensive studies lasting more than a decade, decisions on dietary composition in many cases need to be based on considerations of nutritional physiology. Furthermore, the question was raised whether the use of very rigid evaluation criteria might not unduly delay the application of any product innovation, with potential disadvantages for recipient infants who might then be excluded from the potential benefits of scientific and technological progress.

(ii) *Effects of LCPUFA on growth.* Some studies in which preterm infants were fed with formulas supplying high amounts of n-3 LCPUFA relative to n-6 LCPUFA found reduced growth, which has been associated with poor AA status (22). Results of studies without impairment of infant AA status did not show adverse effects on growth. There is general agreement that modifications of infant formula lipid composition should be tested for potential effects on growth, and that sufficient sample sizes need to be achieved for this purpose.

(iii) *Effects of LCPUFA on antioxidant status and interaction with other formula components.* The potential of enhanced peroxidation by added LCPUFA both in products and in the infant *in vivo* has been discussed. Higher dietary supplies of PUFA and LCPUFA may increase the need for antioxidants such as vitamin E, which was addressed in a recent comment by the ESPGHAN Committee on Nutrition (23). Therefore, one should evaluate effects of each approach of LCPUFA incorporation into infant formula on formula stability and peroxide contents as well as infant antioxidant status. Recent results also indicate the potential of an interaction between LCPUFA and selected amino acids (24).

Moreover, one must consider that effects of LCPUFA contents in formulas may potentially be influenced by other aspects of formula composition, such as the content of precursor essential fatty acids linoleic and α-linolenic acids, the

linoleic/α-linolenic acid ratio, and factors modulating endogenous LCPUFA synthesis such as general nutritional status, trace element status, or infant age (25,26).

(iv) *Efficacy and safety of different LCPUFA sources.* The bioavailability, metabolic effects, and safety of the various LCPUFA sources used for the production of infant formula may differ and need to be characterized. Cost is an important consideration for a manufacturer in the choice of raw materials. It has been concluded that related to kg DHA content, fish oil tends to be the cheapest and egg phospholipids the most expensive choice, whereas the price per kg AA tends to be most reasonable for single-cell oils and most expensive for phospholipids.

Some food sources of LCPUFA such as fish, eggs, and some of the plants providing oils rich in γ-linolenic acid (e.g., black currants) have been part of the human diet for a very long time and are generally regarded as safe. However, the provision of extracted and at times fractionated lipids from such foods in relatively high amounts per kg body weight to newborn infants is a new development, and its safety needs to be documented.

Concern was expressed that the early feeding of diets containing fish oils or egg lipids might sensitize infants and induce a risk for development of allergic reactions against fish or egg proteins. However, manufacturers of raw materials emphasized the purity of extracted lipids without appreciable protein residues. So far, no case of fish or egg allergy caused by sensitization with the feeding of an LCPUFA-enriched formula is known. Quality assurance programs should consider testing oils for residual protein contents.

Single-cell oils with high concentrations of LCPUFA that can be produced with fermentation technology are novel food sources in the human diet, and their safety for use in infant feeding requires thorough evaluation (27–29). Concern was raised that some organisms used for single-cell oil production may produce biotoxic substances and novel sterols (27,30–32). Some scientists proposed that single-cell oils used for infant formula production should preferably be derived from nonpathogenic microorganisms and be free of contaminating substances.

Labeling, health claims and advertisements. In Europe, the available commercial products with LCPUFA may state the content of these fatty acids on the package, but no health claim indicating possible advantages for the infant may be made. The question was raised whether information on the LCPUFA content appearing on the label would be understood by the persons responsible for the feeding of infants, and whether these persons would be induced to ask more information from health workers such as doctors or nurses. Scientists working in industry emphasized that it would be very important that health claims of effects in the recipient infant be based on solid scientific evidence. They felt that the consumer had a right to be informed on scientific results, and the opportunity for such claims would stimulate manufacturers to invest more in product improvements, even if they were costly, and in their scientific investigation. Questions were raised as to what criteria claims could and should be based on, and which body would control and follow up such claims and their implementation, as well as ingredients and safety. There are intense scientific efforts to investigate functional effects of early feeding (33) and these may strengthen the basis for establishing governmental regulations on health claims related to food ingredients.

It was also discussed that even though no health claims related to LCPUFA contents in infant formulas are presently permitted in Europe, advertisements are made on product labels and with parent informations that imply improved visual function, brain development, and cognitive outcome. Representatives of regulatory bodies felt that there is a strong need to control these developments in the consumer interest.

Even though scientific knowledge is universal, the interpretation of the available knowledge as well as the conclusions drawn by recommending and regulating bodies and by the dietary industry varies within and between different parts of the world. This is influenced not only by some difference in the emphasis put on different aspects of basic concepts, but even more so by differences in legal systems, issues of patent protection, and economic aspects. Compared with the LCPUFA issue, the decision to add taurine to infant formulas made several years ago by many manufacturers was relatively easy, since this ingredient was not very expensive. In contrast, the incorporation of LCPUFA into infant formula increases production costs considerably. This increase in cost may be compensated for by an increased retail price in affluent countries with a free market economy, such as Europe, particularly if a proven benefit can be communicated to the consumer. In contrast, it may be a much more difficult decision to make for producers in the United States, where a major proportion of formula is sold to the individual states at low bid for distribution through the Special Supplemental Food Program for Women, Infants and Children (WIC). Hence, the implementation of a costly innovation might considerably reduce the profit of the manufacturers under these circumstances. A particular ethical dilemma might arise for a manufacturer with international presence if a costly innovation with a proven benefit for the recipient infant would be affordable for consumers in affluent countries but not for the majority of consumers in a developing country. How could a company justify selling a "second-class" infant product with a perceived reduced health benefit to a poorer population?

For the time being, the available information indicates the potential of some benefit of a balanced dietary LCPUFA intake for term and particularly preterm infants, but great gaps of knowledge remain with respect to both the possible benefits and safety of different PUFA supplies and the potential dependance of LCPUFA effects on other dietary and biological varaibles. Therefore, it appears reasonable that no unanimous conclusion was reached but a variety of different choices are being followed until further information is accumulated.

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REVIEW

Recent Advances in Brain Cholesterol Dynamics: Transport, Domains, and Alzheimer's Disease

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ABSTRACT: Major advances in understanding cholesterol dynamics and the role that cholesterol plays in vascular disease have recently been made. The brain is an organ that is highly enriched in cholesterol, but progress toward understanding brain cholesterol dynamics has been relatively limited. This review examines recent contributions to the understanding of brain cholesterol dynamics, focusing on extracellular and intracellular lipid carrier proteins, membrane cholesterol domains, and emerging evidence linking an association between cholesterol dynamics and Alzheimer's disease.

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Cholesterol is a molecule that is essential for cellular function. In biological membranes cholesterol contributes to the structure of the membrane, and cholesterol is involved in regulating activity of certain integral proteins. Cholesterol is the precursor of steroids and bile salts. Major advances have been made in understanding how cholesterol contributes to both normal and pathological cell function, particularly with respect to vascular function (1). There has been extensive interest in the dynamics of cholesterol transport, both extracellular and intracellular transport (2–6). Progress has also been made in discerning the structural and functional roles of cellular cholesterol domains (5–7). Liver is the major organ of cholesterol metabolism and the primary site for the synthesis of lipid transport proteins. Brain is highly enriched in cholesterol but brain cholesterol dynamics such as transport and the role of cholesterol domains, are not well understood. Work by Kabara and others in the 1960s and 1970s established the fact that cholesterol was synthesized in brain *in situ* and readers are referred to a review of that work on brain cholesterol metabolism (8). Prior to that time there had been disagreement as to whether cholesterol was synthesized in brain or if brain cholesterol was metabolically stable (8). Subsequent studies have shown that the rate of cholesterol synthesis is high in the fetus and newborn animal and that as the animal matures, brain cholesterol synthesis is very low (9,10). Several recent advances have been made in understanding brain cholesterol dynamics such as identification and potential functional roles of different cholesterol transport proteins, structural and functional properties of membrane cholesterol domains, and the association between alterations in brain cholesterol dynamics and neuropathophysiology. The purpose of this review is to examine recent studies of brain cholesterol dynamics and to discuss the potential role(s) of cholesterol in neuropathophysiology, focusing on Alzheimer's disease.

LIPID TRANSPORT PROTEINS

Lipid transport and the proteins involved in such transport have been studied extensively in systems outside the brain and recently reviewed (3,11,12). While studies of lipid transport in the brain are relatively few when compared with work outside of the central nervous system, there has been an emerging database on both extracellular and intracellular lipid carrier proteins in the brain; those studies are discussed below.

Intracellular proteins. Brain tissue has at least three families of intracellular proteins that may be involved in cholesterol trafficking: the sterol carrier protein-2 (SCP-2) (13,14), caveolin (15,16), and fatty acid binding proteins (17,18). These proteins occur in multiple forms: alternate transcription sites, posttranslational modification, and separate genes. The SCP-2 gene has two initiation sites giving rise to two translation products, a 58 kDa sterol carrier protein-X (SCP-x) and a 15 kDa pro-sterol carrier protein-2 (pro-SCP-2) (17,19). In most tissues, pro-SCP-2 is posttranslationally cleaved by proteolysis to yield the mature 13 kDa SCP-2. A putative 30 kDa SCP-2 gene product arising from alternate mRNA splicing was recently shown not to be present in rat or mouse tissue (19). While SCP-2 was detected in brain tissue by immunoblotting earlier (20), this did not establish whether the brain SCP-2 immunoreactive protein was the

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Abbreviations: Aβ, amyloid-β peptide; AD, Alzheimer's disease; apo, apolipoprotein; APP, amyloid precursor protein; B-FABP, brain fatty acid binding protein; GABA, γ-amino butyric acid; HDL, high density lipoprotein; H-FABP, heart fatty acid-binding protein; LDL, low density lipoprotein; LDL-R, LDL-receptor; L-FABP, liver fatty acid binding protein; NBD-cholesterol, 22-[N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3β-ol; NP-C, Niemann-Pick C disease; NPC1, Niemann-Pick protein; SCP-2, sterol carrier protein-2; SPM, synaptic plasma membrane.

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same as that expressed in other tissues. Recently a cDNA encoding SCP-2 isolated from a mouse brain library, demonstrated for the first time that the brain form of SCP-2 was identical to that found in liver and other tissues (13). Furthermore, it was shown that SCP-2 was present in pinched off nerve endings or synaptosomes (13).

While SCP-2 markedly facilitates transfer of cholesterol as well as other molecules (oxysterols, phospholipids, glycolipids, etc.) between membranes *in vitro*, establishment of ligand binding by this protein has been difficult due to the poor solubility of lipids such as cholesterol. Nevertheless, increasing evidence from ^{3}H-cholesterol, fluorescent [dehydroergosterol, NBD-cholesterol {22-[N-(7 nitrobenz-2-oxa-1,3-diazol-4-yl)amino]-23,24-bisnor-5-cholen-3β-ol}, and ^{13}C-cholesterol binding studies demonstrate that SCP-2 binds cholesterol with high affinity (K_d as low as 6 nM) and equimolar stoichiometry (6). Whether the other SCP-2 gene products, SCP-x and pro-SCP-2, also bind sterols is not known. All three SCP-2 gene products do enhance intermembrane sterol transfer *in vitro*.

Until recently, there was very little physiological evidence demonstrating that SCP-2 gene products modulated cellular cholesterol uptake and intracellular trafficking. However, increasing data from transfected cell lines overexpressing the SCP-2 gene products are highly supportive of these roles. For example, overexpression of 15 kDa pro-SCP-2 in several cell lines (CHO and L-cells) led to increased levels of 13 kDa SCP-2 and increased many aspects of cholesterol metabolism including the following: cholesterol uptake, cellular cholesterol mass, cholesterol esterification, cellular cholesteryl ester mass, transfer of plasma membrane cholesterol to endoplasmic reticulum, and mitochondrial cholesterol oxidation (21–23). The functional role of SCP-2 in brain has not been determined.

Three other intracellular proteins have been identified in brain. Caveolins (a multimember family) are usually thought of as an integral plasma membrane protein (24). However, recent evidence has demonstrated that caveolin forms homo- and hetero-oligomers that bind cholesterol (25) and that caveolin is both membrane-bound and in the cytosol (26). Caveolin is involved in cholesterol trafficking from the endoplasmic reticulum to the plasma membrane (24). Caveolins 1, 2, and 3 have been identified in brain endothelial cells and astrocytes (15,16). A clear role for caveolin in brain cholesterol intracellular trafficking remains to be demonstrated. Finally, two members of the fatty acid binding protein family have recently been isolated from brain: the heart and brain fatty acid binding proteins (H-FABP and B-FABP) (17). Both proteins are found in synaptosomes and, although, these proteins do not bind cholesterol, they do inhibit intermembrane sterol transfer *in vitro* (18).

In summary, the brain contains at least four different proteins (SCP-2, caveolin, H-FABP, and B-FABP) that may play a role in intracellular cholesterol trafficking. Such a role may involve the transport of cholesterol from the neuronal cell body to projecting nerve terminals. Research has shown that phospholipids are synthesized in axons of cultured rat sympathetic neurons but that cholesterol is synthesized only in the cell body and transported to the axon by some unidentified mechanism (27). SCP-2, caveolin, H-FABP, and B-FABP may be part of such a transport mechanism. SCP-2 expression increased significantly in the brain tissue of chronically alcohol-treated mice (13). H-FABP and B-FABP expression in brain tissue significantly decreased with increasing age in mice (18). Changes in the levels of these proteins correlate with chronic ethanol-induced (28,29) and age-related (30) alterations in synaptosomal plasma membrane cholesterol domains and structure. This issue is discussed in the Cholesterol domains section elsewhere in this review.

Apolipoproteins. Apolipoproteins such as apoA-I, apoA-II, apoB, and apoE bind cholesterol, esterified cholesterol, phospholipids, and triglycerides, forming lipoproteins that are the primary means by which cholesterol is transported systemically. The main apolipoprotein of low density lipoproteins (LDL) is apoB. ApoA-I, apoA-II, and apoE are the apolipoproteins associated with high density lipoproteins (HDL). Apolipoproteins in brain include apoA-I, apoA-IV, apoD, apoE, and apoJ (31–33). There has been great interest in the role of apoE in brain as a result of studies indicating an association between the apoE4 allele and Alzheimer's disease (34,35). ApoE is thought to be synthesized in astrocytes and released from there to transport cholesterol and other lipids to neurons (36). ApoE-enriched lipoproteins bind to the low density lipoprotein-receptor (LDL-R), the low density lipoprotein receptor-related protein (LRP), and the apolipoprotein E receptor 2 (apoER2) and these receptors also have been identified in brain tissue (32,37–41). ApoE has been proposed to be an important factor in maintaining the stability of the neuron during aging and brain injury (42–45). In support of that hypothesis was the finding that there was a loss of brain nerve terminals in apoE-deficient mice and that aged apoE-deficient mice displayed the greatest loss of neuronal structure compared to younger apoE-deficient mice and wild-type mice (42). Both apoE3 and apoE4 have been found to increase neurite length in primary cultures of developing rat hippocampal neurons (46). Lesioning of the hippocampus increased apoE expression and increased binding of fluorescent-labeled LDL to hippocampal brain slices (37). That study led to the conclusion that apoE and the LDL receptor were necessary for recycling neuronal cholesterol for membrane biogenesis. Mechanisms of membrane biogenesis and the recycling of cholesterol in neuronal membranes are not known. In skin fibroblasts it was shown that LDL-derived cholesterol is transported by cholesterol carrying "rafts" from the *trans*-Golgi network to the plasma membrane (3). Whether such a mechanism is present in brain tissue is yet to be determined.

ApoE may be required for brain neuronal homeostasis but apoE is not necessary for peripheral nerve homeostasis. Peripheral nerve regeneration and the reutilization of cholesterol occurred in mice that were deficient in apoE and apoA-I (47). Another study reported that axonal regeneration occurred in rat-sympathetic neurons when cholesterol was added alone or with lipoproteins (48). Neither apoE nor apoA-I was necessary for axonal growth. Comparisons between brain tissue

and peripheral neural tissue may not be appropriate with respect to physiological function. Activity of apoE and apoA-I in peripheral nerves may differ when compared with brain tissue. Moreover, there may be other proteins that can efficiently replace the function of apoE and apoA-I in peripheral nerve reutilization.

ApoJ (clusterin) is a sulfated glycoprotein that is found in several different tissues including brain (49). The function of this protein is not well understood but there is evidence suggesting that apoJ may be involved in lipid transport and remodeling, sperm maturation, programmed cell death, and complement activation (50). ApoJ may also be involved in the removal of cholesterol from cells (50). Cholesterol, cholesteryl esters, and phospholipids were removed from foam cells incubated with apoJ. There is some evidence to suggest that apoJ may be associated with certain types of brain lesions. Expression of messenger RNA for apoJ was increased in neurons, glia, and choroid plexus following lesioning of the rat hippocampus (51). In the same study apoE expression increased but mRNAs of LDL-R, LDL-R protein, receptor-associated protein, glycoprotein 330/Megalin, and very low density lipoprotein receptor were not affected.

It is reasonable to conclude that brain apolipoproteins are probably involved in transporting cholesterol to neurons *in vivo.* However, what is not clear is the role of such cholesterol compared to cholesterol that is synthesized in the neuron. For example, could cholesterol incorporated into the neuron by apoE be used to form new membranes in addition to the cholesterol that is synthesized in the neuron? It is possible that neuronal injury might actually result in a net increase in cholesterol content resulting from neuronal cholesterol synthesis and apoE transport of cholesterol into the neuron. However, it has been reported that following entorhinal cortex lesions in 3-mon-old Fisher 344 rats there was an accumulation of nerve terminal-derived cholesterol in both astrocytes and neurons and a reduction in 3-hydroxy-3-methylglutaryl CoA reductase (HMG-CoA reductase), the key enzyme in regulating synthesis of cholesterol (37). It was suggested that the cholesterol taken up by the neurons was then used for membrane biogenesis. What is not clear is the fate of the cholesterol contained in the apoE-lipoprotein complex that is incorporated into the neuron by the LDL-R, LDL-R-related protein, or apoER2 receptors. Moreover, how cholesterol that has been internalized by the neuron is transported to the membrane has not been determined. Raft-mediated transport was discussed earlier. An alternative mechanism is that an intracellular cholesterol carrier protein such as SCP-2 may transport the newly arrived cholesterol into the neuronal membrane. Another intracellular protein, the Niemann-Pick protein (NPC1), whose function appears to be to transport cholesterol within the cell, may also interact with cholesterol derived from lipoproteins (52,53). Further discussion of this protein is contained in the section on Neuropathophysiology and Cholesterol.

MEMBRANE CHOLESTEROL DOMAINS

Cholesterol plays an important role with respect to the physical structure of the membrane. Modification of membrane cholesterol content can alter membrane fluidity, lipid packing, and interdigitation (54,55). Another important role of cholesterol is its interaction with membrane proteins. This interaction has been described in both neuronal and nonneuronal tissue (56–58). Plasma membranes are highly rich in cholesterol; in both erythrocyte membranes and synaptic plasma membranes, cholesterol accounts for over 40 mol% of the total membrane lipid (59,60). Cholesterol in the plasma membrane is not evenly distributed throughout the membrane but is located in different pools or domains (7,61).

Cholesterol lateral domains. Lateral domains of cholesterol have been described and these domains have been identified in neuronal tissue (62–64). To our knowledge, a microscopic picture of cholesterol lateral domains in biological membranes has not appeared. Instead, the existence of lateral domains of cholesterol is based largely on the removal of cholesterol from membranes, kinetic studies of cholesterol exchange, and cholesterol oxidase treatment (6). The large amount of work on lateral cholesterol domains in nonneuronal cells and model membranes has been reviewed previously (6).

Two lateral cholesterol pools that were associated with the acetylcholine receptor (62) were shown in the electroplax membranes of *Torpedo californica*. Approximately 40% of the cholesterol could be easily depleted by incubation with small unilamellar vesicles, whereas the remaining membrane cholesterol was resistant to depletion even at longer incubation periods (62). The pool of cholesterol that was easily removed was thought to be a contributor to the bulk fluidity of the membrane, while the pool that was resistant to depletion may have been closely associated with the acetylcholine receptor (62). Forty percent of cholesterol could be removed from electroplax membranes—results that are similar to what we observed in studies of mouse synaptosomal membranes. We found that there was an exchangeable pool of cholesterol that accounted for 50% of the total synaptosomal membrane cholesterol (63,64) when experiments were conducted at 37°C. Not surprisingly, at 25°C, the size of the exchangeable pool was significantly reduced and the rate of cholesterol exchange was much slower. Chronic ethanol consumption also significantly reduced the rate of cholesterol exchange (64). Another treatment that modified the rate of cholesterol exchange in synaptosomes was the hydrolysis of sphingomyelin. It has been proposed that sphingomyelin may be involved in determining the distribution of cholesterol in membranes (65,66). Sphingomyelin in the exofacial leaflet of the synaptosomal membrane was hydrolyzed by sphingomyelinase. The rate of cholesterol exchange was significantly slower than the rate of exchange in control synaptosomes but the size of the cholesterol exchangeable pool was not affected (63,64). The reduction in the rate of cholesterol exchange that we observed in sphingomyelinase-treated

synaptosomes may have been due to movement of cholesterol from the membrane surface to deeper in the synaptosomal membrane. This idea is supported by data showing that depletion of sphingomyelin by sphingomyelinase in cultured fibroblasts resulted in movement of cholesterol from the cell surface to the intracellular environment (65). It may simply require a longer period of time to move cholesterol from deep within the membrane to the membrane surface where exchange takes place.

There is little known regarding the structure and role of cholesterol lateral domains in brain tissue. Modification of cholesterol lateral domains can alter activity of certain integral proteins (67), and such domains may be important with regard to neuronal functions such as ion transport and receptor function. Mechanisms that regulate cholesterol lateral domains and their contribution to neuronal homeostasis have yet to be determined.

Transbilayer cholesterol domains. Two other important cholesterol domains are the outer or exofacial leaflet and the inner or cytofacial leaflet of the plasma membrane. The two leaflets that make up the bilayer differ in their fluidity, lipid distribution, electrical charge, and active sites of certain proteins (6,68,69). We have shown that the SPM cytofacial leaflet contains over 85% of the total SPM cholesterol (28,30,70). This large difference in the transbilayer distribution of cholesterol is associated with differences in the fluidity of the two leaflets. The cytofacial leaflet that contains almost seven times as much cholesterol as the exofacial leaflet is markedly less fluid compared to the exofacial leaflet (30,70,71). The two leaflets differ in their susceptibility to perturbation. Whereas 25 mM ethanol significantly fluidizes the exofacial leaflet, ethanol at a concentration as high as 400 mM had no effect on the fluidity of the cytofacial leaflet (29,71). Increasing temperature also had a greater effect on fluidity of the exofacial leaflet compared with the cytofacial leaflet (71). It is well known that membrane fluidity is inversely correlated with the cholesterol-to-phospholipid ratio and that increasing the ratio reduces fluidity. We attribute the differences in effects of ethanol and temperature on the two leaflets to the differences in the transbilayer distribution of cholesterol.

SPM transbilayer cholesterol distribution is not fixed or immobile but can be modified *in vivo*. Chronic ethanol consumption altered the transbilayer distribution of cholesterol in SPM of C57BL/6J mice (28). There was approximately a twofold increase in exofacial leaflet cholesterol in the chronic ethanol-treated mice compared with the pair-fed control group. Total amounts of SPM cholesterol did not differ between the ethanol-treated and control groups. We also observed that the exofacial leaflet of the ethanol group was significantly less fluid and the cytofacial leaflet was significantly more fluid than the corresponding leaflets of the control group (29). Increasing age is another condition in which the transbilayer cholesterol distribution of SPM is significantly modified (30). Twenty-five-month-old C57BL/6NNIA mice had approximately 30% of cholesterol in the SPM exofacial leaflet in contrast to mice 14–15 mon and 3–4 mon of age that had approximately 23 and 14% of cholesterol in the exofacial leaflets, respectively. We did not detect a change in the total amount of SPM cholesterol among the three different age groups. Differences in fluidity of the exofacial and cytofacial leaflets were abolished in SPM of the 24–25-mon-old mice. The exofacial leaflet became less fluid. However, fluidity of the cytofacial leaflet of the aged mice was not altered despite a reduction in the amount of cholesterol. Regulation of fluidity in the cytofacial leaflet of aged mice may involve other factors in addition to cholesterol.

Research has consistently indicated that either increasing or decreasing membrane cholesterol can modify membrane proteins. For example, varying the amount of cholesterol in SPM and synaptosomes has had an effect on sodium-dependent γ-aminobutyric acid (GABA) (72). A reduction of cholesterol in the membranes produced a loss in GABA uptake, and the uptake was restored by the addition of cholesterol. Choline uptake was not affected by changes in cholesterol content in that study. Removing or adding cholesterol to the membrane not only modifies the total amount of cholesterol but such procedures would also alter the transbilayer distribution of cholesterol. The transbilayer distribution of cholesterol may be important with respect to regulating the activity of certain membrane-bound proteins; however, this is a topic that has received little attention. Cholesterol enrichment of the erythrocyte exofacial leaflet increased protein sulfhydryl group exposure and antigen exposure (73). We have shown that oxidation of cholesterol in the exofacial leaflet of synaptosomes significantly reduced Ca^{2+} + Mg^{2+}-ATPase activity (74). Na^{+} + K^{+}-ATPase activity was not affected by modification of exofacial leaflet cholesterol. On the other hand, in murine L-cell fibroblasts transfected with liver fatty acid binding protein (L-FABP), Na^{+} + K^{+}-ATPase activity was significantly reduced in the transfected cells (75). This reduction in enzyme activity was associated with a doubling in the percentage of cholesterol in the exofacial leaflet of the transfected cells relative to the control cells. However, there was a marked reduction in the total amount of cholesterol in the L-cell membrane of the transfected cells that may have also contributed to the differences in enzyme activity between the transfected and control cell lines. Changing the distribution of cholesterol between the two leaflets of the membrane could alter the interdigitation or thickness of the two leaflets, which in turn could affect protein activity. Addition of cholesterol to model membranes reduced interdigitation (76). Ca^{2+} + Mg^{2+}-ATPase has been shown to require an optimal thickness or interdigitation and that modification of interdigitation by phosphatidylcholines of different carbon lengths modified enzyme activity (77).

Mechanisms that regulate the transbilayer distribution of cholesterol are poorly understood. There is evidence (78–80) that membrane phospholipid asymmetry is protein-regulated but there has been little progress in understanding the regulation of membrane cholesterol asymmetry. As mentioned in the preceding paragraph, L-cells transfected with the cDNA for L-FABP differed in their transbilayer distribution of cholesterol when compared with control cells. We have not been

able to detect L-FABP in mouse brain although there have been some reports suggesting that L-FABP may be present in rat brain (81). SCP-2 expression was increased in synaptosomes of chronic ethanol-treated mice (13). B-FABP and H-FABP levels were significantly lower in synaptosomes of 25-mon-old mice compared with 4-mon-old mice (18). Changes in the expression of SCP-2, B-FABP, and H-FABP in brain are associated with modification of the transbilayer distribution of cholesterol and fluidity in SPM of chronic ethanol-treated mice and aged mice. These proteins may act on cholesterol that enters the neuron by lipoproteins or contribute to cholesterol transport from the cell body.

SCP-2, B-FABP, and H-FABP are intracellular proteins. Regulation of the transbilayer distribution of cholesterol could involve factors associated with the cell surface such as apolipoproteins, their receptors, and sphingomyelin. We have recently reported that apoE and the LDL-R may play a role in regulating the distribution of cholesterol in the exofacial and cytofacial membrane leaflets (70). The SPM exofacial leaflets of apoE-deficient and LDL-R-deficient mice had two times more cholesterol than the exofacial leaflets of the control mice. These differences in cholesterol distribution were not accounted for by differences in the total amount of SPM cholesterol. ApoE may either add cholesterol by coupling with the LDL-R or other receptors or apoE may remove cholesterol from the membrane which in turn could alter the transbilayer distribution of cholesterol. Worth noting is the fact that although there was a doubling of cholesterol in the exofacial leaflets of the “knockout” mice, cholesterol remained asymmetrically distributed between the two leaflets of the bilayer. While apoE and the LDL-R may be involved in regulating the transbilayer distribution of cholesterol there must be other factors, e.g., apoJ, SCP-2, apoD, NPC1, or sphingomyelin that contribute to maintaining membrane cholesterol asymmetry. Some investigators have proposed that sphingomyelin contributes cholesterol to the distribution of membranes (65,82). Hydrolysis of sphingomyelin in Leydig tumor cells and fibroblasts has resulted in the movement of cholesterol from the cell surface to the cell interior where cholesterol was esterified. Sphingomyelin may be involved in regulating cellular cholesterol distribution, but this regulation may involve exofacial leaflet cholesterol but not cytofacial leaflet cholesterol. Sphingomyelin is primarily if not exclusively located in the exofacial leaflet of erythrocytes and SPM (63,64,83). Sphingomyelin accounts for approximately 2 to 4% of the total SPM phospholipid (29,63,64) but is not present in the SPM cytofacial leaflet (63). In erythrocytes, sphingomyelin content is approximately 25% of total phospholipid (84) content, and the amount in the exofacial leaflet was reported to be between 82 and 100%, depending on the species (83). The percentage of cholesterol in the SPM exofacial leaflet is approximately 13–15% of total SPM cholesterol. Cholesterol content in the erythrocyte exofacial leaflet is approximately 25% of the total membrane cholesterol (59). Thus increasing sphingomyelin content in the exofacial leaflet is positively associated with increasing cholesterol content in that leaflet. The interaction of sphingomyelin and cholesterol might involve binding, complex formation, or changes in membrane structure such as fluidity and lipid packing.

NEUROPATHOPHYSIOLOGY AND CHOLESTEROL

Cholesterol plays a major role in coronary heart disease. The incidence of coronary heart disease is inversely correlated with the distribution of cholesterol in the HDL fraction. In brain, an association between altered cholesterol dynamics and pathophysiology is not as well understood. A notable exception is Niemann-Pick C disease (NP-C), an inherited lipid disorder that is manifest in marked problems in cholesterol homeostasis with the central nervous system a prime target of the disease (52,53). Unesterified cholesterol accumulates in the cells leading to cell dysfunction and cell death. Cells of Niemann-Pick patients are impaired in the capacity to regulate cholesterol, including insertion and deletion of cholesterol from the plasma membrane. Recently it has been shown that mutations in a gene identified as NPC1 are the cause of NP-C (85,86). This gene encodes a protein that would appear to be important in cellular cholesterol transport. Another protein that may be involved in NP-C is apoD. Levels of apoD were significantly increased in brain of NP-C mice, an animal model of NP-C (33). ApoD was also secreted from astrocytes, and the level of apoD was lower in NP-C mice than in the control mice (33). Secretion of apoD may be important in the removal of cholesterol from cells.

NP-C is a disease in which cholesterol homeostasis is clearly disrupted. Alzheimer’s disease (AD) may be a disease in which cholesterol homeostasis is altered. The strongest evidence for such an association is the epidemiological data indicating a relationship between the apoE4 allele and the occurrence of AD. This topic has been examined in several recent reviews (45,87–89). Briefly, the apoE4 allele is a risk factor in late-onset familial and sporadic AD. Individuals with the apoE2 and apoE3 alleles may be at lower risk for AD. There is also evidence to indicate that apoE4 may be more neurotoxic compared with apoE2 and apoE3 (44).

Cholesterol content was slightly but significantly increased in frontal cortex gray matter of AD patients with the apoE4 genotype when contrasted with control subjects who also had the apoE4 genotype (89). The means ± SEM of cholesterol levels were expressed as mg/g wet tissue weight and were 2.04 ± 0.18 and 2.65 ± 0.14 for the control and AD subjects, respectively. However, another study has concluded that brain cholesterol content may actually be lower in AD patients than the cholesterol content in nondemented subjects (90). The cholesterol to phospholipid ratio was decreased by 30% in the temporal gyrus of autopsied brains from AD patients compared to control brains (90). There were no differences in the cholesterol to phospholipid ratio in cerebellum of the two groups. This reduction in the cholesterol to phospholipid ratio was attributed to a reduction of cholesterol content because the phospholipid to protein ratio was similar in brains from Alzheimer’s patients and control subjects. The amounts of

cholesterol were not reported in that study. Much more work is needed to establish if brain cholesterol amounts are affected by AD. Future studies should examine subcellular brain fractions, e.g., synaptic plasma membranes, that would provide important and necessary information about specific lipid changes. In addition, it is possible that there are regions of the brain that are more susceptible to changes in cholesterol homeostasis potentially induced by AD; the data discussed above are consistent with such an interpretation. Differences in the amounts of cholesterol in brain regions could result from cholesterol synthesis, degradation, or transport.

The amount and distribution of membrane cholesterol are important factors that modulate membrane fluidity and in turn affect activity of various proteins (69,91). Reducing the amount of cholesterol in the membrane increases fluidity of the membrane. There have not been any published studies on fluidity of brain membranes of AD patients. However, platelet membranes of AD patients were more fluid than control membranes; differences in fluidity may have resulted from a reduction in membrane cholesterol of AD patients (92,93). One might infer from that study that neuronal membranes of AD patients may be more fluid than membranes of age-matched control individuals and such changes in fluidity could affect protein activity.

Neuritic plaques and neurofibrillary tangles in brain are characteristic neuropathological features of AD (94). The main component of neuritic plaques are aggregates of the amyloid-beta peptide (Aβ). The Aβ peptide is 39–43 amino acids long and is derived from the transmembrane region of the amyloid precursor protein (95,96). There have been an extensive number of studies on the effects of Aβ on membranes, cell function, and behavior (45,97–99). Moreover, recent work has shown that there is an association between Aβ and cholesterol. Using fluorescent-labeled lipids, we have shown that aggregated Aβ binds lipids with an affinity for cholesterol > saturated fatty acids > phosphatidylcholine (100). Aβ was preincubated for different time periods (0, 1, 3, 6, 21, 24 h) and the fluorescent-labeled lipids were then added and fluorescence intensity measured. Binding of lipids was dependent on aggregation of the peptide, particularly with respect to formation of peptide polymers. Lipid binding was not observed in Aβ that had been preincubated for ≤3 h and is similar to an earlier study that reported that cholesterol and phosphatidylcholine did not bind to Aβ that had been incubated for 3 h (101). Cholesterol esterification was inhibited by different amyloid species ($A\beta_{25-35}$, $A\beta_{1-42}$, $A\beta_{1-40}$) in B12 cells and rat neuronal cortex cultures (102). In the same study, Aβ stimulated the removal of cholesterol from rat hippocampal neurons in the presence of 2-hydroxypropyl-β-cyclodextran.

Modification of cholesterol content alters APP and Aβ metabolism. Increasing the cholesterol content in APP 751 stably transfected HEK 293 cells reduced levels of soluble amyloid precursor protein (103). A conclusion of that study was that increased cholesterol levels may be a risk factor for AD, that cholesterol interferes with proteases that act on amyloid precursor proteins (APP), and that such an effect may contribute to neuronal pathology in AD. Whether there is a change in the total amount of brain cholesterol in AD patients has not been demonstrated unequivocally. Administration of cholesterol to mice expressing APP holoprotein and human Aβ-peptide produced a reduction in amounts of brain APP metabolites including $A\beta_{1-40}$ and $A\beta_{1-42}$ (104). In that study it was concluded that changes in cholesterol levels can affect APP metabolism. However, the changes in the total amounts of cholesterol were relatively modest when compared with mice on the control diets. There were no significant differences in the total amount of brain cholesterol between the two groups. In the same study it was found that the frontal cortex of the cholesterol group contained 16 mg of cholesterol, the frontal cortex of the control group contained 13.3 mg of cholesterol, and that this difference was statistically significant. Furthermore, there was more than a twofold increase in frontal cortex apoE in mice on the cholesterol diet compared with the control mice (104). Levels of apoE and not cholesterol per se may be the determining factor in Aβ metabolism. Whereas, increasing cholesterol reduced amounts of brain APP metabolites including $A\beta_{1-40}$ and $A\beta_{1-42}$, it was reported that depletion of cholesterol inhibited the production of Aβ formation in hippocampal neurons (105). Cholesterol was reduced using lovastatin and methyl-β-cyclodextrin. It is apparent that there is disagreement as to the effects of modification of cholesterol amounts on Aβ metabolism. Differences in the procedures to modify cholesterol and the biological systems employed are obvious, and potential explanations for the different effects of cholesterol on Aβ metabolism. However, an alternative explanation is that cholesterol homeostasis differs in different brain regions and/or that Aβ metabolism is brain region-dependent. What is clear is that modification of bulk cholesterol, regardless of the method employed, alters Aβ metabolism.

There is an association between cholesterol and AD, but the role of cholesterol in AD is not understood. Certainly the apoE4 allele data imply that cholesterol transport and possibly other lipids may be modified in brain of some AD patients. Metabolism of brain cholesterol may be altered in AD patients but much more data are needed to establish such a conclusion. APP and Aβ metabolism are affected by cholesterol content; however, the direction of the effect is in dispute.

Cholesterol is absolutely necessary for optimal brain function. Either too much cholesterol or too little can disrupt neuronal structure and function. An understanding of brain cholesterol dynamics is at an early stage of development. For example, it is not known how cholesterol is transported from the cell body of the neuron to the axon and what mechanisms are involved in inserting cholesterol into the plasma membrane. Cholesterol synthesis and degradation are not well understood in brain. There are some data showing that synthesis of brain cholesterol is low compared to other organs and that synthesis is high during development and then synthesis is quite low or stable (9). The half life of cholesterol in a rat brain slice preparation was calculated to be approximately 6 mon (106). Earlier work had indicated a faster turnover of cholesterol in rat (107). It is not known if cholesterol synthesis is the same

for different brain areas. Areas of the brain differ in their lipid composition, including cholesterol; however, an explanation for those differences has not been forthcoming (108). Either differences in the rate of cholesterol synthesis or differences in cholesterol transport mechanisms could explain brain regional differences in cholesterol distribution. Brain cholesterol degradation is another process in which there is little data. Outside of brain, most of the cholesterol is degraded by the liver. Degradation of brain cholesterol may involve transport from brain to the liver or there may be specialized cells in brain that catabolize cholesterol. A conclusion in an earlier review of brain cholesterol was that the sterol was not degraded in brain (109). Recently, a metabolite of cholesterol, 24S-hydroxycholesterol (24-OH-Chol), was identified as being primarily derived from brain cholesterol in both human subjects and Sprague-Dawley rats (110). Much more work is needed on brain cholesterol metabolism.

The function of brain apolipoproteins is only beginning to be understood. Much attention has focused on apoE. It is not clear if transport of cholesterol by apoE to neurons occurs only in response to injury or if such transport is required for normal neuronal cholesterol homeostasis. Involvement of other apolipoproteins in brain has not been characterized well. Another important topic that has not been examined is the role of brain lipoproteins in cellular cholesterol efflux. HDL have been shown to remove cholesterol from peripheral cells (111) and this mechanism of "reverse cholesterol transport" may be another factor involved in the regulation of cholesterol homeostasis in brain. Lipoproteins from human cerebrospinal fluid reduced cholesterol levels in fibroblasts loaded with cholesterol (112). It has been reported, however, that lipoproteins of the cerebrospinal fluid and those secreted by rat astrocytes differ in shape and lipid composition (113). Whether lipoproteins of the cerebrospinal fluid and those present in brain cells are similar in shape and composition from the same species has not been determined. Differences in lipoproteins could alter lipid transport. Optimal membrane structure and integral protein function require that cholesterol as well as other lipids be asymmetrically distributed between the two leaflets. Mechanisms that are involved in regulating the transbilayer distribution of brain membrane lipids including cholesterol are poorly understood.

The association between brain cholesterol and Alzheimer's disease is intriguing. While epidemiological data indicate that apoE4 allele may be a risk factor for AD, the molecular role of cholesterol in AD is yet to be determined. There is an interaction among APP, Aβ, and cholesterol. It has not been convincingly demonstrated whether too much or too little cholesterol drives this interaction. Areas of brain that differ in their cholesterol content may be more or less susceptible to the neurotoxic effects of Aβ.

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ARTICLE

Evidence That the *trans*-10,*cis*-12 Isomer of Conjugated Linoleic Acid Induces Body Composition Changes in Mice

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ABSTRACT: We investigated the effects of conjugated linoleic acid (CLA) preparations, which were enriched for the *cis*-9,*trans*-11 CLA isomer or the *trans*-10,*cis*-12 CLA isomer, on body composition in mice. Body composition changes (reduced body fat, enhanced body water, enhanced body protein, and enhanced body ash) were associated with feeding the *trans*-10,*cis*-12 CLA isomer. In cultured 3T3-L1 adipocytes, the *trans*-10,*cis*-12 isomer reduced lipoprotein lipase activity, intracellular triacylglycerol and glycerol, and enhanced glycerol release into the medium. By contrast, the *cis*-9,*trans*-11 and *trans*-9,*trans*-11 CLA isomers did not affect these biochemical activities. We conclude that CLA-associated body composition change results from feeding the *trans*-10,*cis*-12 isomer.

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CLA is the acronym for a class of positional and geometric conjugated dienoic isomers of linoleic acid. The term originated in 1987 when we reported biological activity (i.e., anticarcinogenic activity) associated with CLA isolated from grilled ground beef or produced from linoleic acid by base-catalyzed isomerization (1). Since then substantial interest has developed in the biochemical actions of CLA and its potential application to foods, feeds, and pharmaceuticals (2; for a current listing of the scientific literature on CLA since 1987, see http://www.wisc.edu/fri/clarefs.htm).

Dietary CLA has been shown to affect body composition (reduction in body fat, enhancement of fat-free mass) in mice (3,4), rats (5,6), and pigs (7–10). Previously we (3) reported that CLA exerts direct effects on adipocytes, which are the principal sites of fat storage, and skeletal muscle cells, which are the principal sites of fat combustion. We found that adding CLA to the culture medium of mouse 3T3-L1 adipocytes produced a dose-dependent reduction in lipoprotein lipase (LPL) activity and apparently induced lipolysis as well in this cell line. Additionally, skeletal muscle from mice fed CLA exhibited elevated carnitine palmitoyltransferase (CPT) activity. Evidence was also presented indicating that CLA enhanced whole body protein accretion. Based on these findings we proposed that the physiological mechanism of body fat reduction in mice by CLA involved inhibition of fat storage in adipocytes coupled with both elevated β-oxidation in skeletal muscle and an increase in skeletal muscle mass (3).

A central question concerns the biochemical mechanism(s) whereby CLA induces these physiological effects on adipocytes and skeletal muscle. This complex matter logically begins with the issue of CLA metabolism. The CLA preparations used to date in this research consisted principally of two isomers present in similar amounts: *cis*-9,*trans*-11 CLA, and *trans*-10,*cis*-12 CLA (1,3,11–13). Hence it is possible that either, or both, of these isomers could be involved in inducing body composition change. Previously (14) we provided evidence indicating that the *trans*-10,*cis*-12 CLA isomer decreased the expression of hepatic stearoyl-CoA desaturase mRNA in mice.

We now report on the effects, on body composition in mice, of feeding CLA preparations that contained both *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA in approximately equal amounts, and preparations that were enriched for one or the other of these isomers. Body composition change was associated with the *trans*-10,*cis*-12 CLA isomer content of the preparations. A commercial preparation that was highly enriched for the *trans*-10,*cis*-12 isomer was also active in reducing LPL activity in cultured 3T3-L1 adipocytes and enhancing triacylglycerol release from these cells. By contrast, commercial preparations highly enriched for either the *cis*-9,*trans*-11 or *trans*-9,*trans*-11 CLA isomers did not affect these biochemical activities.

MATERIALS AND METHODS

Materials. Linoleic acid was purchased from Nu-Chek-Prep Corporation (Elysian, MN); triolein, [9,10-^{3}H(N)], (specific activity 12 Ci/mmol) from American Radiolabeled Chemicals (St. Louis, MO); and [1-^{14}C]linoleic acid (specific activity 55 mCi/mmol) from Amersham Life Science (Arlington

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Abbreviations: CLA, conjugated linoleic acid; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; LPL, lipoprotein lipase;.

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Heights, IL). The isomer contents of the CLA preparations used in this study are presented in Table 1. UW-CLA was prepared as described (12). UW-CLA-1 and UW-CLA-2 were prepared by low-temperature crystallization of UW-CLA as described below. Natural Lipids (Hovdebygda, Norway) kindly provided CLA-SF (prepared by base isomerization of safflower seed oil), CLA-DC (prepared from dehydrated castor oil), and *trans*-10,*cis*-12 CLA. The *cis*-9,*trans*-11 CLA and *trans*-9,*trans*-11 CLA preparations were purchased from Matreya Inc. (Pleasant Gap, PA). 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Rockville, MD).

Low temperature crystallization. Low-temperature crystallization of UW-CLA was performed as described (15) with slight modification. Methyl esters of UW-CLA were prepared with 4% HCl in methanol (12). The CLA methyl esters were dissolved in acetone, and the solution was cooled to a range of −60 to −70°C. The solvent was removed by evaporation from the supernatant fluid. The supernatant fluid was redissolved in methanol and subjected again to recrystallization; the resulting supernatant fluid, which was enriched for *cis*-9,*trans*-11 CLA, was designated UW-CLA-1. The crystalline fraction from the first crystallization was redissolved in acetone and recrystallized at the same temperature; this preparation, which was enriched for the *trans*-10,*cis*-12 CLA isomer, was designated UW-CLA-2. UW-CLA-1 and UW-CLA-2 were hydrolyzed with 1 M KOH in methanol and extracted with hexane after acidification. Fractions were analyzed with gas chromatography (GC); the extent of methylation or hydrolysis was determined with thin-layer chromatography. GC was conducted with a Hewlett-Packard 5890 series II (Wilmington, DE) fitted with a flame-ionization detector and 3396A integrator. A Supelcowax-10 fused-silica capillary column (60 m × 0.32 mm i.d., 0.25 µm film thickness) (Bellefonte, PA) was used, and oven temperature was programmed from 50 to 200°C with an increase of 20°C per min, held for 50 min, increased 10°C per min to 225°C, and held for 20 min.

TABLE 1
Isomer Distribution of the Conjugated Linoleic Acid (CLA) Preparations Used in this Study[a]

	Fatty acid (%)			
	c-9,*t*-11[b]	*t*-10,*c*-12	*t*-9,*t*-11/*t*-10,*t*-12	Other CLA isomers[c]
UW-CLA[d]				
(used in Exp. 1)	41.1	43.5	10.0	3.04
(used in culture)	45.7	47.6	1.71	2.79
UW-CLA-1[d]	72.4	13.0	3.51	9.22
UW-CLA-2[d]	16.2	79.2	3.06	1.56
cis-9,*trans*-11 CLA[e]	96.3	N.D.[g]	2.60	1.08
trans-10,*cis*-12 CLA[f]	1.61	92.8	1.16	1.64
trans-9,*trans*-11 CLA[e]	N.D.	N.D.	100[h]	N.D.
CLA-SF[f]	41.9	43.5	1.78	1.47
CLA-DC[f]	29.1	2.63	8.44	20.1[i]

[a]Methylated with 4% HCl/methanol at room temperature for 30 min to minimize artifact formation.
[b]*c*-9,*t*-11, *cis*-9,*trans*-11; *t*-10,*c*-12, *trans*-10,*cis*-12; *t*-9,*t*-11, *trans*-9,*trans*-11; *t*-10,*t*-12, *trans*-10,*trans*-12.
[c]Mostly *cis*-9,*cis*-11 or *cis*-10,*cis*-12 isomers.
[d]CLA, prepared as described in Ref. 12; for preparation of UW-CLA-1 and UW-CLA-2 see the Materials and Methods section.
[e]Purchased from Matreya, Inc. (Pleasant Gap, PA).
[f]Materials and analysis provided by Natural Lipids (Hovdebygda, Norway). CLA-SF and CLA- DC were made from safflower seed oil and dehydrated castor oil, respectively.
[g]N.D., not detected.
[h]*trans*-9,*trans*-11 isomer only.
[i]Additionally includes 8,10 and 11,13 isomers.

Animal studies and body composition analyses. Weanling ICR mice (females for experiment 1, males for experiment 2) and semipurified diet (TD94060, 99% basal mix) were purchased from Harlan Sprague-Dawley (Madison, WI). The diet was composed as follows (ingredient, g/kg): sucrose, 476; casein, "vitamin-free" test, 210; corn starch, 150; DL-methionine, 3; corn oil, 60; cellulose, 50; mineral mix, AIN-76, 35; vitamin mix, AIN-76A, 10; calcium carbonate, 4; choline bitartrate, 2; and ethoxyquin, 0.1. For the treatment diets, 5 g UW-CLA, 3 g UW-CLA-1, or 2.5 g UW-CLA-2 (for Experiment 1), 5 g CLA-SF, 5 g or 9 g CLA-DC (for Experiment 2) were added at the expense of corn oil to maintain 6% fat. Diet was stored at −20°C until use. Mice were housed individually in a windowless room with a 12-h light-dark cycle in strict accordance to guidelines established by the Research Animal Resources Center of University of Wisconsin-Madison. Diet and water, available *ad libitum*, were freshly provided three times per week. After a 5-d adaptation period mice were randomly separated into groups and fed control diet, or diet supplemented with 0.5% UW-CLA, 0.3% UW-CLA-1, or 0.25% UW-CLA-2 (Experiment 1); or 0.5% CLA-SF, 0.5% CLA-DC, or 0.9% CLA-DC (Experiment 2).

For body composition analyses, animals were sacrificed, gut contents were removed (to obtain empty carcass weight), and the carcasses frozen at −20°C. Frozen carcasses were chopped and then freeze-dried to determine water content. Each dried carcass was ground to give a homogeneous sample before further analysis. Total nitrogen was analyzed by the Kjeldahl method (16). Carcass fat content was measured by extraction with diethyl ether overnight using a Soxhlet apparatus. Total ash content was determined by incineration (500–600°C, overnight).

Adipocyte cell culture. 3T3-L1 preadipocytes were cultured as described (17). Briefly, 3T3-L1 preadipocytes were grown to confluence at 37°C in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS). At 2-d postconfluence (designated "day 0") cell differentiation was induced with a mixture of methylisobutylxanthine (0.5 mM), dexamethasone (0.25 µM), and insulin (1 µg/mL) in DMEM containing 10% FBS. On day 2 this medium was replaced with DMEM medium containing 10% FBS and insulin only. On day 4 and thereafter the medium consisted of DMEM plus 10 %FBS only; this medium was subsequently replaced with fresh medium at 2-d intervals. Fatty acid–albumin complexes were prepared as described (3). All culture media including control had a final concentration of 100 µM albumin.

Free and esterified glycerol were determined using a Sigma Diagnostic Kit (Sigma Chemical Co., St. Louis, MO).

Heparin-releasable LPL activity (EC 3.1.1.34, 10 U heparin/mL media for 1 h at 37°C) was measured as described (18). Recovery of free fatty acid was estimated at 71% by using [^{14}C]linoleic acid. Protein was determined using Bio-Rad DC Protein assay kit (Hercules, CA).

Statistical analyses. Data were subjected to analysis using the Statistics Analysis System (SAS Users Guide: Statistics, SAS Institute Inc., Cary, NC). Data in Tables 2–4, and Figures 1 and 2 were analyzed with one-way analysis of variance (ANOVA). Two-way ANOVA (treatments and experiments) were performed on data as log values for Figures 3 and 4. If the interaction between treatment and experiment was significant, this interaction was then used as the error term in the Least Square Means analysis. For Figure 4, standard errors were computed using a nested model including variability between experiments and variability among dishes with an experiment as described (19).

RESULTS

Table 1 shows the relative isomer content of the CLA preparations employed in this study. UW-CLA was characterized primarily by two peaks with retention times corresponding to the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 CLA isomers. UW-CLA-1 was enriched for the *cis*-9,*trans*-11 isomer, whereas UW-CLA-2 was enriched for the *trans*-10,*cis*-12 CLA isomer. Preparations designated *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and *trans*-9,*trans*-11 CLA, respectively, contained more than 90% of the indicated isomer. CLA-SF was similar to UW-CLA. CLA-DC contained a number of CLA isomers including the *cis*-9,*trans*-11 isomer, but very little of the *trans*-10,*cis*-12 isomer.

In Experiment 1, mice were fed control diet, or diet containing 0.5% UW-CLA, 0.3% UW-CLA-1, or 0.25% UW-CLA-2. Body weight gains during the course of this experiment are shown in Figure 1; total feed intake is shown in Table 2. Mice fed diets supplemented with UW-CLA-2 gained significantly less body weight than controls. Mice fed diets supplemented with UW-CLA or UW-CLA-1 appeared to gain less weight than controls, but the differences were not statistically significant (however, the mean empty carcass weight of mice fed diet supplemented with UW-CLA was significantly different from controls, see Table 3). Feed intake was significantly reduced for mice fed UW-CLA and UW-CLA-2, but there was no difference in feed intake between controls and mice fed diet supplemented with UW-CLA-1.

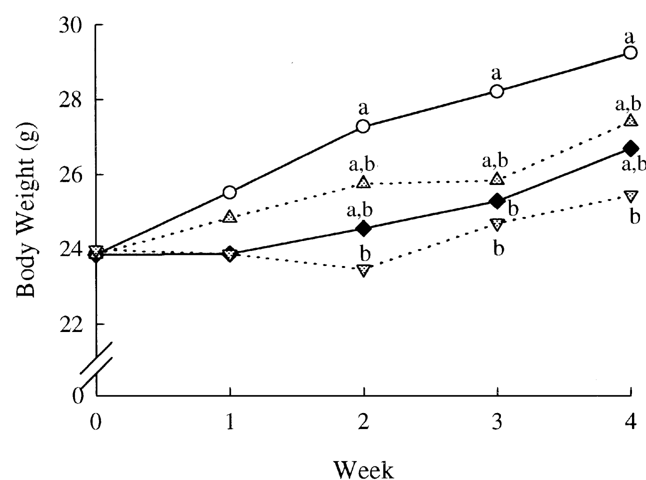

FIG. 1. Body weights, Experiment 1. Animals were fed one of the following diets for 4 wk: control (circle); UW-CLA, 0.5% (diamond); UW-CLA-1, 0.3% (triangle); UW-CLA-2, 0.25% (inverted triangle). Reported values are means ± SE for 5 or 6 animals. At each time point, means with different roman letters are significantly different at $P < 0.05$. UW-CLA, diet devised at University of Wisconsin containing conjugated linoleic acid (Ref. 12). For preparation of UW-CLA-1 and UW-CLA-2, see the Materials and Methods section.

TABLE 2
Food Intake[a]

	Food Iintake (g/mouse/4 wk)
Experiment 1	
Control	129.2[a] ± 2.5
UW-CLA	110.6[b] ± 4.5
UW-CLA-1	131.7[a] ± 1.5
UW-CLA-2	113.9[b] ± 6.8
Experiment 2	
Control	129.5[a] ± 2.0
CLA-SF	112.9[c] ± 1.7
CLA-DC 0.5%	117.9[b,c] ± 5.2
CLA-DC 0.9%	122.8[a,b] ± 1.8

[a]Female (Experiment 1) or male (Experiment 2) mice were fed treatment diet for 4 wk. Reported values are mean ± SE (n = 5 or 6 for Experiment 1, and 7 or 8 for Experiment 2). For each experiment, means with different superscript roman letters are significantly different ($P < 0.05$).

Body composition results from Experiment 1 are shown in Table 3. The percentage of body fat of the mice fed diet supplemented with UW-CLA was reduced by 70% relative to controls, whereas the percentages of whole body water, protein, and ash were significantly increased. This is consistent with our previous observations (3). A similar reduction in percentage of whole body fat, accompanied by increased percentages of whole body water, protein, and ash, was observed for mice fed diet containing UW-CLA-2. By contrast, mice fed diet containing UW-CLA-1 exhibited a smaller reduction in percentage of body fat, and smaller increases in percentages of whole body water, protein, and ash relative to controls.

In Experiment 2, mice were fed control diet or diet containing 0.5% CLA-SF, 0.5% CLA-DC, or 0.9% CLA-DC. Feeding CLA-DC at 0.9% of the diet provided an amount of the *cis*-9,*trans*-11 isomer that was equivalent to that in diet supplemented with 0.5% CLA-SF; however, the diet supplemented with 0.5% CLA-SF contained about 10 times more *trans*-10,*cis*-12 CLA than diet supplemented with 0.9% CLA-DC (Table 1).

Body weight changes during the course of this experiment are shown in Figure 2; feed intake data are indicated in Table 2. Animals fed diet containing CLA-SF exhibited reduced body weight gain relative to controls, whereas mice fed diet supplemented with CLA-DC at 0.5% or 0.9% exhibited

TABLE 3
Body Composition, Experiment 1[a]

	ECW (g)[b]	Fat (%)	Water (%)	Protein (%)	Ash (%)
Control	27.43[a] ± 1.21	22.27[a] ± 1.80	54.30[a] ± 1.35	16.26[a] ± 0.49	3.29[a] ± 0.13
UW-CLA	24.28[b] ± 0.76	6.69[c] ± 0.86	65.59[c] ± 0.68	19.04[b] ± 0.24	3.78[b] ± 0.10
UW-CLA-1	25.53[a,b] ± 0.59	13.08[b] ± 1.66	60.99[b] ± 1.14	18.09[b] ± 0.50	3.54[a,b] ± 0.13
UW-CLA-2	23.44[b] ± 0.92	6.80[c] ± 1.26	65.35[c] ± 1.13	19.33[b] ± 0.29	3.83[b] ± 0.08

[a]Female mice were fed treatment diet for 4 wk. Reported values are means ± SE (n = 5 or 6). In each column, means with different superscript roman letters are significantly different ($P < 0.05$).
[b]ECW, empty carcass weight.

TABLE 4
Body Composition, Experiment 2[a]

	ECW (g)[b]	Fat (%)	Water (%)	Protein (%)	Ash (%)
Control	31.4[a] ± 0.4	15.31[a] ± 1.66	58.3[a] ± 1.2	19.57[a] ± 0.41	3.35[a] ± 0.11
CLA-SF	27.6[b] ± 0.6	4.78[c] ± 0.57	66.1[c] ± 0.4	22.59[c] ± 0.17	3.84[b] ± 0.11
CLA-DC 0.5%	30.7[a] ± 1.3	12.27[a,b] ± 1.84	60.5[a,b] ± 1.5	20.58[a,b] ± 0.47	3.32[a] ± 0.08
CLA-DC 0.9%	30.4[a] ± 0.9	10.06[b] ± 0.99	61.9[b] ± 0.7	20.93[b] ± 0.24	3.31[a] ± 0.09

[a]Male mice were fed treatment diet for 4 wk. Reported values are mean ± SE (n = 7 or 8). In each column, means with different superscript roman letters are significantly different ($P < 0.05$).
[b]ECW, empty carcass weight.

similar weight gain to controls. Mice fed CLA-SF ate significantly less feed than controls. Mice fed a diet supplemented with 0.5% CLA-DC also appeared to eat significantly less feed than controls, but this difference was not evident for mice fed diet supplemented with 0.9% CLA-DC.

The data of Table 4 indicate that the mice fed a diet supplemented with CLA-SF exhibited significantly greater changes in body composition relative to controls than mice fed diet supplemented with CLA-DC at either 0.5 or 0.9%.

We previously reported (3) that CLA inhibited heparin-releasable LPL activity in 3T3-L1 adipocytes. Using this model,

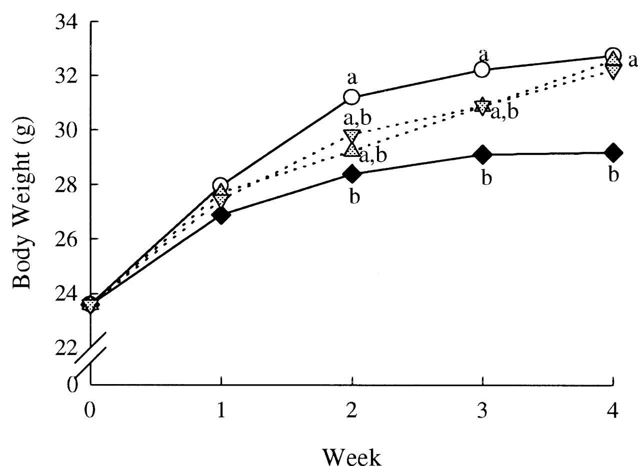

FIG.2. Body weights, Experiment 2. Animals were fed one of the following diets for 4 wk: control (circle); CLA-SF, 0.5% (diamond); CLA-DC, 0.5% (triangle); CLA-DC, 0.9% (inverted triangle). Reported values are means ± SE from 7 or 8 animals. At each time point, means with different roman letters are significantly different at $P < 0.05$. CLA-SF diet (Natural Lipids, Hovdesygda, Norway), prepared by base isomerization of safflower seed oil; CLA-DC (Natural Lipids), prepared from dehydrated castor oil.

we studied the effects of UW-CLA, *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and *trans*-9,*trans*-11 CLA on LPL activity (Fig. 3). UW-CLA and *trans*-10,*cis*-12 CLA significantly reduced LPL activity, whereas *cis*-9,*trans*-11 CLA and *trans*-9,*trans*-11 CLA were without effect (Fig. 3A). UW-CLA and *trans*-10,*cis*-12 CLA also significantly enhanced glycerol release (Fig. 3B), and significantly reduced intracellular triacylglycerol (Fig. 3C) and glycerol (Fig. 3D). By contrast, *cis*-9,*trans*-11 CLA and *trans*-9,*trans*-11 CLA appeared to produce no effect on intracellular triacylglycerol (Fig. 3C) or glycerol (Fig. 3D). Both *trans*-9,*trans*-11 CLA and *cis*-9,*trans*-11 CLA appeared to induce significant enhancements in glycerol release at the concentrations tested (Fig. 3B).

We determined the dose-response for *trans*-10,*cis*-12 CLA on LPL activity and intracellular triacylglycerol (Fig. 4). Both of these biochemical markers were reduced in a dose-dependent fashion with addition of *trans*-10,*cis*-12 CLA.

Previously (3) we published data on the inhibition of LPL activity in 3T3-L1 adipocytes by CLA. The CLA preparation employed in that study (3) was identical to UW-CLA in this study; accordingly, it was not possible to determine which isomer was responsible for the inhibitory effect. Figure 5 shows these previously published data (3) plotted as total CLA (top axis) or normalized for the *trans*-10,*cis*-12 or *cis*-9,*trans*-11 isomer content of UW-CLA (bottom axes) (the scales of the bottom axes are similar because the concentrations of the *trans*-10,*cis*-12 and *cis*-9,*trans*-11 isomers in UW-CLA are similar—Table 1). Figure 5 also includes the new data from Figure 4, which were obtained with *trans*-10,*cis*-12 CLA, and additional new data which were obtained with *cis*-9,*trans*-11 CLA. It is clear from this plot of data from independent experiments that the inhibition of LPL activity is effected by the *trans*-10,*cis*-12 isomer, not the *cis*-9,*trans*-11 isomer.

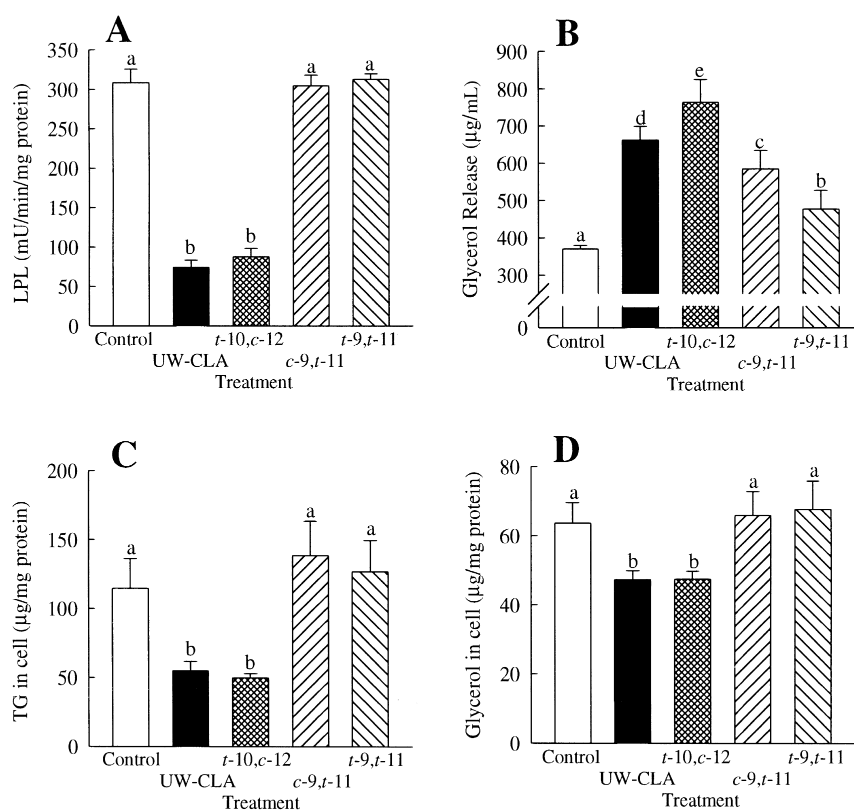

FIG. 3. Effects of CLA on heparin-releasable lipoprotein lipase (LPL) activity (A), glycerol release (B), cellular triacylglycerol (TG) (C), and cellular glycerol (D) in 3T3-L1 adipocytes. Final concentrations are 100 μM for UW-CLA, 43.6 μM for *trans*-10,*cis*-12 isomer (*t*-10,*c*-12), 44.0 μM for *cis*-9,*trans*-11 isomer (*c*-9,*t*-11), and 40.0 μM for *trans*-9,*trans*-11 isomer (*t*-9,*t*-11). Albumin (100 μM) was included in all cases, and incubations were for 48 h. Reported values are mean ± SE (n = 12–16, collected from three independent experiments). Data were analyzed as log value with a two-way analysis of variance using fatty acid (as treatments) and experiment. If the interaction between treatment and experiment was significant, this interaction was used as the error term. Means with different roman letters are significantly different ($P < 0.05$). For other abbreviation see Figure 1.

DISCUSSION

Our present results indicate that the effects of CLA on body composition are associated with the *trans*-10,*cis*-12 CLA isomer. Enrichment of dietary supplements for the *cis*-9,*trans*-11 isomer was not correlated with body composition change. The small amounts of *trans*-10,*cis*-12 CLA in UW-CLA-1 and CLA-DC (Table 1) are most likely responsible for the modest effects of these CLA preparations on body composition (Tables 3,4). In addition, in cultured 3T3-L1 mouse adipocytes, a preparation that was highly enriched for *trans*-10,*cis*-12 CLA significantly reduced LPL activity and enhanced triacylglycerol release. By contrast, preparations highly enriched for either the *cis*-9,*trans*-11 or *trans*-9,*trans*-11 CLA isomers did not affect these biochemical activities (Fig. 3–5). Accordingly, we conclude that CLA-associated body composition change is effected by the *trans*-10,*cis*-12 isomer. It is important now to determine if the 20-carbon metabolites of *trans*-10,*cis*-12 CLA identified by Sebedio *et al.* (20) will induce body composition changes in mice and other species. We have presented preliminary data indicating that conjugated eicosadienoic acid is biologically active in this regard (21).

Our working hypothesis (3) is that the CLA-induced changes in body composition are largely the result of effects of CLA on adipocytes (the major site of fat storage) and skeletal muscle cells (the major site of fat oxidation), although CLA-induced reductions in feed intake (Table 2) may also play an important role. Although the exact relationship between these different factors is unclear, they appear to correlate closely with other CLA-induced effects on lipid metabolism (14,22), indicating that hepatocytes may also be affected by CLA. Indeed, we have already demonstrated that UW-CLA (but not enzymatically synthesized *cis*-9,*trans*-11 CLA) decreased the expression of hepatic stearoyl-CoA desaturase mRNA in mice (14). Accordingly, the conclusion now that body composition changes are effected by the *trans*-10,*cis*-12 CLA isomer indicates a central role for this isomer in mediating many (but not necessarily all) of the biochemical effects attributed to CLA. Given the array of reported

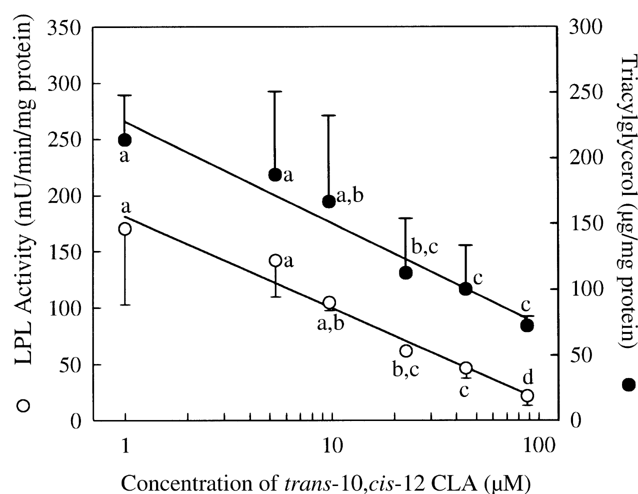

FIG. 4. Dose-response of *trans*-10,*cis*-12 CLA on heparin-releasable LPL (open circle) and amount of cellular TG (closed circle) in 3T3-L1 adipocytes. Albumin (100 μM) was included in all cases, and incubations were for 48 h. Reported values are mean ± SE (n = 4–8, collected from two independent experiments). Data were analyzed as log value with a two-way analysis of variance using fatty acid (as treatments) and experiment. If the interaction between treatment and experiment was significant, this interaction was used as the error term. Means with different roman letters are significantly different ($P < 0.05$). (To avoid zero values of log, the value of 1 was added to all data.) For abbreviations see Figures 1 and 3.

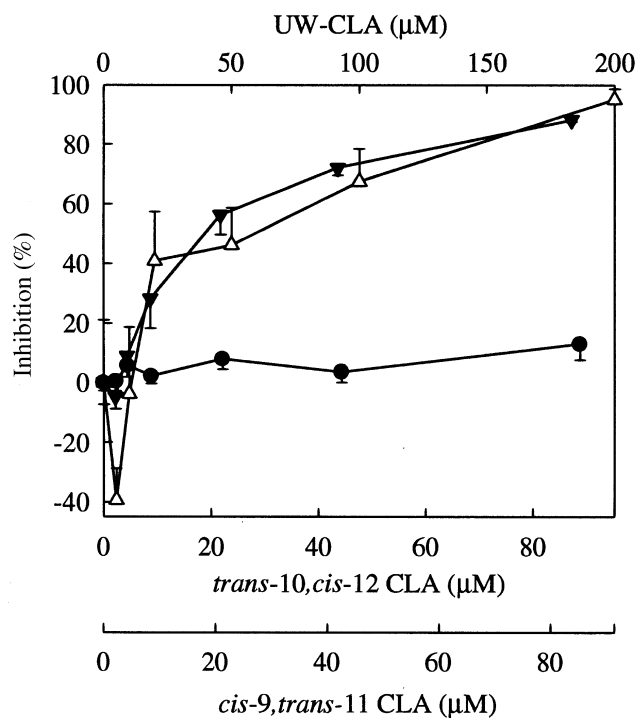

FIG. 5. Effect of UW-CLA, *trans*-10,*cis*-12 CLA, and *cis*-9,*trans*-11 CLA on heparin-releasable LPL activity in 3T3-L1 adipocytes. The top axis indicates the concentration of total CLA and refers to previously published data (3) using UW-CLA (open triangle). The bottom axis indicates the *trans*-10,*cis*-12 isomer concentration, or the *cis*-9,*trans*-11 isomer concentration and refers to data obtained with *trans*-10,*cis*-12 CLA (closed inverted triangle, data from Fig. 4), *cis*-9,*trans*-11 CLA (closed circle), or UW-CLA normalized for *trans*-10,*cis*-12 or *cis*-9,*trans*-11 isomer content. Albumin (100 μM) was included in all cases, and incubations were for 48 h. Reported values are means ± SE of 3–16 culture dishes, normalized against control.

physiological and biochemical effects of CLA, it is likely that the *cis*-9,*trans*-11 CLA isomer (and possibly other CLA isomers as well) will also be found to exert significant biological effects.

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Changes in Body Composition in Mice During Feeding and Withdrawal of Conjugated Linoleic Acid

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ABSTRACT: Two experiments were conducted. In Experiment 1, 8-wk-old mice were fed control diet or diet supplemented with 0.5% conjugated linoleic acid (CLA) to study the effect of CLA on body composition (CLA: 40.8–41.1% *c*-9,*t*-11 isomer, 43.5–44.9% *t*-10,*c*-12 isomer). The data for CLA-fed mice vs. controls described parallel but significantly distinct responses for both absolute and relative changes in body fat mass (reduced in CLA-fed mice) and for relative changes in whole body protein and whole body water (both of which were increased in CLA-fed mice). In the CLA-fed mice, the effect on whole body protein appeared to precede the reduction in body fat mass. In Experiment 2, weanling mice were fed control diet or diet supplemented with 0.5% CLA for 4 wk (test group), at which time all mice were fed control diet devoid of added CLA. The test group exhibited significantly reduced body fat and significantly enhanced whole body water relative to controls at the time of diet change. Time trends for changes in relative body composition were described by parallel lines where the test group exhibited significantly less body fat but significantly more whole body protein, whole body water, and whole body ash than controls. Tissue CLA levels declined following the withdrawal of CLA from the diet. In skeletal muscle of mice fed CLA-supplemented diet, the *t*-10,*c*-12 isomer was cleared significantly faster than the *c*-9,*t*-11 CLA isomer.

Paper no. L8012 in *Lipids 34*, 243–248 (March 1999).

CLA is the acronym for a class of positional and geometric conjugated dienoic isomers of linoleic acid. The term dates from 1987 when we reported biological activity (i.e., anticarcinogenic activity) associated with CLA produced from linoleic acid by base-catalyzed isomerization (1). Since then substantial interest has developed in the biochemical actions of CLA and its potential application to foods, feeds, and pharmaceuticals (2; for a current listing of the scientific literature on CLA since 1987, see our web page [http://www.wisc.edu/fri/clarefs.htm).]

Dietary CLA has been shown to affect body composition (reduction in body fat, enhancement of fat-free mass) in a number of animal species including mice (3,4), rats (5,6), and pigs (7–10). Previously we (3) reported that CLA exerts direct effects on adipocytes, which are the principal sites of fat storage, and skeletal muscle cells, which are the principal sites of fat combustion. We found that adding CLA to the culture medium of mouse 3T3-L1 adipocytes produced a dose-dependent reduction in lipoprotein lipase activity, and apparently induced lipolysis as well in this cell line. Additionally, skeletal muscle from mice fed CLA exhibited elevated carnitine palmitoyltransferase activity. Evidence was also presented indicating that CLA enhanced whole body protein accretion. Based on these findings we proposed that the physiological mechanism of body fat reduction in mice by CLA involved inhibition of fat storage in adipocytes coupled with both elevated β-oxidation in skeletal muscle and an increase in skeletal muscle mass (3).

We now report the results of two experiments designed to confirm and expand upon these previous observations. Experiment 1 was designed to study body composition at various times after initiating CLA feeding. Experiment 2 was conducted to determine the effect on body composition of subsequently withdrawing CLA from the diet.

The findings confirm that mice fed CLA-supplemented diet exhibited enhanced whole body protein and whole body water content, coupled with reduced fat mass. The effect on whole body protein appeared to precede body fat reduction in mice fed diet supplemented with CLA. Changes in body composition were still evident 8 wk after the cessation of CLA feeding, by which time tissue CLA levels had returned to control levels.

EXPERIMENTAL PROCEDURES

Animals and Diets. Female ICR mice were purchased from Harlan Sprague-Dawley (Madison, WI) and housed in a windowless room on a 12-h light/dark cycle, in strict accordance with guidelines established by the Research Animal Resources Center of the University of Wisconsin-Madison. Fresh diet was provided *ad libitum* three times per week; body weights were determined weekly. Diet ingredients [Composition (ingredient, g/kg): sucrose, 500; casein, "vitamin-free" test, 200; corn starch, 150; DL-methionine, 3; corn oil, 55; cellulose, 50; mineral mix, AIN-76, 35; vitamin mix, AIN-76A, 10; choline bitartrate, 2; ethoxyquin, 0.1. The foregoing diet was used in Experiment 1 (11). The diet in Experi-

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Abbreviations: ANOVA, analysis of variance; CLA, conjugated linoleic acid.

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ment 2 was identical except for sucrose (481 g/kg diet), casein (210 g/kg) and calcium carbonate (4 g/kg). When the diet was supplemented with conjugated linoleic acid (CLA), it contained 5 g CLA plus 50 g corn oil.] or semipurified diet (TD 94060) were purchased from Harlan-Teklad (Madison, WI). Control diets contained 5.5% corn oil whereas CLA-supplemented test diets contained 0.5% CLA plus 5% corn oil. CLA was prepared by alkali isomerization (12,13) of linoleic acid purchased from Nu-Chek-Prep Corporation, Elysian, MN. CLA produced by this method has been extensively characterized (12–14). The preparation used in this investigation was distinguished primarily by two peaks with retention times corresponding to the *c*-9,*t*-11 (40.8–41.1%) and *t*-10,*c*-12 (43.5–44.9%) CLA isomers, with other CLA isomers containing double bonds at the delta 9,11 or 10,12 positions as minor components (*t*-9,*t*-11/*t*-10,*t*-12, 4.6–10.0%).

Determination of body composition. Animals were sacrificed by CO_2 suffocation, and gut contents were removed prior to determining empty whole body weight. The whole bodies were then frozen at −20°C, chopped, ground, and freeze-dried to determine water content. Total protein was calculated by the Kjeldahl method (15). Whole body fat was measured following extraction overnight with diethyl ether using a Soxhlet apparatus. Total body ash was determined by incineration (500–600°C, overnight).

Fatty acid analyses. In Experiment 2, tissues (muscle, liver, and fat pad) were collected from two animals at each time point and subjected to fatty acid analysis as described previously (13). Fatty acid methyl esters were prepared by reaction with 4% HCl in methanol for 20 min at 60°C and identified by comparison with standards (Sigma Chemical Co., St. Louis, MO, and Nu-Chek-Prep Corporation) by gas chromatography (GC). GC analysis was conducted with a Hewlett-Packard 5890 series II gas chromatograph fitted with a flame-ionization detector and 3396A integrator. A Supelcowax-10 fused-silica capillary column (60 m × 0.32 mm i.d., 0.25 μm film thickness) was used, and oven temperature was programmed from 50 to 200°C, increased 20°C per min, held for 50 min, increased 10°C per min to 230°C, and held for 20 min.

Statistics. Data in Figures 1 and 4 were subjected to analysis of variance (ANOVA) using the General Linear Models procedure of the SAS Institute (SAS Users Guide: Statistics, SAS Institute Inc., Cary, NC). For Figures 2, 3, 5, 6, and 7, data were subjected to ANOVA using the mixed procedure which included a two-factor ANOVA with factors of time and diet (for Figs. 2 and 3, data from week 0 were excluded). The resulting ANOVA table contains three portions for treatments, one for diet, one for time, and one for the interaction between diet and time. The sum of squares for time was further divided into two portions, the first representing a linear time trend and the second reflecting time effects beyond the linear. The sum of squares for the treatment and time interaction was similarly divided, with the first piece representing the difference in linear trends for the two diets and the second piece representing the difference in effects beyond the linear between the two diets. In all analyses in which data were obtained on individual mice within a cage, we used PROC MIXED to explore the significance of cage effects. If such effects were not significant for all response variables of interest within an experiment, the cage term was dropped from the analysis of that experiment. If cage effects were significant for at least some response variables within an experiment, the PROC MIXED results were used for all analyses. However, in a small number of cases, we determined that *P*-values for comparisons of particular interest differed when the cage effect was included vs. when it was excluded. If for the variable in question the cage effect was not significant, we report the *P*-value for the situation with the cage effect excluded.

RESULTS

We previously established that mice fed diet supplemented with 0.5% CLA exhibited significantly reduced body fat and significantly increased whole body protein relative to mice fed a control diet (3). To confirm and expand upon these findings we conducted two experiments. The first (Experiment 1) was designed to study body composition at various times after initiating CLA feeding. The second (Experiment 2) was conducted to determine the effect on body composition of subsequently withdrawing CLA from the diet.

Figure 1 shows body weight and feed intake over the course of Experiment 1. In comparison with controls, the mice fed diet supplemented with CLA exhibited slightly reduced body weight gain. The CLA-fed mice also ate significantly less feed than control mice ($P < 0.0001$). These findings are in agreement with our previous report (3).

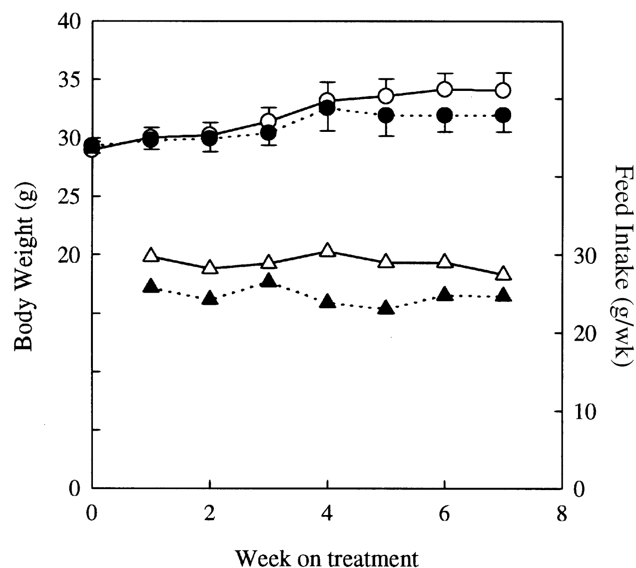

FIG. 1. Body weights and feed intake of mice in Experiment 1. Closed symbols, conjugated linoleic acid (CLA)-fed mice; open symbols, control mice. Circles, mean body weight/mouse ± S.E.; there was no cage effect ($P > 0.20$ for all points). Triangles, mean feed intake/mouse/wk determined from 3–10 cages each holding 1–3 mice; feed intakes for the CLA-fed and control groups were significantly different ($P < 0.0001$).

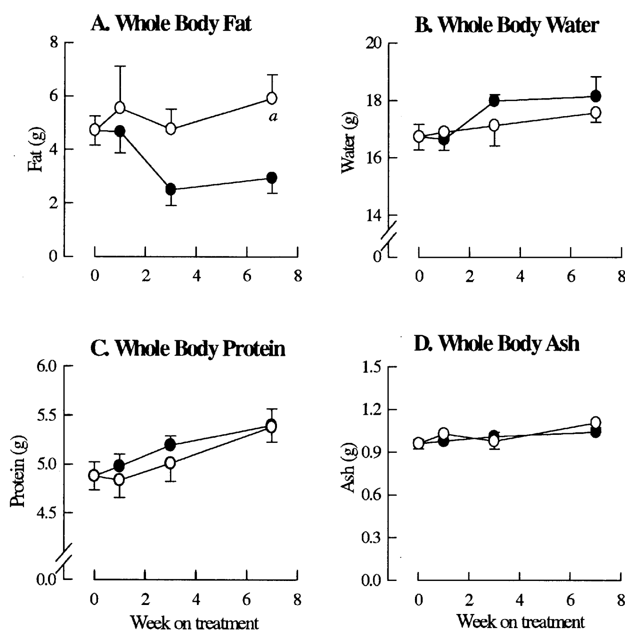

FIG. 2. Absolute changes in whole body fat, whole body water, whole body protein and whole body ash for mice of Experiment 1. Closed circles, CLA-fed; open circles, controls. Each data point represents the mean ± S.E. of 6 animals. $^aP < 0.05$ when compared to control at the same time point when cage effects, which were not significant, were ignored. For abbreviation see Figure 1.

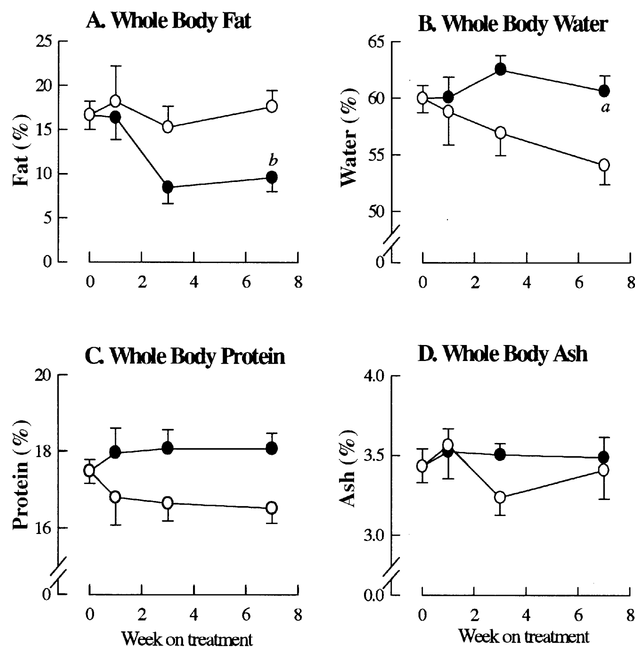

FIG. 3. Relative changes in whole body fat, whole body water, whole body protein, and whole body ash for mice of Experiment 1. Closed circles, CLA-fed; open circles, controls. Each data point represents the mean ± S.E. of 6 animals. $^aP < 0.05$ when compared to control at the same time point. $^bP < 0.05$ when compared to control at the same time point when cage effects, which were not significant, were ignored. See Figure 1 for abbreviation.

Figure 2 shows changes in absolute body composition over the course of Experiment 1. At 3 wk a reduction in whole body fat was evident in the CLA-fed mice relative to controls (Fig. 2A); the difference was significant at week 7. Overall, the whole body fat data for CLA-fed vs. controls (Fig. 2A) were described by parallel but significantly distinct responses ($P = 0.0274$). There were no significant differences in whole body water (Fig. 2B), whole body protein (Fig. 2C), or whole body ash (Fig. 2D), at any time studied. However, the CLA-fed mice appeared to exhibit small though nonsignificant elevations in whole body water (Fig. 2B) and whole body protein (Fig. 2C) at some of the time points.

To explore further the possible biological significance of the data on Figure 2, we calculated the changes in relative body composition of the mice of Experiment 1. The data are shown in Figure 3. Differences in both the percentage whole body fat (Fig. 3A) and whole body water (Fig. 3B) were apparent at week 3 and significant at week 7. At 1, 3, and 7 wk, respectively, differences in relative whole body protein (Fig. 3C) between CLA-fed and control animals approached significance ($P = 0.186$, $P = 0.109$, and $P = 0.085$, respectively). When the data for CLA-supplemented mice at these times (1, 3, and 7 wk, respectively) were summed and compared with the summed control data for the same time period, the difference in percentage whole body protein was highly significant (18.0 ± 0.3% for CLA-fed, and 16.7 ± 0.3% for controls, $P = 0.0045$). No significant differences in whole body ash between controls and mice fed CLA-supplemented diet were observed (Fig. 3D).

An analysis of overall time trends for the data of Figure 3 indicated that the slopes of the data for CLA-fed and control mice were not different. However, for whole body fat (Fig. 3A), whole body water (Fig. 3B), and whole body protein (Fig. 3C), the linear intercepts for CLA and control data were significantly different from each other ($P = 0.0251$ for whole body fat, $P = 0.0207$ for whole body water, and $P = 0.0125$ for whole body protein). This indicates that in each instance the data describe parallel but significantly distinct responses. No time trend was observed for the whole body ash data (Fig. 3D).

In Experiment 2, CLA-supplemented diet was fed for 4 wk and then withdrawn. At times thereafter body compositions were determined and compared with mice fed control diet throughout the study.

Figure 4 shows body weight and feed intake over the course of Experiment 2. During the time when CLA was fed, the mice fed diet supplemented with CLA exhibited significant reductions in feed intake (weeks 1 and 3) which were possibly accompanied by small nonsignificant reductions in body weight gain relative to controls. After withdrawal of dietary CLA, no significant differences in feed intake or body weight were evident although at week 6 and thereafter the test animals that had previously been fed CLA-supplemented diet appeared to eat slightly more feed than controls that had never been fed CLA-supplemented diet.

The absolute changes in body composition following withdrawal of dietary CLA are shown in Figure 5. For body fat (Fig. 5A), the overall time trends for the data of the CLA-fed

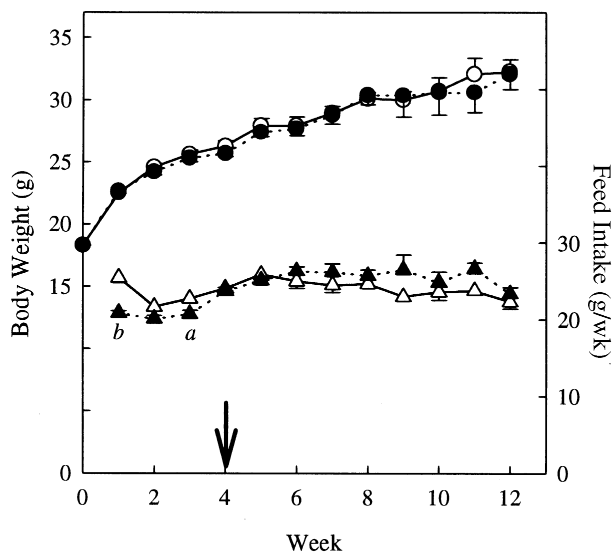

FIG. 4. Body weights and feed intake of mice in Experiment 2. Female ICR mice were fed either control or CLA-supplemented diet for 4 wk, then switched to control diet (arrow). Closed symbols, CLA-fed mice; open symbols, control mice. Circles, mean body weight/mouse ± S.E.; triangles, mean feed intake/mouse/wk ± S.E. determined from 2–10 cages each holding 5 mice under the assumption that each mouse acted independently. $^{a}P < 0.05$; $^{b}P < 0.0001$. For abbreviation see Figure 1.

and control mice exhibited parallel but significantly distinct responses ($P = 0.0016$), indicating that body fat increased in both groups but remained lower in the mice that had been fed CLA-supplemented diet. There appeared to be no significant differences in the overall time trends for whole body protein (Fig. 5B), whole body water (Fig. 5C), or whole body ash (Fig. 5D) between controls and mice that had been fed CLA-supplemented diet. However, whole body water and protein appeared to remain elevated in the mice that had been fed CLA-supplemented diet.

Figure 6 shows the relative body composition for the mice of Experiment 2. The group that had been fed diet supplemented with CLA exhibited increased whole body protein, which was significant at 1 and 2 wk (Fig. 6C), as well as increased whole body water and reduced body fat, which were significant at 0, 1, 2, and 8 wk (Fig. 6B and 6A). Relative whole body ash appeared to be higher in the CLA-fed group at every time point but the differences were not significant at any time point (Fig. 6D).

The overall time trends for the data of Figure 6 can be statistically described as parallel lines where the mice that had been fed CLA exhibited significantly less body fat ($P = 0.0001$) (Fig. 6A) and significantly more whole body protein ($P = 0.0014$) (Fig. 6C), whole body water ($P = 0.0001$) (Fig. 6B), and whole body ash ($P = 0.0448$) (Fig. 6D).

Figure 7 shows the changes in the amounts of the *c*-9,*t*-11 and *t*-10,*c*-12 CLA isomers in liver, fat pad, and muscle dur-

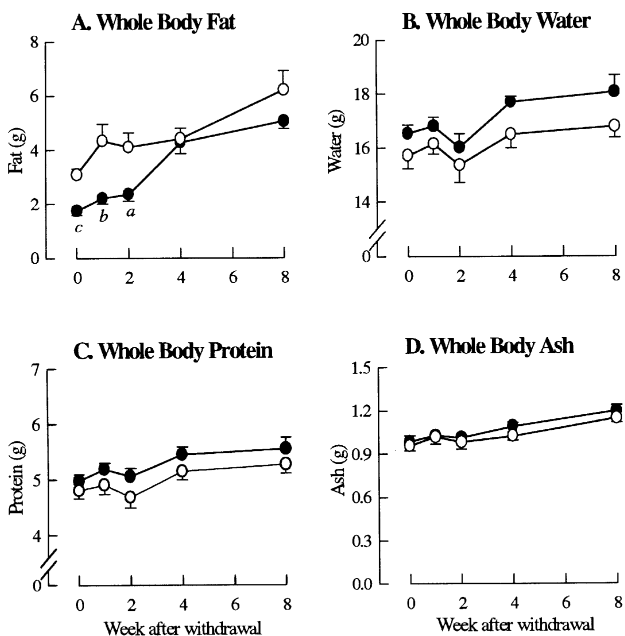

FIG. 5. Absolute changes in whole body fat, whole body water, whole body protein, and whole body ash for mice of Experiment 2. Closed circles, mice that had been fed CLA; open circles, controls. Each data point represents the mean ± S.E. of 7 or 8 animals. $^{a}P < 0.05$; $^{b}P < 0.01$ when compared to control at the same time point. $^{c}P < 0.05$ when compared to control at the same time point when cage effects, which were not significant, were ignored. For abbreviation see Figure 1.

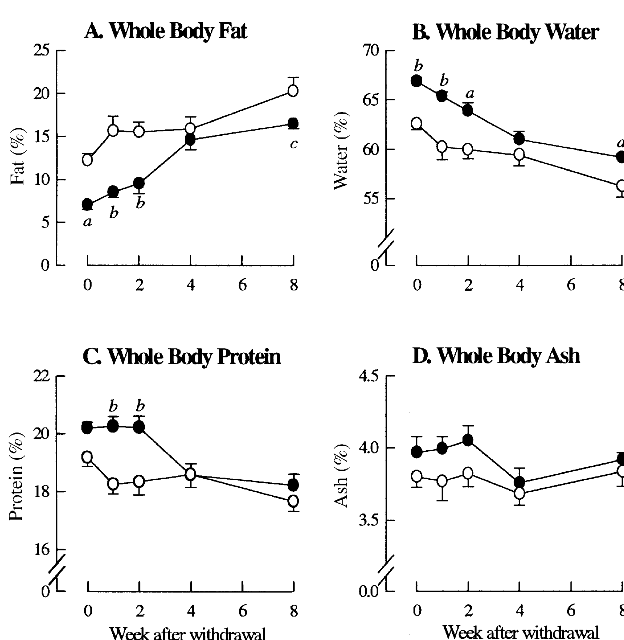

FIG. 6. Relative changes in whole body fat, whole body water, whole body protein, and whole body ash for mice of Experiment 2. Closed circles, mice that had been fed CLA; open circles, controls. Each data point represents the mean ± S.E. of 7 or 8 animals. $^{a}P < 0.05$; $^{b}P < 0.01$, when compared to control at same time point. $^{c}P < 0.05$ when compared to control at the same time point when cage effects, which were not significant, were ignored. For abbreviations see Figure 1.

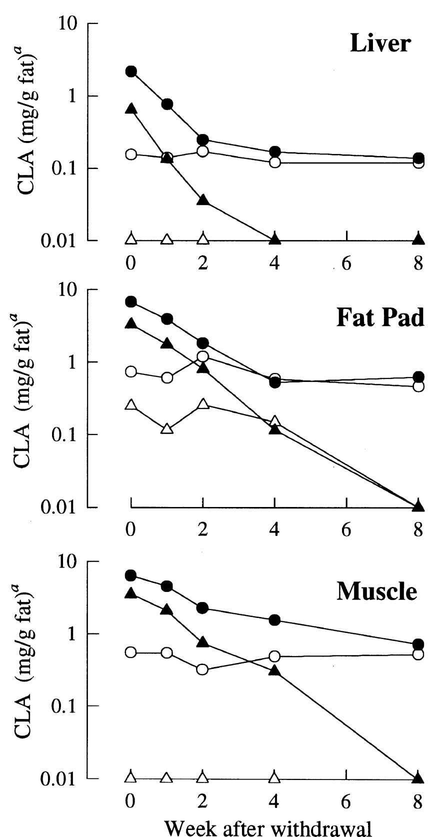

FIG. 7. CLA concentration of liver, fat pad, and muscle from animals in Experiment 2. Closed symbols, mice that had been fed CLA; open symbols, controls. Circles, *c*-9,*t*-11 CLA isomer; triangles, *t*-10,*c*-12 CLA isomer. Ten control and 10 CLA-fed animals were sacrificed as indicated (2 each per time point). [a]To avoid a zero log value when tissue CLA was undetectable, 0.01 was added to data points. For abbreviation see Figure 1.

ing the CLA withdrawal period of Experiment 2. In mice fed CLA, the levels of these CLA isomers in muscle and fat pad were similar and about three times greater than the levels in liver. Following CLA withdrawal from the diet, the *c*-9,*t*-11 isomer was virtually undetectable above background in liver after 2 wk, in fat pad after 4 wk, and in muscle after 8 wk, whereas the *t*-10,*c*-12 isomer was virtually undetectable in liver after 1 wk, fat pad after 2 wk, and muscle after 4 wk. In skeletal muscle, the *t*-10,*c*-12 CLA isomer was cleared significantly faster than the *c*-9,*t*-11 CLA isomer ($P = 0.0001$) (Fig. 7).

The CLA preparation that was fed contained both of these isomers in about equal amounts. It is noteworthy, then, that in all three tissues from mice fed CLA, the *t*-10,*c*-12 isomer was initially present at about half the concentration of the *c*-9,*t*-11 isomer.

DISCUSSION

Dietary CLA affects body composition in several animal species (3–10). Direct effects are found in adipocytes and skeletal muscle cells (3). Specifically we (3) reported that adding CLA to the culture medium of 3T3-L1 adipocytes produced a dose-dependent reduction in lipoprotein lipase activity (lipoprotein lipase hydrolyses fatty acids from circulating triacylglycerides; the free fatty acids are then taken up by the adipocytes and re-esterified). Adding CLA to the culture medium also appeared to induce lipolysis in this cell line. Additionally, skeletal muscle from mice fed CLA exhibited elevated carnitine palmitoyltransferase activity (carnitine palmitoyltransferase is the rate-limiting enzyme in fatty acid β-oxidation). Evidence was also presented indicating that CLA enhanced whole body protein accretion in mice.

Accordingly, we proposed that the physiological mechanism of body fat reduction in mice by CLA involved inhibition of fat storage in adipocytes coupled with both elevated β-oxidation in skeletal muscle and an increase (both relatively and absolutely) in skeletal muscle mass. The data presented in the present report are consistent with this model.

In addition to confirming and expanding upon our previous findings, the data of Experiment 1 provide limited evidence that an effect on whole body protein may precede body fat reduction in mice fed diet supplemented with CLA. At 1 wk of CLA feeding an apparent increase in (or maintenance of) whole body protein as percentage of body mass was evident (Fig. 3C). By contrast, reduced body fat was not clearly evident until 3 wk of CLA feeding (Fig. 2A, 3A). It is possible that the CLA-induced effect on whole body protein (relative to controls) is causally related to the (subsequent or accompanying) reduction in body fat, in that skeletal muscle is the principal site of fat combustion. The issue of energy balance may also be complicated by an apparent reduction in feed intake in CLA-fed animals, which occurred in Experiment 1 (Fig. 1) but to a much lesser extent in Experiment 2 (Fig. 4). In this regard it should be noted that fat free mass is conserved in direct proportion to food intake, e.g., more lean is conserved as more food is consumed (16). Further research is indicated to fully resolve these issues.

The data of Experiment 2 indicate that upon cessation of CLA feeding the percentage of whole body fat increased in the mice that had been fed CLA-supplemented diet (Fig. 5A and 6A). However, during the 8-wk study period, the overall level of body fat in the test group appeared to remain consistently lower than the body fat of control animals that had not been fed CLA, even though feed intake was virtually identical between the control and test mice (Fig. 4). Additionally, during this time, whole body protein (Fig. 6C) and whole body water (Fig. 6B) tended to remain elevated in the mice that had been fed the CLA-supplemented diet. Hence CLA appears to induce effects on body composition that continue for at least some time after clearance of the supplemental dietary CLA from tissue, presumably as a result of CLA metabolism (Fig. 7).

The data of Figures 4, 5A, and 6A do not appear to be consistent with the proposal (5, 17) that CLA induces adipocyte differentiation in rodents. If CLA had induced adipocyte differentiation (and, thereby, adipocyte number), then following CLA withdrawal, at which time feed intake was also similar for test and control mice (Fig. 4), we would have expected that in mice previously fed the CLA-supplemented diet, body fat accumulation would have achieved and possibly even sur-

passed control levels. This would have been expected because, if the hypothesis were correct, there would have been more fat cells in the CLA-fed mice than in the control mice, owing to the hypothetical stimulation of adipocyte differentiation by CLA and to the fact that energy would not be limiting in the mice that had been fed CLA (Fig. 4). Clearly, however, excessive body fat accumulation was not observed (Fig. 5A, 6A). To the contrary, body fat accumulation in the mice previously fed CLA lagged behind the controls and did not appear to return to control levels even after 8 wk (Fig. 6A), indicating that CLA may actually have blocked adipocyte differentiation during the time when CLA-supplemented diet was fed. Alternative explanations are possible, of course, indicating the need for further research.

The data of Figure 7 indicate that the levels of both the *c*-9,*t*-11 and *t*-10,*c*-12 CLA isomers decline at similar rates in liver and fat pad when supplemental CLA is withdrawn from the diet. However, in skeletal muscle, the *t*-10,*c*-12 CLA isomer was cleared significantly faster than the *c*-9,*t*-11 CLA isomer. The data of Figure 7 also indicate that in liver, fat pad, and muscle, the *t*-10,*c*-12 isomer was initially present at about half the concentration of the *c*-9,*t*-11 isomer, even though the CLA preparation that was fed contained both of these isomers in about equal amounts. The difference between tissue and dietary levels of these isomers could be due at least in part to the apparent preferential metabolism of *t*-10,*c*-12 CLA by skeletal muscle and possibly other tissues sites as well.

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Pharmacokinetic Advantages of a Newly Developed Tacrolimus Oil-in-Water-Type Emulsion *via* the Enteral Route

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ABSTRACT: We developed an oleic acid oil-in-water (o/w)-type emulsion of a new tacrolimus formulation that presented an improvement in the delivery of the drug for oral absorption. This investigation was undertaken to assess a sustained release drug delivery system and selective drug transfer into the lymphatic system. The whole blood concentration profiles after oral administration at a dose of 2mg/kg and bone marrow, spleen, liver, lung, small intestine, kidney, brain, and whole blood distribution after oral administration at a dose of 1 mg/kg of o/w emulsion formulation of tacrolimus (O/W group) were compared with those of commercially available formulation (T group) in the rat. The mean diameter of the o/w emulsion droplets was 0.47 μm immediately after preparation. The tacrolimus entrapping efficiency of o/w emulsion was 71.3 ± 5.0% in 12 h and did not change for 2 d. The area under the whole blood concentration-time curve (AUC) in the O/W group was significantly higher ($P < 0.01$) than that in the T group. In contrast, the values of constant elimination rate and total clearance in the O/W group were significantly lower ($P < 0.01$) than those in the T group, with a comparative bioavailability of 115.9%. The tissue concentration of tacrolimus in the O/W group was significantly higher levels in the bone marrow, spleen, liver, lung, and small intestine, and significantly lower in the brain and kidney, relative to the T group. The o/w emulsion of tacrolimus may be an improved dosage form *via* the enteral route.

Paper no. L7956 in *Lipids 34,* 249–254 (March 1999).

Tacrolimus has become an established immunosuppressant in the management of organ transplant patients. Tacrolimus has a molecular weight of 822, and is highly lipophilic. It is very soluble in methanol, ethanol, chloroform, and propyleneglycol and this seems to be associated with a markedly variable bioavailability and pharmacokinetics following oral administration (1). Much of this variability appears to result from poor absorption (1). We have been particularly interested in the development of sustained release drug delivery systems (2,3) and in selectively transferring drugs into the lymphatic system (4). Oil-in-water (o/w)-type emulsions are a system of dispersed-oil drops containing lipophilic drugs in aqueous fluids. There have been a few applications of this type of emulsion as a drug carrier *via* the enteral route. Sandimmune Neoral (5) (Sandoz Pharma Ltd., Basel, Switzerland) has been developed in an attempt to incorporate cyclosporine into a microemulsion to improve intestinal absorption. Compared to standard formulation, the absorption rate and systemic availability of cyclosporine were greater for Sandimmune Neoral at several dose levels (5). On the other hand, tacrolimus as well as cyclosporine causes adverse effects beyond systemic immunosuppression, including neurotoxicity and nephrotoxicity (6). The neurotoxicity and nephrotoxicity of tacrolimus appear to be dose-dependent (7,8).We proposed that o/w emulsion of tacrolimus was unlikely to be distributed in the brain and kidney, with their poor reticuloendothelial system (RES), and was likely to be distributed in the bone marrow and spleen, with their rich RES. To reduce the adverse effects of tacrolimus while enhancing the immunosuppressive efficacy to the site of the target organs, we developed a new emulsion formulation of tacrolimus. In the present study, an o/w emulsion based on oleic acid was used as a model for the delivery of tacrolimus. The pharmacokinetic advantages and potential usefulness of the emulsion as a carrier were evaluated.

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Abbreviations: α, constant distribution rate; AUC, area under the whole blood concentration-time curve; AUC_{tr}, AUC calculated by trapezoidal rule; C_{max}, peak level; CL_{tot}, total clearance; F, extent of bioavailability; FEBA, comparative bioavailability; k_{21}, intercompartmental transfer rate constant; ka, rate constant of absorption; Kel, constant elimination rate; o/w, oil-in-water; RES, reticuloendothelial system; T_{max}, peak time; Vc, apparent volume of the central compartment; Vd(β), apparent volume of distribution.

MATERIALS AND METHODS

Materials. Both tacrolimus and oleic acid were purchased from Fujisawa Pharmaceutical Company Ltd. (Osaka, Japan). Tween 20 (Wako Pure Chemical Ltd., Osaka, Japan) and casein sodium (Katayama Chemical Ltd., Osaka, Japan) were used as surface agents.

Preparation of emulsion. This study used tacrolimus solution (100 mg/1 mL) in ethanol. Tacrolimus solution (100 mg/1 mL) was diluted with 9 mL oleic acid. To prepare the o/w emulsion, 2 mL of the tracrolimus solution diluted with oleic acid (100 mg/10 mL) was blended with a mixture of 189 mL distilled water, 2 mL Tween 20, 2 g casein sodium, and 5

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g glycerol and stirred for about 15 min in an ice bath using an ultrasonic homogenizer (V-Level 200, Nippon Seiki Co., Tokyo, Japan). O/W emulsions were checked microscopically by microphotography. The diameter of the o/w emulsion droplets was measured by a Shimazu Laser Diffraction Particle Size Analyzer, SALD-2001 (Kyoto, Japan). We measured the particle diameters of the emulsions once immediately after and then again 24 h after preparation of the emulsions to find out the variability between batches. We administered the o/w emulsion formulation within 24 h after preparation. To determine the entrapping efficiency of tacrolimus in the oil phase of o/w emulsion, o/w emulsion was dialyzed. The dialyzer used was hemofiltration FH-66D (Gambro Co., Germany). The external water of the dialyzer was prepared using o/w emulsion without tacrolimus. The dialysis was continued at room temperature for 12 h ($n = 3$) and 24 h ($n = 3$). The tacrolimus concentration in the external water of dialyzer was assayed by microparticle enzyme immunoassay (9), which is described subsequently in the Measurement of tacrolimus levels section. The entrapping efficiency was calculated by the equation

$$\text{entrapping efficiency (\%)} = 100 \cdot (1 - C_{\text{ext}} \cdot V \cdot R/A) \qquad [1]$$

where A represents the value of total amount of tacrolimus in the o/w emulsion, C_{ext} represents the value of tacrolimus concentration in the external water of dialyzer, V represents the volume of o/w emulsion and the external water of the dialyzer, and R represents the dilution rate of the external water of the dialyzer.

Animals. All animals were housed, fed, and handled in accordance with the Guidelines for Animal Experimentation of Hamamatsu University School of Medicine, with approval of the institution review board for animal experiments. Male DA/Slc rats weighing 180–220 g (SLC, Shizuoka, Japan) were used. The experiments described in this report were conducted according to the *Guide for the Care and Use of Laboratory Animals*, in strict adherence to the Declaration of Helsinki.

Experimental design. Animals were separated into two experimental groups. The first (O/W group) consisted of rats given the o/w emulsion of tacrolimus. The second group contained rats given the commercially available formulation (solid dispersion formulation containing 20% active ingredient, 20% hydroxypropyl methylcellulose, 20% croscarmellose sodium, and 40% lactose), which was dispersed with isotonic sodium chloride solution (T group). Tacrolimus was administered orally without sedation into the stomach at a dose of 2.0 mg/kg (O/W group; $n = 6$, T group; $n = 6$) and 1.0 mg/kg (O/W group, $n = 45$; T group, $n = 45$). The animals were fasted from 24 h before administration until 48 h afterward in both groups. The test solution contained 0.1 mg tacrolimus in 1.0 mL in each group. Venous blood samples were obtained predose and then 15 min, 30 min, and 1, 2, 4, 8 12, 24, and 48 h after 2.0 mg/kg tacrolimus administration. Venous blood samples and tissue samples were obtained predose ($n = 5$) and then 15 min ($n = 5$), 1 h ($n = 5$), 3 h ($n = 5$), 6 h ($n = 5$), 9 h ($n = 5$), 12 h ($n = 5$), 24 h ($n = 5$), and 48 h ($n = 5$) after 1 mg/kg tacrolimus administration. The blood samples (50 μL) were taken from blood vessels of the tail at each time point and were collected in EDTA-containing tubes. At the end of the experiment, bone marrow, brain, liver, spleen, kidney, small intestine, and lung samples were excised. These samples were immediately frozen at −20°C.

Measurement of tacrolimus levels. Concentration of tacrolimus in whole blood and tissue samples was assayed by microparticle enzyme immunoassay method (9) using anti-tacrolimus monoclonal antibody. This assay for the IMx analyzer (Dainabot Co., Tokyo, Japan) utilizes four reagents: a precipitation reagent to extract tacrolimus from whole blood; a capture reagent consisting of latex microparticles to which tacrolimus antibodies have been attached; a tacrolimus alkaline phosphatase conjugate reagent; and an enzyme substrate reagent consisting of 4-methylumbelliferyl phosphate. Rat whole blood standard and tissue samples standards in the range of 1.5–30 ng/mL were prepared using blank rat whole blood and homogenates of tissue samples. Several tacrolimus metabolites formed in the systemic circulation are not detected by this microparticle enzyme immunoassay allowing kinetic analysis of distribution of tacrolimus alone. SOFT max PRO, which is a program made by Molecular Devices Corp. for a personal computer, is used for collection and analysis of data. The standard curves were shown by a four-parameter fitting equation:

$$\mathrm{Y} = (\mathrm{A} - \mathrm{D})/[1 + (\mathrm{X}/\mathrm{C})^{\mathrm{B}}] + \mathrm{D} \qquad [2]$$

where Y represents the value of optimal density; X represents the value of concentration of tacrolimus; and A, B, C, and D represent the values of each parameter. The correlation coefficients for the standard curves of whole blood and tissue samples were more than 0.995 in both the free and emulsion forms of tacrolimus, in the range of 1.5–30 ng/mL. Precision analysis gave coefficients of variance of 13.3, 4.9, and 8.1% for quality control samples in intraassay of 5, 10, and 20 ng/mL. The accuracy for 5, 10, and 20 ng/mL samples was 93.3, 97.7, and 98.5% of their values, respectively. The detection range was 3–30 ng/mL judging from the intravalidation data. For the determination of tissue levels of tacrolimus, tissue samples were homogenized in 10 vol of isotonic sodium chloride solution and analyzed. Tissue tacrolimus levels were calculated as the amount of tacrolimus in 1 g of tissue sample. Whole blood tacrolimus concentration profiles were analyzed by population pharmacokinetic modeling. Nonlinear multiple regression analysis was performed using MULTI 2 (10). The tacrolimus concentration (C) to time (t) data were fitted to the following equation:

$$\begin{aligned} C = &-[F \cdot k_a \cdot D \cdot (k_a - k_{21})/V_c \cdot (\alpha - k_a) \cdot (k_{el} - k_a)] \cdot \exp(-k_a \cdot t) \\ &+ [F \cdot k_a \cdot D \cdot (k_{21} - \alpha)/V_c \cdot (k_a - \alpha) \cdot (k_{el} - \alpha)] \cdot \exp(-\alpha \cdot t) \\ &+ [F \cdot k_a \cdot D \cdot (k_{21} - k_{el})/V_c \cdot (k_a - k_{el}) \cdot (\alpha - k_{el})] \cdot \exp(-k_{el} \cdot t) \qquad [3] \end{aligned}$$

where F, V_c, k_a, k_{21}, α, k_{el}, and D are extent of bioavailability, apparent volume of the central compartment, rate constant of

absorption, intercompartmental transfer rate constant, constant distribution rate, constant elimination rate, and oral administration dose of 2 mg/kg, respectively. Pharmacokinetic parameters—peak time (T_{max}), peak level (C_{max}), apparent volume of distribution [$V_{d(\beta)}/F$], area under the whole blood concentration–time curve (AUC), and total clearance (CL_{tot})—were estimated by MULTI 2. Area under the whole blood concentration–time curve (AUC_{tr}) was also calculated by the trapezoidal rule for the mean whole blood levels. Therefore, the values of AUC estimated from MULTI 2 were used for further analysis. Comparative bioavailability (F_{EBA}) was calculated by the following equation:

$$F_{EBA} = (CL_{tot}^{O/W} \cdot AUC_{tr}^{O/W}/D)/(CL_{tot}^{T} \cdot AUC_{tr}^{T}/D) \quad [4]$$

where superscripts O/W and T represent the O/W group and T group, respectively.

Statistics. Tacrolimus levels are presented as means ± standard deviation, and compared between the groups using the unpaired Student's *t*-test and *F*-test. The analysis of variance for the particle diameters of the emulsions was performed by *F*-test. $P < 0.05$ was considered significant.

RESULTS

The mean diameters of the o/w emulsion droplets were 0.47 µm immediately after preparation and 0.49 µm 24 h afterward. The particle distributions in the emulsion immediately after preparation and 24 h afterward were not significantly different (Table 1). The entrapping efficiency of tacrolimus in emulsion formulation was 71.3 ± 5.0% in 12 h and 71.9 ± 3.2% in 24 h. The stability of o/w emulsion formulation did not change when the emulsion was stored at room temperature for 2 d.

Concentration of tacrolimus in blood. Whole blood concentration profiles of drug after oral administration of O/W group and T group at a dose of 2.0 mg/kg are shown in Figure 1. Whole blood profiles were best accommodated by a two-compartment model for both O/W group and T group. Table 2 lists the pharmacokinetic parameters based on whole blood concentration. The value of AUC calculated by the trapezoidal rule in the O/W group was significantly higher ($P < 0.01$) than in the T group. In contrast, the values of k_{el} and CL_{tot} in the O/W group were significantly lower ($P < 0.01$) than those in the T group. There were no significant differences in C_{max}, T_{max}, $V_{d(\beta)}/F$, and V_c values between the O/W group and T group. The value of F_{EBA} was 115.9%. The interindividual variabilities for pharmacokinetic parameters of O/W group were not significantly reduced compared with the T group.

Tacrolimus levels of organs. As shown in Table 3, the tacrolimus levels of the bone marrow in the O/W group were significantly higher (15 min, 1 h, 3 h, and 6 h: $P < 0.05$; 9 h, 12 h, 24 h, and 48 h: $P < 0.01$) than in the T group. The tacrolimus levels in the spleen at 12, 24, and 48 h were significantly higher ($P < 0.01$) in the O/W group than in the T group. The tacrolimus levels of the liver in the O/W group were also significantly higher (15 min, 9 h, and 48 h: $P < 0.05$; 6 h: $P < 0.01$) than in the T group. The small intestinal concentrations of tacrolimus at 15 min, 1, 12, 24, and 48 h were significantly higher ($P < 0.01$) in the O/W group than in the T group. The lung concentrations at 12, 24, and 48 h were significantly higher ($P < 0.01$) in the O/W group than in the T group. In contrast, the tacrolimus levels in the brain were quite low compared with the levels in other organs. The brain concentrations of tacrolimus were significantly lower (6 and 9 h: $P < 0.05$; 15 min, 1, 3, and 12 h: $P < 0.01$) in the O/W group than in the T group. The tacrolimus levels in the kidney in the O/W group were significantly lower (6 and 9 h: $P < 0.05$; 12 and 24 h: $P < 0.01$) than in the T group. There were no significant differences between the whole blood concentrations of tacrolimus at the dose of 1.0 mg/kg.

TABLE 1
The Diameter of the Oil-in-Water-Type Emulsion Droplet Was Measured by a Shimazu Laser Diffraction Particle Size Analyzer, SALD-2001

Particle diameter (µm)	Particle volume ratio (%)[a]					
	Immediately after the preparation[b]			After 24 h[c]		
	#1	#2	#3	#1	#2	#3
–0.22	0.1	1.0	0.2	0.6	0.7	0.2
0.22–0.32	14.2	14.8	14.4	8.4	13.0	9.3
0.32–0.47	34.5	29.8	32.6	35.7	36.6	33.2
0.47–0.67	35.2	37.3	36.0	39.5	34.9	40.1
0.67–0.97	12.4	13.6	14.4	12.4	13.2	14.0
0.97–1.39	3.3	2.9	2.2	3.0	1.0	2.0
1.39–2.01	0.2	0.4	0.1	0.2	0.5	1.0
2.01–	0.1	0.2	0.1	0.2	0.1	0.2

[a]The values of particle volume ratio were calculated as percentage in three batches (#1, #2, #3). The values are not significantly different between batches.
[b]$P < 0.05$. The values are not statistically different from that immediately after the preparation within a batch.
[c]$P < 0.05$.

DISCUSSION

This is the first study to document the pharmacokinetic advantages of an orally administered oleic acid o/w emulsion incorporating tacrolimus. An o/w emulsion was used as a carrier of tacrolimus *via* the enteral route since numerous emulsions for oral administration have already been studied (11), and lipophilic drugs can be incorporated easily and efficiently within emulsions. The behavior of an emulsion is largely dependent on the lipid that constitutes the oil phase (12). If the emulsion is composed of unsaturated fatty acids, an absorption-promoting action may be obtainable (13). Emulsions enter the lymphatic system easily (4). However, we have no data to show that the drug could reach the systemic circulation incorporated in the oil-in-water-type emulsion.

Following oral administration, tacrolimus is absorbed erratically and incompletely (1). Tacrolimus is fairly stable in the gastrointestinal juice and is absorbed predominantly from the upper part of the small intestine (14). Oleic acid mixed

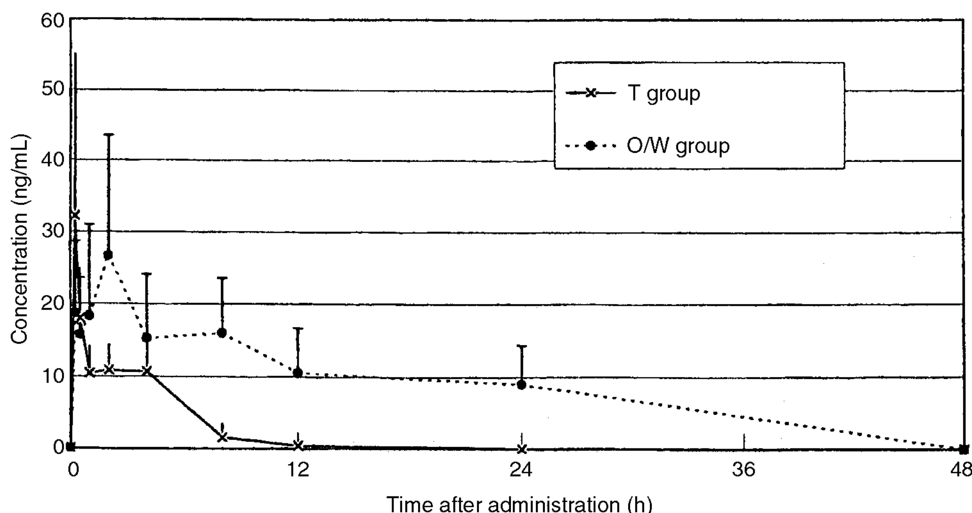

FIG. 1. Whole blood tacrolimus concentration profiles of O/W group (rats given the oil-in-water emulsion of tacrolimus) and T group (rats given the commercially available formulation) following orally administered doses of 2 mg/kg to rats. (●), O/W group (n = 6); (X), T group (n = 6). Each point represents the mean ± SD of six rats.

micelles markedly increase the mucosal membrane permeability to drugs and enhance their intestinal absorption (12). The oleic acid o/w emulsion of tacrolimus we obtained had very small particle size and good stability *in vitro*. However, no significant difference was seen in whole blood tacrolimus concentrations between O/W group and T group after oral administration at a dose of 1.0 mg/kg because whole blood tacrolimus concentrations were low in both groups, compared with the assay detection range of more than 3.0 ng/mL. On the other hand, pharmacokinetic parameters based on whole blood tacrolimus concentrations after 2.0 mg/kg oral administration in the O/W group indicated increased bioavailability relative to the T group.

In our view, some of the most important criteria of a good sustained release formulation are a bioavailability of at least 80% relative to the commercially available formulation (15), higher value of AUC, and lower values of k_{el} and CL_{tot} than in the commercially available formulation, whereas the V_c and $V_{d(\beta)}$ values should not be different between two formulations. In the present study, when an oleic acid o/w emulsion formulation of tacrolimus was administered orally at a dose of 2.0 mg/kg, the bioavailability of tacrolimus from the O/W group was 115.9%, the AUC value was significantly higher, and the k_{el} and CL_{tot} values were significantly lower compared to those from the commercially available formulation. There were no significant differences in V_c and $V_{d(\beta)}$ values between the O/W group and T group. Increased bioavailability appears to result from reduced CL_{tot} and smaller first-pass effect. The variability was much larger in the O/W group, in part due to inadequate precision in the delivered dose. The release pattern of a sustained release preparation should be independent of pH, enzyme, agitation, and any other variables that might be encountered in the gastrointestinal tract. Further, *in vitro* evaluation of these parameters is needed.

TABLE 2
Pharmacokinetic Parameters[a] Based on Whole Blood Concentrations of Tacrolimus After 2 mg/kg Oral Administration

Group	C_{max}[b] (ng/mL)	T_{max}[c] (h)	AUC_{tr} (0–48)[d] (ng/mL/h)	AUC (0–48)[e] (ng/mL/h)	CL_{tot}[f] (L/h/kg)	k_{el}[g] (1/h)	V_c[h] (L/kg)	$V_{d(\beta)}/F$[i] (L/kg)
O/W[j]	32.1 ± 9.6	1.6 ± 0.8	312.3 ± 41.0[k]	299.5 ± 133.7	8.5 ± 5.7[k]	0.01 ± 0.01[k]	27.7 ± 17.6	2345.7 ± 2658.2
T[j]	36.3 ± 18.3	0.48 ± 0	80.1 ± 39.9	73.5 ± 16.5	28.6 ± 7.4	0.17 ± 0.02	14.8 ± 8.7	169.7 ± 30.8

[a]Values represent mean ± SD of six animals.
[b]C_{max}, peak level.
[c]T_{max}, peak time.
[d]AUC_{tr} (0–48), area under the concentration-time curve calculated by the trapezoidal rule.
[e]AUC (0–48), area under the concentration-time curve estimated by MULTI 2.
[f]CL_{tot}, total clearance.
[g]k_{el}, constant elimination rate.
[h]V_c, apparent volume of the central compartment.
[i]$V_{d(\beta)}$/F, apparent volume of distribution.
[j]O/W, oil-in-water-type emulsion of tacrolimus; T, commercially available formulation of tacrolimus. Tacrolimus (2 mg/kg) was administered as each formula. Significantly different from T group.
[k]$P < 0.01$.

TABLE 3
Changes in Time of Tacrolimus Levels (ng/1 g tissue) in Organs[a]

Organ	Group	15 min	1 h	3 h	6 h	9 h	12 h	24 h	48 h
Bone	O/W[b]	1240 ± 223[c]	1370 ± 223[c]	1707 ± 101[c]	4080 ± 684[c]	3920 ± 212[d]	800 ± 80[d]	1040 ± 40[d]	870 ± 52[d]
marrow	T[b]	673 ± 200	920 ± 131	1553 ± 76	1513 ± 81	2160 ± 144	347 ± 130	360 ± 72	387 ± 160
Spleen	O/W[b]	1067 ± 83	660 ± 302	500 ± 100	846 ± 153	840 ± 104	386 ± 42[d]	413 ± 31[d]	580 ± 44[d]
	T[b]	953 ± 50	913 ± 140	653 ± 142	793 ± 16	833 ± 58	266 ± 14	253 ± 25	200 ± 10
Liver	O/W[b]	607 ± 91[c]	353 ± 50	300 ± 60	427 ± 12[d]	417 ± 65[c]	53 ± 20	70 ± 13	85 ± 17[c]
	T[b]	403 ± 95	317 ± 76	280 ± 44	213 ± 23	273 ± 25	80 ± 10	72 ± 21	58 ± 4
Small	O/W[b]	1113 ± 81[d]	1080 ± 35[d]	893 ± 310	380 ± 46	453 ± 84	270 ± 20[d]	327 ± 55[d]	180 ± 20[d]
intestine	T[b]	817 ± 15	493 ± 31	760 ± 225	467 ± 76	500 ± 50	14 ± 12	5 ± 9	14 ± 16
Lung	O/W[b]	677 ± 12	677 ± 12	673 ± 64	503 ± 15	477 ± 15	303 ± 38[d]	297 ± 38[d]	187 ± 12[d]
	T[b]	540 ± 53	530 ± 70	610 ± 105	483 ± 29	530 ± 113	72 ± 8	93 ± 41	87 ± 8
Brain	O/W[b]	16 ± 3[d]	15 ± 6[d]	16 ± 3[d]	44 ± 20[c]	49 ± 21[c]	17 ± 2[d]	16 ± 3	3 ± 0
	T[b]	72 ± 3	64 ± 4	64 ± 12	71 ± 4	71 ± 4	27 ± 2	23 ± 5	4 ± 1
Kidney	O/W[b]	503 ± 21	520 ± 0	590 ± 75	427 ± 21[d]	373 ± 25[c]	110 ± 10[d]	113 ± 8[d]	57 ± 33
	T[b]	503 ± 15	507 ± 21	500 ± 40	467 ± 15	493 ± 60	146 ± 5	168 ± 14	63 ± 3
Whole	O/W[b]	6.0 ± 2.2	3.4 ± 7.5	2.5 ± 2.0	1.6 ± 1.5	0 ± 0	0 ± 0	0 ± 0	0 ± 0
blood	T[b]	8.5 ± 4.8	7.3 ± 3.2	4.0 ± 1.3	1.8 ± 0.3	0 ± 0	0 ± 0	0 ± 0	0 ± 0

[a]Values represent mean ± SD of five animals. The values of whole blood level were calculated as ng/mL alone. See Table 2 for abbreviations.
[b]Tacrolimus (1 mg/kg) was administered as each formula. The value is significantly different from T group.
[c]$P < 0.05$.
[d]$P < 0.01$.

The present pharmacokinetic study of the O/W group of emulsion demonstrated significantly increased tacrolimus levels in the bone marrow, spleen, liver, lung, and small intestine, and significantly decreased tacrolimus levels in the brain and kidney, compared to the T group. This suggests that the main site of o/w emulsion uptake after oral administration was the RES; brain and kidney are known to contain little in the way of RES elements. Increased delivery of oleic acid o/w emulsion incorporating tacrolimus into the lymphoid organs may markedly alter alloantigen presentation, proliferation and differentiation of allosensitive lymphocytes, thereby increasing the effectiveness of this drug in delaying graft rejection. From the viewpoint of adverse effects, decreased tacrolimus levels in the brain and kidney with this emulsion formulation of tacrolimus may logically be expected to result in less tendency to cause neurotoxicity and nephrotoxicity but this point was not addressed in the present study. The evaluation of the host's systemic immune function as well as the effect of local immunosuppression on tolerance induction and the avoidance of neurotoxicity and nephrotoxicity by the use of an o/w emulsion formulation need to be explored.

In conclusion, our o/w emulsion formulation of tacrolimus has very small particle size, good *in vitro* stability, a sustained release of drug, and selective drug transfer into the lymphatic system comparable to the characteristics of a commercially available formulation. It is considered that o/w emulsion might have advantages as an alternative oral dosage form for tacrolimus.

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Susceptibility of Serum Lipids to Copper-Induced Peroxidation Correlates with the Level of High Density Lipoprotein Cholesterol

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ABSTRACT: As a first step in evaluating the significance of our recently developed method of monitoring the kinetics of copper-induced oxidation in unfractionated serum, we recorded the kinetics of lipid oxidation in the sera of 62 hyperlipidemic patients and analyzed the correlation between oxidation and lipid composition of the sera [high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglycerides]. We used six factors to characterize the kinetics of oxidation, namely, the maximal absorbance of oxidation products (OD_{max}), the maximal rate of their production (V_{max}), and the time at which the rate was maximal (t_{max}) at two wavelengths (245 nm, where 7-ketocholesterol and conjugated dienic hydroperoxides absorb intensely, and 268 nm, where the absorbance is mostly due to dienals). The major conclusions of our analyses are that: (i) Both OD_{max} and V_{max} correlate positively with the sum of concentrations of the major oxidizable lipids, cholesterol, and cholesteryl esters. (ii). The value of t_{max}, which is a measure of the lag preceding oxidation and therefore reflects the resistance of the serum lipids to copper-induced oxidation, exhibits a negative correlation with HDL cholesterol. Although this finding accords with the observation of shorter lags for HDL than for LDL, it is apparently inconsistent with the role of HDL as an antirisk factor in coronary heart diseases.

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Oxidation of low density lipoprotein (LDL) in the subendothelial space of major arteries is believed to play a key role in atherogenesis (1–3). The possible involvement of high density lipoprotein (HDL) in this process is not clear. On one hand, several *in vitro* studies have shown that HDL is more susceptible to oxidation than LDL (4–6) and that in mixtures of HDL and LDL the lag preceding oxidation of LDL lipids is longer than the lag preceding the oxidation of pure HDL but shorter than the lag preceding the oxidation of pure LDL (6). On the other hand, in several other studies HDL appeared to be less susceptible to oxidation than LDL and to protect LDL against copper-induced oxidation, presumably owing to the activity of the HDL-associated enzymes platelet-activating factor-acetyl hydrolase (PAF-AH) and paraoxonase (7–10).

Previously we developed a method for monitoring the kinetics of copper-induced lipid oxidation in unfractionated serum (6,11). Although this optimized assay is only slightly dependent on the experimental processing of the serum, we do not know whether the recorded kinetic profiles, measured under nonphysiological conditions, are relevant to *in vivo* oxidation. As a first step in evaluating the possible significance of our assay, we investigated the relationship between the kinetics of lipid oxidation and the lipoprotein composition of hyperlipidemic patients, for whom lipid composition, as measured routinely by clinical laboratories, varies markedly with respect to the concentrations of triglycerides (TG), LDL-cholesterol, and HDL-cholesterol (Table 1). Specifically, we recorded continuously the time course of copper-induced oxidation of serum samples of 62 patients and analyzed the correlations between the characteristics of oxidation of the sera and the concentrations of TG, HDL-cholesterol and LDL-cholesterol. Because the patients differed in their dyslipidemia, treatment, sex, and age, we did not expect the results to yield any conclusion regarding the correlation between the kinetics of oxidation and the treatment given to the patients. Nonetheless, given the large variation of the serum lipid concentrations we attribute significance to the correlations described below between the concentrations of the different components of the serum and the factors that can be used to characterize its copper-induced oxidation *in vitro*.

MATERIALS AND METHODS

The study is based on the lipid composition and oxidizability of the serum of 62 randomly chosen, nonobese, hyperlipidemic patients of the lipid outpatient clinic of Rabin Medical Center. It was carried out according to the principles of the Declaration of Helsinki approved by the Human Experimentation Committee of the Hospital. All participants gave their written informed consent. Obese patients and patients suffering from inflammatory conditions (white blood cell count ≥ 12,000 per dL) were excluded from the study. Nine of the participating patients suffered from insulin–independent diabetes. Eleven of the patients were smokers. Venous blood was drawn after 12 h of fasting, as part of the regular checkup examinations. Serum was prepared by centrifugation (at 1000

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; OD_{max}, maximal absorbance of oxidation products; PAF-AH, platelet-activating factor-acetyl hydrolase; PUFA, polyunsaturated fatty acids; t_{max}, time at which the rate of oxidation was maximal; TG, triglycerides; V_{max}, maximal rate of oxidation products accumulation.

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TABLE 1
Composition and Parameters of Oxidation Kinetics in Sera of Hyperlipidemic Subjects[a]

Treatment	Diet	Fibrates	Statins	All
Number (M/F)	17 (7/10)	15 (8/7)	30 (16/14)	62 (31/31)
Age (yr)	50.47 ± 9.73	55.67 ± 9.25	57.30 ± 10.64	55.03 ± 10.33
Cholesterol (mg/dL)	245.5 ± 41.0	232.1 ± 40.9	243.3 ± 64.9	241.2 ± 53.5
LDL cholesterol (mg/dL)	129.7 ± 51.3	121.2 ± 35.7	150.6 ± 57.0	137.8 ± 51.9
HDL cholesterol (mg/dL)	47.4 ± 14.4	41.1 ± 11.5	54.1 ± 12.1	49.1 ± 13.5
Triglycerides	342.0 ± 256.1	340.7 ± 142.2	180.4 ± 68.2	263.5 ± 175.0
Kinetics at 245 nm				
OD_{max}[a]	0.631 ± 0.108	0.641 ± 0.149	0.585 ± 0.109	0.611 ± 0.120
V_{max} (OD/min)	0.0054 ± 0.0013	0.0050 ± 0.0011	0.0058 ± 0.0016	0.0055 ± 0.0014
t_{max} (min)	86.8 ± 21.7	94.2 ± 20.2	78.1 ± 19.0	84.4 ± 20.8
Kinetics at 268nm				
OD_{max}[a]	0.379 ± 0.053	0.396 ± 0.064	0.381 ± 0.060	0.384 ± 0.059
V_{max} (OD/min)	0.0026 ± 0.0005	0.0026 ± 0.0005	0.0029 ± 0.0006	0.0027 ± 0.0006
t_{max} (min)	91.5 ± 19.1	100.0 ± 18.3	80.5 ± 13.7	88.2 ± 18.1

[a]OD_{max} denotes the (nearly constant) optical density (OD) level, as measured after 5 h of exposure of the serum to copper (see the Materials and Methods) section. Statin, hydroxymethylglutaryl-CoA-reductase inhibitor; M/F, male/female; LDL, low density lipoprotein; HDL, high density lipoprotein; V_{max}, maximal rate of oxidation products accumulation, *t*, time.

× *g*) of the blood (collected into vacutainers) at 4°C, 30 min after collection. Isolation of serum lipoproteins was performed by sequential ultracentrifugation according to our earlier report (6). The cholesterol and TG concentrations in the serum and isolated lipoproteins were determined using commercially available kits (Boehringer-Mannheim, Mannheim, Germany).

The kinetics of lipid oxidation in each serum was monitored through continuous recording of the absorbance at two wavelengths (245 and 268 nm) following 50-fold dilution of the serum in a phosphate-buffered saline containing sodium citrate (0.72 mM) and $CuCl_2$ (0.1 mM) (6,11). Figure 1 depicts the results of a typical time course of the absorbance under these conditions. The time-dependent increase of absorbance at 268 nm, to which dienals make the major contribution, lags only slightly behind the increase of absorbance at 245 nm, to which the major contribution is that of lipid hydroperoxides and 7-ketocholesterol (12). Each of these time dependencies was characterized by three factors, namely, the maximal absorbance (OD_{max}), the maximal rate of increase of absorbance (V_{max}), and the time at which the rate was maximal (t_{max}). The last factor has been shown previously to correlate with the more commonly used lag time (13). Because t_{max} can be unequivocally determined from the first derivative of the time course of accumulation (Fig. 1, lower panel), we prefer using this factor (rather than the lag) to characterize the resistance of serum lipids to oxidation.

Data analysis was performed by the standard procedures provided by Microsoft Excel 7 (Redmond, WA) and Microcal Origin 4.0 (Northampton, MA) software.

RESULTS

Of the 62 patients that participated in this study, 17 were treated for hyperlipidemia by a low-fat diet only, 15 were treated with fibric acid derivatives (fibrates), and 30 were treated with hydroxymethylglutaryl-CoA-reductase inhibitors (statins) at varying doses. The concentrations of serum lipids

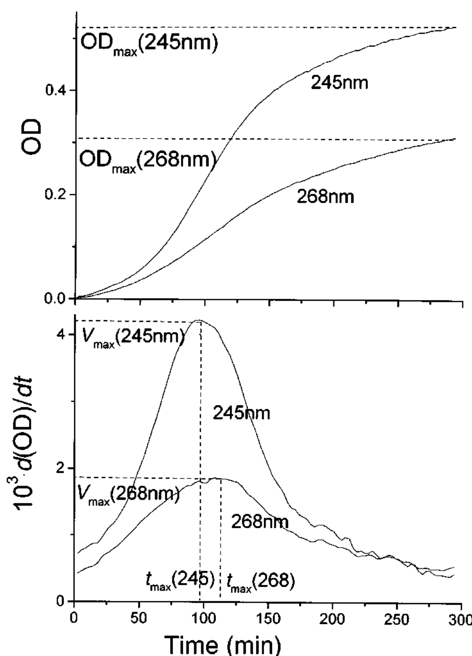

FIG. 1. Time course of lipid peroxidation in unfractionated serum, as monitored by continuous recording of absorbance at 245 and 268 nm. A serum sample (30 µL) was added to a phosphate-buffered solution (pH 7.4) containing 150 mM NaCl, 0.72 mM sodium citrate, and 0.1 mM $CuCl_2$, preequilibrated at 37°C. The absorbance, recorded at intervals of 3 min, using a Kontron (Model 933) spectrophotometer equipped with an automated cell changer, is depicted in the upper panel. The lower panel depicts the derivative of the time dependencies given in the upper panel. The values of OD_{max} were derived from the time dependencies of accumulation (upper panel). The values of V_{max} (in OD units/min) and of t_{max} (in min) were derived from the respective coordinates of the peaks in the time courses of rate (lower panel). OD, optical density; V_{max}, maximal rate of oxidation products accumulation.

in these three groups of patients (Table 1) differed accordingly. The lipid compositions of the sera of those patients who were treated by diet or fibrates were very similar with respect to both cholesterol and TG. Furthermore, the total cholesterol content of the sera of the hypercholesterolemic patients treated with statins was not significantly different from that of the sera of the other two groups. However, both the experimentally determined HDL cholesterol and the calculated LDL cholesterol levels were somewhat higher in the statin group than in the other two groups ($P < 10^{-2}$). By contrast, the patients of the statin groups had much lower TG levels than those of the other two groups ($P < 10^{-3}$).

Also presented in Table 1 are the results of our kinetic studies, given in terms of the mean values and standard deviations of V_{max}, t_{max} and OD_{max}, as measured both at 245 and 268 nm for the sera of patients of the three groups. These data indicate that oxidation of the serum of statin-treated patients was preceded by somewhat shorter lag times than the oxidation of the other patients ($P < 0.02$). Interestingly, even the largest mean value of t_{max}, as measured at 245 nm for the group of hyperlipidemic patients treated by fibrates (94.2 min), was shorter than that observed for 15 normolipidemic individuals as determined by the same assay (107 ± 17 min; Fainaru, O., Pinchuk, I., Fainaru, M., Assali, A. and Lichtenberg, D., in preparation). The question remains whether these results relate to the lipid composition and/or to the treatment. The following analyses concerning the correlation between the oxidation parameters and serum composition for all the tested sera provide a partial answer to this question.

Figure 2 presents the correlations observed between the various kinetic factors and the composition of the individual sera. Because peroxidation of serum lipids depends on many factors, it is not surprising that none of these correlations was very high. Nonetheless, the following correlations were highly statistically significant ($P < 10^{-3}$). (i) Both OD_{max} (observed range of values 0.369–0.948 OD units) and V_{max} (observed range of values 0.0024–0.0101 OD units/min), as measured at 245 nm, depended linearly on the cholesterol concentration (range of values 138–457 mg/dL). (ii) The correlations between cholesterol level and either OD_{max} (observed range of values 0.260–0.517 OD units) or V_{max} (observed range of values 0.0017–0.0042 OD units/min) at 268 nm were statistically significant but somewhat weaker. (iii) The value of OD_{max} at 245 nm (but not at 268 nm) correlated negatively with the concentration of HDL cholesterol (range of values 22–80 mg/dL). (iv) The value of t_{max}, as measured at either 245 (observed range of values 48–135 min) or 268 nm (observed range of values 54–135 min) correlated negatively with the level of HDL cholesterol.

Before evaluating these results we tested whether the correlations observed for the whole population exist for subgroups of the 62 patients studied. First, we analyzed separately the data on the 31 male and 31 female patients who participated in the study and found the same trends as those obtained for all the sera taken together. Second, we repeated these analyses for the group of 53 nondiabetic patients as well

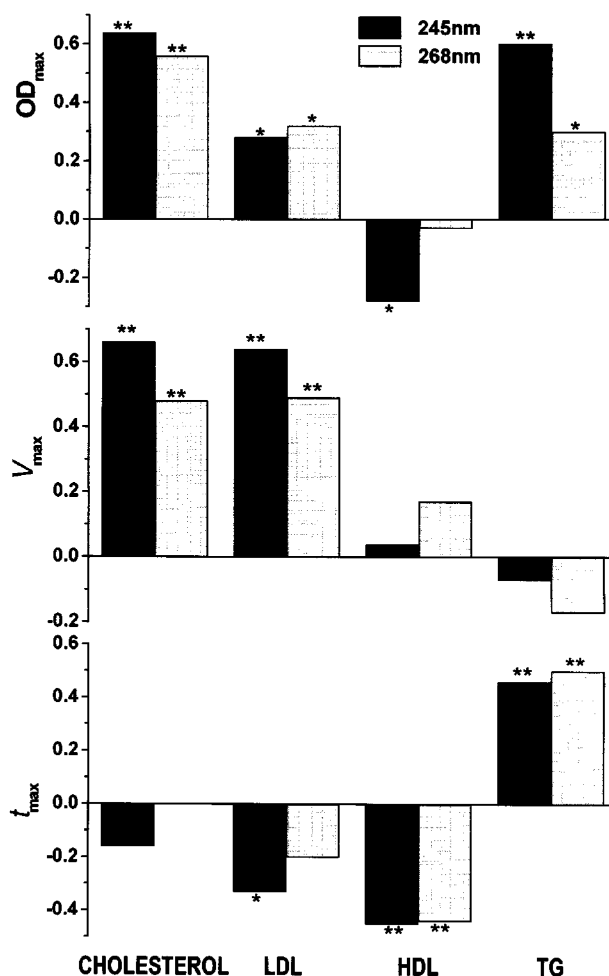

FIG. 2. The relation between serum composition and oxidation parameters. Correlation coefficients are given between the serum concentrations of cholesterol (total), low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, and total triglycerides (TG), on one hand, and the oxidation parameters, OD_{max}, V_{max} and t_{max}, on the other. The black bars relate to oxidation kinetics as recorded at 245 nm, whereas the gray bars relate to the kinetics as recorded at 268 nm. The symbol (*) corresponds to a P-value smaller than 0.05; the symbol (**) corresponds to P-values smaller than 10^{-3}.

as for the group of 51 nonsmokers among the studied patients and found in both cases the same correlations observed for all the patients, with comparable correlation coefficients. These analyses showed that in each of these groups both V_{max} and OD_{max} correlated with the lipid composition of the serum as described above. Furthermore, the positive correlation between t_{max} and TG was similar for each of the studied groups.

By contrast, the correlation between t_{max} and HDL in the statin-treated group differed markedly from that observed for the patients of the two other groups. Linear regression analyses of the dependence of t_{max} on HDL exhibited negative correlations for each of the three groups, but the influence of HDL on t_{max} was less pronounced for the statin-treated patients than for the other groups. Specifically, for the statin group the dependence of t_{max} on HDL did not reach the 5% limit of significance, the correlation coefficient was very low

($R = -0.13$), and the computed slope of the dependence was -0.21 min/mg% HDL cholesterol. By contrast, in the other two groups the same analysis indicated that the dependence of t_{max} on HDL is very significant ($R = -0.59$, $P = 4 \times 10^{-4}$) and much stronger (-0.94 min/mg% HDL cholesterol). This difference may possibly indicate that the HDL of patients treated by statins is different from that of patients of the two other groups, as a result either of the difference in the dyslipidemia or of the statin treatment. We are unaware of any data that may either support or rule out any of these possibilities.

DISCUSSION

The dependencies of both OD_{max} and V_{max} on serum composition can be interpreted as follows: (i) The linear dependence of OD_{max}, measured at 245 nm after 5 h of exposure to copper, on the total cholesterol is expected because hydroperoxides are intermediates in the time course of oxidation (12,14). Hence, oxidized cholesterol is likely to make the major contribution to the maximal level of OD at 245 nm, as measured after most of the cholesterol in the lipoproteins had already oxidized while most of the hydroperoxides had already decomposed. Furthermore, V_{max}, expressed in terms OD units per minute, is proportional to the concentration of the major oxidizable lipids (cholesterol and cholesteryl esters); its correlation with the total cholesterol level is not surprising. (ii) To explain the correlation between cholesterol level and either OD_{max} or V_{max} at 268 nm, it should be noted that much of the polyunsaturated fatty acids (PUFA), from which dienals are formed (*via* hydroperoxides) is esterified with cholesterol. However, this correlation was weaker, probably because free PUFA and PUFA esterified into phospholipids and TG, which do not necessarily correlate with the level of cholesterol, also contribute to the production of dienals. (iii) The negative correlation between OD_{max} at 245 nm (but not at 268 nm) and the concentration of HDL cholesterol can be understood as being a result of the significant correlation between OD_{max} at 245 nm and total cholesterol, on one hand, and the negative correlation between HDL cholesterol and the total cholesterol level, on the other. By contrast, no correlation could have been expected between OD_{max} at 268 nm and HDL cholesterol because the contribution of cholesterol oxidation products to the absorbance at this wavelength is marginal.

The most intriguing result of this study is the negative correlation between t_{max} (as measured either at 245 or at 268 nm) and HDL cholesterol concentration. This correlation accords with our previous finding that the lag obtained in the time course of oxidation of mixtures of HDL and LDL decreases upon increasing the concentration of HDL in the mixture (6), although it is apparently inconsistent with the observations of other authors that oxidative changes in LDL–HDL mixture occur later than in isolated LDL (10).

The pathological relevance of these results, as well as those of any other *in vitro* assay of lipid oxidation of either LDL or unfractionated serum or plasma, is questionable. In fact, HDL is an established anti-risk factor in coronary heart diseases. Hence, our results may imply that the significance of the reverse transport of cholesterol by HDL exceeds its enhancing effect on the rate of oxidation. In addition, HDL oxidation may have a more direct effect on oxidation-induced atherogenesis in the subendothelial space, where LDL oxidation is involved in foam cell formation and the concentration of HDL is smaller than that of LDL (15,16). First, HDL may play a protective role by being oxidized prior to LDL (17,18). Second, oxidized HDL itself may either be atherogenic (19) or anti atherogenic, possibly owing to the activity of the HDL-associated enzymes paraoxonase and/or PAF-AH (16,20,21).

Further effort will be devoted to elucidate these possibilities.

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Activation of Acyl-CoA Cholesterol Acyltransferase: Redistribution in Microsomal Fragments of Cholesterol and Its Facilitated Movement by Methyl-β-Cyclodextrin

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ABSTRACT: Acyl-CoA cholesterol acyltransferase (ACAT) (EC 2.3.1.26) in the yolk sac membrane of chicken eggs plays an important role in the transport of lipids, which serve as both structural components and as an energy source during embryogenesis. ACAT from the yolk sac membrane of chicken eggs 16 d after fertilization has higher activity and better stability than its mammalian liver counterpart. During our study of the avian enzyme, ACAT was found to be activated up to twofold during storage at 4°C. The activation was investigated, and data suggest that redistribution of cholesterol within microsomal vesicles leads to the increase. Methyl-β-cyclodextrin (MβCD) increases activation an additional twofold, possibly by facilitating the movement of cholesterol within microsomal fragments and allowing redistribution of cholesterol in lipid bilayers to a greater extent. Treatment of microsomes with MβCD removes cholesterol from the membranes. Controlled amounts of cholesterol can be restored to the membranes by mixing them with cholesterol-phosphatidylcholine liposomes in the presence of MβCD. Under these conditions, the plot of ACAT vs. cholesterol mole fraction in the liposomes is sigmoidal. The finding that MβCD can enhance cholesterol transfer between liposomes and microsomes and reduce the limitation of slow movement of nonpolar molecules in aqueous media should make cyclodextrins more useful in *in vitro* studies of apolar molecule transport between membrane vesicles.

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Acyl-CoA cholesterol acyltransferase (ACAT) (EC 2.3.1.26) catalyzes intracellular esterification of cholesterol with long-chain fatty acyl coenzyme A. ACAT is an integral membrane protein and resides in the cytoplasmic side of the rough endoplasmic reticulum (ER) (1). ACAT activity has been observed in many kinds of tissues and plays an important role in the maintenance of intracellular cholesterol homeostasis. In the liver, the production and secretion of lipoproteins, which deliver various lipid molecules to peripheral tissues, require a lipid core of triacylglycerols and cholesteryl esters. Dietary absorption of cholesterol requires the function of intestinal ACAT, which controls the rate-limiting step of absorption (2). Overaccumulation of cholesteryl esters in macrophages and smooth muscle cells in large arteries leads to formation of fatty streaks and atherosclerosis. During embryogenesis, ACAT of the yolk sac membrane of chicken eggs functions significantly in providing essential lipid nutrients to the developing embryo (3,4). More than fivefold higher ACAT activity was observed at day 16 in the yolk sac membrane compared with that in the liver or intestine (3). Despite its biological significance, ACAT has not been isolated in pure form, and study of the enzyme is limited to microsomal preparations or detergent extracts.

It is known that cellular membranes are not homogeneous structures of randomly distributed lipids with embedded proteins. Rather, they contain microorganizations that differ from each other by the size of domains, by lipid and protein compositions, and by the kinetic properties of each component (5). Cholesterol-rich and cholesterol-poor domains have been shown to exist in the plane of the membrane, and the distribution of the sterol in the two leaflets of the bilayer and among different membrane compartments is known to be asymmetrical (5). In an early study, Hashimoto and Fogelman (6) showed that ACAT in rat liver microsomes is concentrated in the RNA-rich fraction, whereas the bulk of the cholesterol ester is found in smooth microsomes. When acyl-CoA was added to mixtures of the two kinds of membranes, a disproportionate amount of the cholesterol ester formed was in the smooth membrane fraction. It was suggested that cholesterol in the smooth microsomes may have been esterified by ACAT on the surface of the RNA-rich membranes; neither cholesterol nor cholesterol ester needed to be transported between the membranes. Transport of cholesterol among different membrane structures and maintenance of concentration gradients require lipid carrier proteins and transporting vesicles. Cholesterol oxidase susceptibility indicates that about 90% of cellular free cholesterol is associated with the plasma membrane (7); and many studies have shown that, under physiological conditions, ACAT is not saturated with cholesterol. Therefore, transfer of the sterol from other cellular fractions

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Abbreviations: ACAT, Acyl-CoA cholesterol acyltransferase; CE, cholesteryl ester; ER, endoplasmic reticulum; MβCD, methyl-β-cyclodextrin; PC, phosphatidylcholine; PL, phospholipid; TAG, triacylglycerol.

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to the ACAT substrate pool can be a limiting factor in the activity of the enzyme.

Cyclodextrins are a family of cyclic molecules composed of varying numbers of glucose units. Among the commercially available cyclodextrins, β-cyclodextrin and its derivatives have been found to be the most efficient molecules in extracting cholesterol from lipid bilayers (8,9). Atger *et al.* (10) showed that cyclodextrins can facilitate the transfer of cholesterol from cells to phospholipid vesicles. A complex of cholesterol with methyl-β-cyclodextrin (MβCD) can be used as an efficient delivery tool to provide cells or other biological systems with cholesterol (11).

In this paper the effect of prolonged incubation of microsomes and the addition of a cyclodextrin derivative, both allowing cholesterol redistribution, on ACAT activity are reported.

MATERIALS AND METHODS

Materials. Chicken eggs, 16 d after fertilization, were obtained from Hy-Vac (Adel, IA). 2-[*N*-Morpholino]ethanesulfonic acid (MES), cholic acid sodium salt, Triton X-100, 4-aminoantipyrine, sodium 3,5-dichloro-2-hydroxybenzene sulfonate, MβCD, cholesterol, oleoyl coenzyme A, horseradish peroxidase, and cholesterol oxidase were all from Sigma (St. Louis, MO). Phosphatidylcholine, 20 mg/mL in chloroform, was prepared from chicken eggs following a procedure by White *et al.* (12). *p*-Methylaminophenol sulfate was purchased from Aldrich Chemical Co. (Milwaukee, WI). Tris(hydroxymethyl)aminomethane Na_2EDTA, sucrose, sodium bisulfite, sodium sulfite, perchloric acid, and 7-mL polyethylene scintillation vials were obtained from Fisher Scientific (Fair Lawn, NJ). Bovine serum albumin, fatty acid poor, was purchased from Calbiochem Co. (La Jolla, CA). Ecolume scintillation fluid was obtained from ICN Pharmaceuticals, Inc. (Costa Mesa, CA). 1-^{14}C-Oleoyl CoA was from DuPont NEM (Boston, MA). [1α,2α(n)-^{3}H] Cholesteryl oleate was from Amersham Life Science, Inc. (Arlington, IL). Silica gel (40–140 mesh) was a product of J.T. Baker, Inc. (Phillipsburg, NJ). Plates for thin-layer chromatography, silica gel 60 precoated, 20 × 20 cm, were from Merck (Darmstadt, Germany). Packard Tri-carb 1600TR liquid scintillation analyzer with a dual radioisotope counting program was used for radioactivity counting. LiposoFast extrusion device from Avestin, Inc. (Ottawa, Canada) was employed for liposome preparation.

Preparation of yolk sac membrane microsomes from fertilized chicken eggs. The procedure described by Shand *et al.* (3) was followed. Yolk sac membranes were isolated from 15- or 16-d-old chick embryos, which were reported to have maximal ACAT activity (3). The membrane was washed in ice-cold NaCl (0.9 wt%) four or five times to remove excess yolk. The following steps were performed at 4°C. Yolk sac membrane was homogenized by a mechanical homogenizer in about 3 vol of medium I (0.25 M sucrose, 1 mM EDTA, 10 mM Tris-Cl, pH 7.4), and then was centrifuged at 10,200 × *g* for 15 min. The reddish supernatant was then centrifuged at 106,500 × *g* for 30 min. The pellet was suspended in medium II (50 mM MES, 50 mM Tris-Cl, pH 7.2) and centrifuged at 106,500 × *g* for another 30 min. The new pellet was resuspended in medium II. The microsomes had protein concentrations of 20 to 50 μg/μL and were stored in aliquots at –70°C.

Protein concentration was determined by the method of Lowry *et al.* (13).

ACAT assay. The assay for ACAT followed procedures reported by Doolittle and Chang (14) and Chautan *et al.* (15). Microsomes (160 μL) containing 15 to 250 μg protein were preincubated at 37°C for 10 min. To start the esterification reaction, 40 μL of ^{14}C-oleoyl CoA solution (250 μM ^{14}C-oleoyl CoA, 25 dpm/pmole and 12.5 mg/mL BSA,) was added to the assay mixture. After 5 min, 2 mL of hexane/2-propanol (3:2, vol/vol) was added, followed by 10 μL of cholesteryl-^{3}H-oleate (about 20,000 dpm), serving as an internal standard. The organic layer was separated and dried under a stream of N_2. The solid residue was redissolved in 200 μL hexane/diethyl ether (98:2, vol/vol) and applied to a column of 0.7 g silica gel (40–140 mesh) that had been prewashed with 3 mL hexane/diethyl ether. The cholesteryl oleate was eluted from the column with 6 mL hexane/diethyl ether and counted by a dual channel radioisotope counter.

Quantitation of synthesis of cholesteryl ester (CE), triacylglycerol (TAG), and phospholipids (PL) Microsomes (5 μL) containing 220 μg protein were incubated with 155 μL medium II or MβCD- medium II at room temperature for 30 min. The mixture was incubated at 37°C for 10 min prior to the addition of 40 μL ^{14}C-oleoyl CoA. Two milliliters chloroform/MeOH (2:1, vol/vol) was added to the mixture after a 5-min reaction time at 37°C followed by the addition of 10 μL ^{3}H-cholesteryl oleate. The top aqueous layer was extracted one more time with chloroform/MeOH (2:1, vol/vol), and the organic extracts were combined and dried under N_2. The solid residue was redissolved in 50 μL chloroform/MeOH (2:1, vol/vol) and applied onto a thin-layer chromatographic plate that had been prewashed in MeOH for 5 min and air-dried completely. For the quantitation of neutral lipids, the plate was developed in petroleum ether/diethyl ether/acetic acid (80:20:0.5, by vol) (16), and for complex lipids in chloroform/methanol/water/acetic acid (70:30:4:2, by vol) (17). Following comparison with standards, the spots corresponding to CE, TAG, or PL were scraped into a scintillation vial and counted for radioactivity.

Cholesterol depletion. Microsomes (1 mL) were mixed with 5 mL MβCD solution and stirred slowly at 4°C for 1 h. The amount of protein in the microsomes was 44 mg. The mixture was centrifuged at 106,500 × *g* at 4°C for 30 min. The pellet was suspended in 1.3 mL medium II and subjected to the same treatment with MβCD two more times and one wash with medium II. Gentle homogenization was sometimes needed during resuspension, and the final microsomes were prepared in a small volume of medium II to have a concentrated protein solution.

Preparation of liposomes. The liposomes were prepared

following the procedure of MacDonald *et al.* (18). Cholesterol was recrystallized twice from methanol and dissolved in chloroform. A solution of phosphatidylcholine (PC), 20 mg/mL in chloroform, was mixed with different amounts of cholesterol in chloroform so that varying mole ratios of cholesterol to PC were obtained. The combined solution was dried first under a stream of N_2 and then under vacuum at 40–45°C for 30 min. The waxy residue was suspended in medium II, and the final PC concentration was 10 mg/mL. The mixture was then frozen and thawed 10 times, using a dry ice–ethanol bath and a room temperature water bath, respectively. The multilamellar vesicles were extruded 19 times through two polycarbonate membranes (100-nm pore size) mounted on an extrusion device. The unilamellar liposomes were stored under N_2 at 4°C and used within 2 d. The concentrations of cholesterol and PC were determined for each liposome preparation.

Determination of cholesterol concentration. The procedure described by Sale *et al.* (19) was followed with moderate modification. Cholesterol was extracted three times from microsomes or liposomes using hexane/2-propanol (3:2, vol/vol). The solvent was evaporated under N_2, and the solid residue was redissolved in ethanol. Reagent A contained 822 μM 4-aminoantipyrine, 1.9 mM Na 3,5-dichloro-2-hydroxybenzene sulfonate, 200 IU/L peroxidase, and 50 mM phosphate buffer (3 mM Na cholate–0.5 vol% Triton X-100, pH 7.0), and was prepared on the day of the assay. To a 200-μL sample or standard containing 5 to 20 μg cholesterol in ethanol was added 1 mL reagent A. After a 10-min incubation at 37°C, the absorbance at 505 nm was measured and taken as blank. Ten microliters of 25 IU/mL cholesterol oxidase dissolved in 50 mM phosphate buffer was added to the sample prior to a second incubation at 37°C for 15 min. The absorbance at 505 nm was measured again, and the difference between the two readings was used to calculate the amount of cholesterol.

Determination of PL concentration. PL concentration was determined according to Harris and Popat (20), and the assumption was made that the majority of PL in the microsomes or liposomes had 1 mol of phosphorus per mol of PL. An aliquot of microsomes or liposomes in a final volume of 120 μL was mixed with 1 mL 70% perchloric acid. For the supernatant in the cholesterol depletion experiment, a larger volume of sample, e.g., 500 μL, was used to ensure a large enough sample of phosphorus, and the sample was first evaporated to dryness and then redissolved in 120 μL water. After digestion, 200 μL of digest was diluted with 1.7 mL H_2O followed by 200 μL 5 wt% ammonium molybdate and 400 μL Elon reducing reagent (20). Absorbance at 795 nm was measured 10 min later.

RESULTS

ACAT of yolk sac membrane was found to be quite tolerant of repeated freezing and thawing: specific activity (SA) of 753 ± 4.4 pmole/min/mg after the first cycle vs. 842 ± 16.0 after the sixth cycle, an increase of 12%. Freezing and thawing is generally destructive of protein structure, and enzymes tend to become inactive after repeated freezing and thawing. To investigate this phenomenon further, microsomes were thawed and stored at 4°C for 10 d. The ACAT activity was measured on the day the membrane preparation was thawed (d 0) and every other day thereafter. The experiment was carried out three times: the first experiment started the next day after the microsomes were prepared, the second experiment was after 3.5 wk storage at −70°C, and the third after 7 wk storage at −70°C. The results of these three experiments are shown in Figure 1. Storage of microsomes at −70°C slowly inactivated ACAT. When the microsomes were newly made and stored at −70°C overnight, the ACAT SA was 504 ± 6.4 pmole/min/mg (100%) (value at d 0). After 3.5 wk storage at −70°C, the SA decreased to 466 ± 7.9 pmole/min/mg (92%). After 7 wk at −70°C, the SA decreased to 395 ± 11.2 pmole/min/mg (78%). During storage at 4°C, the activity first increased, then slowly declined. Control experiments (not shown) showed that protease inhibitors had no effect on the initial rise in activity during 4°C storage although they slowed the later loss in activity. Oxidation with H_2O_2 or O_2 plus Cu^{2+} also had no appreciable effect on ACAT activity, nor did addition of various lipid hydrolysis products.

Incubation of microsomes with MβCD at concentrations up to 1 mM stimulated the enzyme activity: ACAT SA was 1088 ± 33.7 (1 mM) vs. 278 ± 4.6 (0 mM) pmole/min/mg, a 3.9-fold increase (Fig. 2). The synthesis of CE, TAG, and PL in the presence of 1 mM MβCD was measured (Fig. 3). The synthesis of CE was elevated 3.8-fold: 1782 ± 5.4 vs. 471 ± 24 pmole/min/mg, whereas synthesis of TAG and PL did not change significantly: TAG: 64.7 ± 2.2 vs. 92.6 ± 12

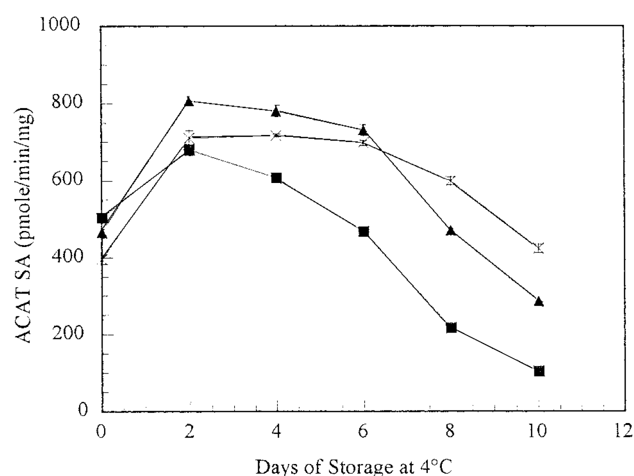

FIG. 1. Effect of storage at 4°C on acyl CoA-cholesterol acyltransferase (ACAT) activity. The microsomes were taken out of −70°C freezer, thawed, and stored at 4°C. ACAT activity was measured on the day they were thawed (day 0) and every other day thereafter. The line with ■ represents the experiment carried out the next day after the microsomes were prepared, and the lines with ▲ and × represent experiments done 3.5 and 7 wk later, respectively. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol.

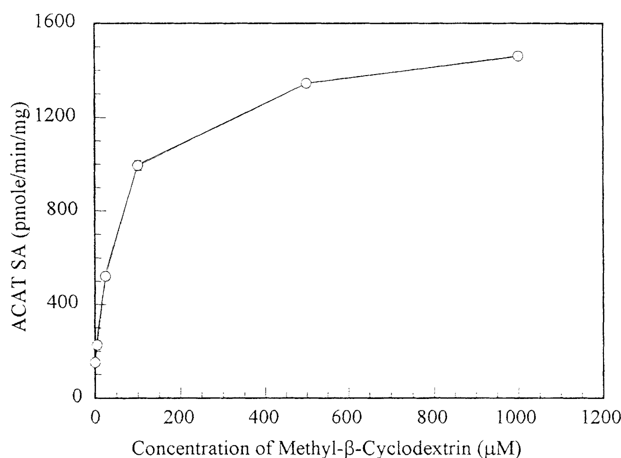

FIG. 2. Effect of methyl-β-cyclodextrin (MβCD) on ACAT activity. Microsomes (5 μL) containing 220 g protein were incubated with 155 μL MβCD-medium II (50 mM MES, 50 mM Tris-Cl, pH 7.2) at room temperature for 30 min followed by the regular ACAT assay. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol. For abbreviation see Figure 1.

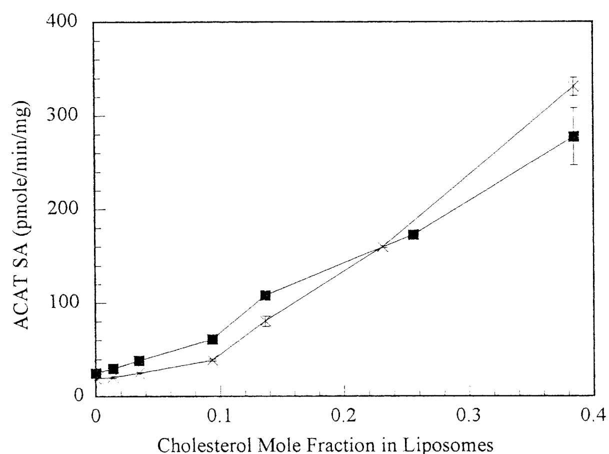

FIG. 4. Effect of cholesterol mole fraction on ACAT activity. Microsomes were taken out of the −70°C freezer and stored at 4°C for 2 d before the depletion experiment. Treated microsomes (5 μL) were incubated with 155 μL cholesterol–phosphatidylcholine liposomes of different mole fractions prior to the regular ACAT assay. In the experiment represented by the line with solid squares, microsomes were treated with 25 mM MβCD three times and incubated with liposomes at room temperature for 30 min. In the experiment represented by the line with ×, microsomes were treated with 25 mM MβCD twice and stored at 4°C overnight followed by the third treatment and wash, and the treated microsomes were incubated with liposomes at 4°C overnight. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol. For abbreviations see Figures 1 and 2.

dpm/min/μg, and PL: 76.2 ± 1.2 vs. 57.5 ± 9.6 dpm/min/μg in the presence and absence, respectively, of MβCD.

Cholesterol in the microsomes was depleted by treatment with 25 mM MβCD, from initial levels of 0.067 to 0.016 μg/μg protein (Expt. 1) and 0.062 to 0.015 μg/μg protein (Expt. 2). The ACAT activity in the microsomes was assayed in the presence of increasing cholesterol mole fraction in PC-cholesterol liposomes (Fig. 4). In the control assays, the depleted microsomes were mixed with liposomes containing only PC, so the residual cholesterol from the microsomes represented mole fractions of 0.0018 and 0.0015 in the two experiments. Cholesterol in the liposomes stimulated ACAT activity 11-fold in the first experiment: 278 ± 30.6 (0.38 cholesterol mole fraction) vs. 25 ± 1.1 (zero cholesterol mole fraction) pmole/min/mg, and 17-fold in the second experiment: 331 ± 9.9 (0.38 cholesterol mole fraction) vs. 19 ± 1.1 (zero cholesterol mole fraction) pmole/min/mg (Fig. 4). Despite the substantial increase in the specific activity, the enzyme was not saturated with cholesterol at the highest cholesterol concentration used in the experiments.

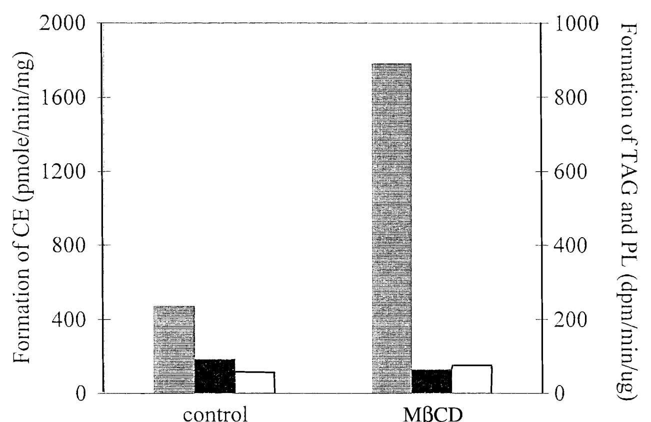

FIG. 3. Effect of MβCD on the synthesis of cholesteryl ester (CE: gray bar), triacylglycerol (TAG: black bar), and phospholipids (PL: open bar). Microsome preparations with 220 μg protein were incubated with 1 mM MβCD at room temperature for 30 min. The formation of CE, TAG, and PL was quantitated by a thin-layer chromatographic method as described in the Materials and Methods section. In all cases, the range is less than 17% of the mean. For abbreviation see Figure 2.

Cholesterol in microsomes was depleted to 0.007 μg/μg protein by treatment with 50 mM MβCD (final amount 0.0012 mole fraction in the ACAT assay when the microsomes were mixed with liposomes containing no cholesterol). The enzyme was again assayed in the presence of liposomes with varying amounts of cholesterol, with or without MβCD (Fig. 5). In the absence of MβCD, cholesterol stimulated ACAT activity 14-fold: 338 ± 5.1 (0.54 cholesterol mole fraction) vs. 24 ± 0.4 (zero cholesterol mole fraction) pmole/min/mg, but in the presence of MβCD, cholesterol stimulated the enzyme activity 63-fold: 1395 ± 7.3 (0.39 cholesterol mole fraction) vs. 22 ± 0.1 (zero cholesterol mole fraction) pmole/min/mg, and saturation of ACAT was observed.

DISCUSSION

The function of ACAT in the yolk sac membrane is to convert cholesterol and fatty acids in the yolk to CE, which can be packaged into lipoproteins and stabilize the assembly and structure of the particles secreted into circulation (4). Experiments with chicken embryos indicate that CE are synthesized extensively (about 80% of the free cholesterol is converted to

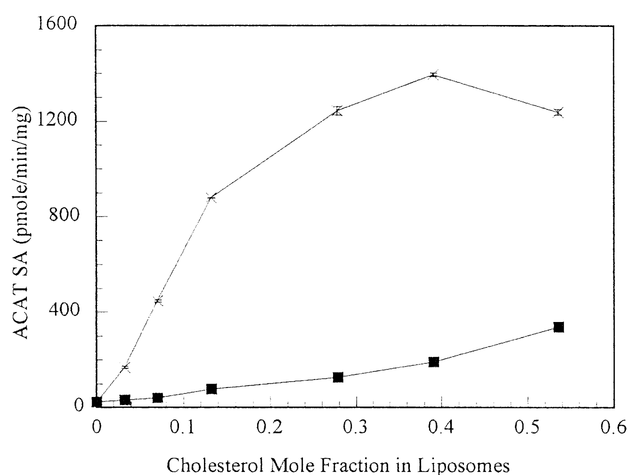

FIG. 5. Effect of cholesterol mole fraction on ACAT activity in the absence or presence of 1 mM MβCD. Microsomes were thawed and stored at 4°C for 2 d. After being depleted with 50 mM MβCD three times, 5 μL of the microsomes was incubated at room temperature for 30 min with cholesterol–phosphatidylcholine liposomes of different mole fractions in the absence or presence of 1 mM MβCD followed by the regular enzyme assay. The line with solid squares represents the assay in the absence of MβCD and the line with × represents the assay in the presence of 1 mM MβCD. Each value is a mean of duplicates with a bar showing the range; where no bar is shown, it is smaller than the symbol. For abbreviations see Figures 1 and 2.

CE) in the yolk sac membrane, and that esterification of cholesterol, which exists primarily as the free sterol in the yolk, facilitates the absorption of free fatty acids as well as cholesterol and contributes importantly to the absorption and transportation of lipid from yolk to embryos (4). Thus, ACAT plays an important role in the supply of lipid molecules as structural components and energy sources during embryo development (3,4). The properties of the avian enzyme were found to be comparable to those of the mammalian counterpart, including catalytically related histidine and cysteine residues and detergent-extractable activity (data not shown). The higher specific activity found in the yolk sac membrane (3) and better stability upon storage (data not shown) make this an attractive system for the study of ACAT.

Recent progress in molecular cloning of human macrophage ACAT (21) has accelerated the process of ACAT investigation, but study of the enzyme is still limited to microsomal preparations or detergent extracts because the enzyme has resisted purification. During the study of the avian ACAT, a nearly twofold activation of the enzyme was observed during storage at 4°C for 10 d (Fig. 1), and the following series of experiments was designed to investigate this phenomenon.

Mounting evidence suggests that under physiological conditions ACAT is not saturated with substrate cholesterol and that addition of exogenous cholesterol to microsomes can increase the synthesis of CE. It has been shown that cholesterol-rich and -poor domains are present in lipid bilayers inside the cell, and transport of the sterol among different membrane structures and the maintenance of the gradient of cholesterol require lipid carrier proteins and transporting vesicles (5). Upon break-up of the cell and subsequent cellular fractionation, the nonequilibrium condition of cholesterol is disrupted, and redistribution of the sterol among membrane fragments may now be able to proceed. Without adding cholesterol exogenously, Corton and Hardie (22) showed that redistribution of endogenous cholesterol among microsomal vesicles could increase ACAT activity and, in this case, that sterol carrier proteins possibly induced and facilitated the redistribution. The activation of ACAT observed in the 4°C storage experiment could be due to a redistribution of cholesterol in the microsomes during the storage period, with the redistribution leading to an increase in the ACAT substrate pool and resulting in the activation seen in the first 2 d of the experiment. To test the hypothesis, a means of systematically varying the cholesterol concentration in the substrate pool was required.

Cyclodextrins are macrocyclic molecules composed of varying numbers of glucose units. With a hydrophilic surface and hydrophobic interior, cyclodextrins can form inclusion complexes with hydrophobic molecules and enhance the solubility of lipophiles in aqueous solutions (23). Utilization of cyclodextrins in delivery of nonpolar drug molecules and supplementation of natural lipid carriers in circulation and cell cultures has been very important in pharmaceutical, clinical, and basic research (24–27), and the usefulness of these lipid-solubilizing sugar oligomers in cholesterol research is increasing. Studies of reverse cholesterol transport and intracellular cholesterol trafficking use cyclodextrins as effective cholesterol acceptors (28–31). Among the commercially available cyclodextrins, β-cyclodextrin with seven glucose units is the most effective molecule to solubilize cholesterol from lipid bilayers (8,9). Cyclodextrins themselves are quite toxic to animals, and their solubilities in aqueous solutions are limited. Derivatives of cyclodextrins have been synthesized chemically and found to be much less toxic and more soluble (25). In their study of reverse cholesterol transport, Kilsdonk *et al.* (28) found that the methyl derivative of β-cyclodextrin was more effective than the 2-hydroxypropyl derivative or β-cyclodextrin itself in extracting cholesterol from the plasma membrane of mouse L-cell fibroblasts. Not only are cyclodextrins used as acceptors in cholesterol depletion experiments, but also complexes of cholesterol with cyclodextrins can efficiently provide the sterol to membranes or cells in cholesterol repletion experiments (11,32).

Addition of MβCD to microsomes enhanced ACAT activity greatly, nearly a fourfold increase in ACAT SA (Fig. 3). The results of these experiments suggest that MβCD can shuttle cholesterol among microsomal fragments, from cholesterol-rich microdomains to cholesterol-poor microdomains, or from non-ACAT substrate pools to the ACAT substrate pool, and the enhanced redistribution of cholesterol results in increased enzyme activity. Other membrane-associated activities, synthesis of PL and TAG, did not increase in the presence of MβCD, strengthening the conclusion that enhanced access to cholesterol is the basis for the stimulation of ACAT. Thus, redistribution of cholesterol among microsomal vesicles during the storage period was very likely the mechanism

that increased ACAT activity seen in the first 2 d of the experiments (Fig. 1). During the course of this study, Liza *et al.* (33) reported an approximately fourfold activation of rat liver microsomal ACAT in the presence of 6 mM 2-hydroxypropyl-β-cyclodextrin and explained the activation as a result of the facilitated redistribution of cholesterol in the presence of the cyclodextrin molecule. Therefore, increases in ACAT activity because of cholesterol redistribution may be quite general. An increase in ACAT activity during storage of mammalian microsomes may not be apparent because the mammalian enzyme is less stable than the one from egg yolk membrane used in this study and loses activity even though cholesterol redistribution is occurring. In one instance, Cheng *et al.* (34) observed a nearly twofold increase in ACAT activity when they compared cell extracts incubated for 30 min at 37°C or at 4°C. It is plausible to suggest that a faster redistribution of cholesterol at the higher temperature was the factor leading to the increase. The activation of ACAT by redistribution of cholesterol in the absence of MβCD did not exceed twofold, whereas in the presence of the cyclodextrin, a nearly fourfold increase in the activity was observed (Fig. 2). ACAT remained fully active for about 4 d before beginning to lose activity. Thus, different extents of activation of the enzyme do not depend upon loss of enzyme under the same conditions.

To study the effect of cholesterol concentration on avian ACAT activity, microsomes of yolk sac membrane were depleted of cholesterol by repeated treatments with MβCD and then assayed with liposomes with varying ratios of cholesterol to PC. As shown in Figure 4, ACAT activity increases progressively with increasing cholesterol mole fraction, without reaching a plateau. At the highest concentration used, 0.38 mole fraction, and with overnight incubation with the liposomes, ACAT was not saturated with cholesterol. According to Yeagle (35), plasma membranes normally contain about 0.45 mole fraction of cholesterol, and ER membranes have 0.10–0.12 mole fraction of the sterol. The cholesterol mole fraction in microsomes of yolk sac membrane was measured to be from 0.099 to 0.12, in very good agreement with the literature. At around 0.10 mole fraction of cholesterol, the human macrophage ACAT activity was shown to reach a plateau (34), but the mouse liver enzyme reached a plateau at a higher cholesterol mole fraction (about 0.3) (36). Perhaps the avian enzyme has a very high K_m, or a change in membrane properties caused by increasing concentrations of cholesterol prevents saturation of the enzyme. A third possibility is that the transfer of cholesterol from cholesterol–PC liposomes to microsomes is slow, limiting the access of the sterol to the ACAT substrate pool. Since MβCD is capable of facilitating cholesterol redistribution among microsomal vesicles, it should enhance the transfer of cholesterol from liposomes, the source of substrate, to the microsomes, the site of enzyme catalysis. Indeed, when the depleted microsomes were assayed with liposomes of increasing cholesterol mole fractions in the presence of 1 mM MβCD, the enzyme activity reached a maximum around 0.4 cholesterol mole fraction (Fig. 5). Thus, failure to saturate the enzyme with a high concentration of cholesterol (Fig. 4) is the result of limitation of substrate movement, and the use of MβCD accelerated the transfer of cholesterol from liposomes to the enzyme substrate pool and minimized that restraint.

The usefulness of MβCD in the study of ACAT is clearly seen in Figure 5 where the enzyme shows saturation kinetics in the presence of the cyclodextrin but not in its absence. The ACAT assay solution was a mixture of microsomal vesicles and small unilamellar liposomes (in the extrusion device used to prepare liposomes, the membrane pore size was 100 nm). Cholesterol in the microsomal membranes was depleted to about 1/10 of the usual content in the ER membrane, so that the major supply of the sterol was from liposomes. Encounter of cholesterol in the liposomes with ACAT on the ER membrane could be achieved in two ways. It could diffuse through the aqueous medium, but desorption of the sterol from lipid bilayers and aqueous diffusion would be very slow considering the solubility of the sterol in aqueous solution. In pure water, the solubility of cholesterol is 1.8 μg/mL or 4.7 μM (37). Or, movement of the sterol between membranes could be promoted by collision or contact of ER membranes with liposomes, and the efficiency of cholesterol transfer from one bilayer to another may very well depend on the duration and distance of the contact and may not be high. Cholesterol transport inside the cell among different membrane compartments is proposed to proceed through three possible mechanisms: aqueous diffusion, vesicle-mediated transport, and soluble protein or lipid carriers. Vesicular transport and cytosolic carriers are most likely the major mechanisms (38,39). Artificial vesicles like liposomes lack the possible protein and lipid factors that mediate cellular lipid transfer between bilayers or vesicle–membrane fusion, so artificial vesicles may not function effectively in some *in vitro* studies (5). The access of cholesterol to the ACAT substrate pool is a problem in the study of regulation of ACAT, which the use of MβCD can mitigate.

Very recently, Chang *et al.* (40) reported purification of the recombinant ACAT of human origin and the sigmoidal response of ACAT specific activity toward cholesterol concentration. An allosteric effect of the sterol on the enzyme was proposed. In his book, however, Segel (41) described another kinetic mechanism in which a sigmoidal profile of activity vs. concentration does not associate cooperative substrate binding. In this mechanism, the enzyme catalyzes a Random Bi Bi reaction, and the formation of the complex—enzyme-substrate 1-substrate 2—is much slower than the conversion to products and one route to products is favored over the other. The possibility of a slow formation of ACAT-cholesterol-acyl-CoA complex is not unlikely and may be worth investigation.

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Transformation of Bile Acids and Sterols by Clostridia (fusiform bacteria) in Wistar Rats

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ABSTRACT: The effects on bile acid and sterol transformation of clostridia (fusiform bacteria), the dominant intestinal bacteria in rodents (*ca.* 10^{10} counts per g wet feces) were examined in Wistar rats. After inoculation of clostridia into germ-free rats and into rats previously inoculated solely with *Escherichia coli*, most of the endogenous bile acids were deconjugated, and cholic acid and chenodeoxycholic acid were 7α-dehydroxylated to deoxycholic acid and lithocholic acid, respectively. Tauro-β-muricholic acid, another major bile acid in rats, was deconjugated, but only part of it (*ca.* 30%) was transformed into hyodeoxycholic acid. Cholesterol and sitosterol were also reduced to coprostanol and sitostanol, respectively. *Escherichia coli* transformed neither bile acids nor sterols. These data suggest that clostridia play an important role in the formation of secondary bile acids and coprostanol in rats.

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Intestinal bacteria biotransform bile acids (1,2). Deconjugation, dehydroxylation, oxidation–reduction, and epimerization are the major reactions in bile acid metabolism, whereas deconjugation and 7α-dehydroxylation are the most important. Since the major bile acids found in human feces are deconjugated and 7α-dehydroxylated, deoxycholic acid and lithocholic acid arise from taurine- or glycine-conjugated primary bile acids. In the case of rats and mice, taurine-conjugated cholic acid and β-muricholic acid are major bile acids synthesized in the liver. Cholic acid is transformed to deoxycholic acid, and β-muricholic acid to β-hyocholic acid (previously called as ω-muricholic acid) and further to hyodeoxycholic acid by intestinal bacteria.

Deconjugation activity is found in many species of intestinal bacteria (3) but those possessing 7α-dehydroxylation activity are limited. 7α-Dehydroxylating activity is found in *Clostridium* (4–7), *Bacteroides* (8), *Lactobacillus* (9), *Eubacterium* (4,5,10,11) and in as yet unclassified intestinal bacteria (4,5). The population of these 7α-dehydroxylating bacteria is not large, and their counts are only 10^5–10^6 per g wet feces, while the counts of dominant intestinal bacteria possessing no 7α-dehydroxylation activity (12) are around 10^{11}–10^{12} per g wet feces.

Previously, we found that fecal deoxycholic acid and coprostanol formed from cholesterol by intestinal bacteria were markedly decreased in cecectomized (13) or totally colectomized rats (14). Eyssen *et al*. (15) also reported that production of coprostanol is abolished in cecectomized rats. On the other hand, clostridia, or fusiform bacteria, grow only in the cecum of rats and mice, and the population is 10^{10} counts or more per g feces (16).

Therefore, we suspected that clostridia participated in the biotransformation of bile acids and cholesterol in rats, and found that a significant formation of secondary bile acids and coprostanol was achieved in the clostridia monoassociated germ-free rats.

MATERIALS AND METHODS

Animals and treatments. Germ-free Wistar rats (*ca*. 4 mon old) bred in our laboratory (Shionogi Aburahi Laboratories, Shiga, Japan) were housed individually under germ-free conditions and inoculated orally with clostridia (fusiform bacteria) and *Escherichia coli* isolated from conventional rats kept in our laboratory.

Clostridia were collected from the large intestinal content after treatment with chloroform (only spores survive) by procedures described previously (16). Since a culture system for clostridia has not yet been established, the chloroform-treated intestinal contents were suspended in Trypticase Soy Broth (BBL, Cockeysville, MD) and directly administered to rats. *Escherichia coli* was cultured for 72 h at 37°C in GAM semifluid media (Nissui Co., Tokyo, Japan). After thorough mixing, a 1-mL aliquot containing *ca*. 10^8 bacteria was administered to each rat.

Germ-free rats were divided into three groups, each consisting of two males and two females. The first group was inoculated with clostridia, the second group was with *E. coli* and then clostridia 1 wk after the inoculation of *E. coli*, and the third group was given the feces of conventional rats.

The rats were fed a commercially available chow diet (CMF Diet; Oriental Kobo Co., Tokyo, Japan) sterilized by

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γ-irradiation at 50 kGy. The feces were collected every day for 6 wk after inoculation of clostridia. At the end of the experiment, the rats were killed and the numbers of viable bacteria in the fresh feces were examined (16,17).

Determination of fecal bile acid and sterols. A portion of feces (usually *ca*. 1 g) was extracted three times with 20 mL ethanol at 85–90°C for 1 h. After filtration, a portion of the extract was subjected to ion-exchange chromatography on a piperidinohydroxypropyl Sephadex-LH-20 (PHP-LH-20, Shimadzu, Kyoto, Japan) gel column (18) to obtain the fractions of free, glycine-conjugated, taurine-conjugated, and sulfated bile acids. The sulfate fraction was hydrolyzed using sulfatase and β-glucuronidase (19) and then subjected to piperidinohydroxypropyl Sephadex-LH-20 column chromatography to obtain the fractions of free, glycine-conjugated, and taurine-conjugated bile acids. The bile acids were analyzed by high-performance liquid chromatography on a reversed-phase column (Develosil ODS-HG-5, 15 cm × 4.6 mm i.d.; Nomura Chemical, Seto, Japan) at room temperature and quantified using an immobilized 3α-hydroxysteroid dehydrogenase column with postcolumn fluorescent detection (19,20). Tauro-23-nordeoxycholic acid was used as an internal standard.

Fecal sterols were reextracted with *n*-hexane from the ethanol extract, converted to trimethylsilyl ether derivatives with trimethyl silyl imidazole, and quantified by a Shimadzu gas–liquid chromatograph Model GC-14A (Shimadzu, Kyoto, Japan) equipped with a methylsilicon capillary column (Hicap CBP-1, 25 m × 0.25 mm i.d., Shimadzu), a flame-ionization detector, a solventless injector, and a Chromatopak C-R4A integrator (Shimadzu). The column temperature was kept at 260, the injector port at 305, and the detector at 300°C.

RESULTS

Bacterial counts in the feces after 6 wk of inoculation are shown in Table 1. Only clostridia were detected in the first group; *E. coli* and clostridia were detected in the second group, and a usual pattern of intestinal flora was established in the third group. The counts of clostridia reached on the order of 10^{10} per g feces in all the rats.

TABLE 1
Fecal Bacterial Counts in Germ-Free Male Rats Inoculated with Clostridia, *Escherichia coli,* and Conventional Rat Feces

	Clostridia		*E. coli* + clostridia		Rat feces	
Bacteria	1[a]	2	1	2	1	2
Enterobacteriaceae	N.D.[b]	N.D.	7.9[c]	7.8	7.6	7.8
Enterococci	N.D.	N.D.	N.D.	N.D.	7.5	7.6
Lactobacilli	N.D.	N.D.	N.D.	N.D.	10.0	10.1
Bifidobacteria	N.D.	N.D.	N.D.	N.D.	9.5	N.D.
Bacteroidaceae	N.D.	N.D.	N.D.	N.D.	10.3	10.3
Clostridia	10.0	10.0	10.1	10.1	10.0	10.1
Total count	10.0	10.0	10.1	10.1	10.6	10.6

[a]Rat number.
[b]N.D., not detected (less than 10^2 counts/g feces).
[c]Log counts/g feces, 6 wk after inoculation.

The bile acids were analyzed after fractionating into free, glycine-conjugated, taurine-conjugated, and sulfated bile acids; but only a trace amount of bile acids, less than 0.01 μmol/g feces, was detected in the glycine-conjugate and sulfate fractions.

The fecal bile acids were composed of taurine-conjugated primary bile acids under germ-free conditions (0 wk), but most of the bile acids (>97%) were deconjugated and the secondary bile acids were formed as early as 1 wk after the inoculation of clostridia. The composition ratio of fecal bile acids remained almost unchanged thereafter, resembling that in the ex-germ-free rats given conventional rat feces 6 wk before.

In the second experiment, clostridia were inoculated 1 wk after the first inoculation with *E. coli*. This action was expected to enhance the growth of clostridia by making the intestinal lumen more anaerobic. *Escherichia coli* caused neither deconjugation nor formation of secondary bile acids, but the changes in the fecal bile acid composition after inoculation of clostridia were very similar to those in clostridia monoassociated rats.

Since no difference was found between the first and second experiments in the changes of the fecal bile acid composition after clostridia inoculation, both data were combined and are shown in Table 2. Three days after inoculation, most of the bile acids were deconjugated, and considerable amounts of unconjugated primary bile acids, such as cholic acid and β-muricholic acid, were detected. Unconjugated cholic acid decreased markedly 1 wk after inoculation, but unconjugated β-muricholic acid remained high. Secondary bile acids such as deoxycholic, lithocholic, and β-hyocholic acids were at relatively low levels after 3 d, but, after at least a week, the levels increased. 7-Oxo-deoxycholic acid levels were high after 3 d but decreased thereafter.

Table 3 shows the fecal bile acid composition in male and female germ-free rats, those inoculated with clostridia (with and without an *E. coli* preinoculation) and those with rat feces 6 wk before. The fecal bile acid composition in the clostridia-inoculated rats was very similar to that in the conventional rats, suggesting that clostridia are primarily responsible for the formation of secondary bile acids in rats.

Subsequently, changes in the composition ratio of deoxycholic acid in 12α-hydroxyl bile acids (cholic acid group), of coprostanol in C-27 sterols (cholesterol plus coprostanol), and of sitostanol in C-29 sterols (sitosterol plus sitostanol) in the feces after clostridia inoculation were examined. As shown in Figure 1, the formation of deoxycholic acid increased to values of 60–75% after 1 wk in rats inoculated with only clostridia and with *E. coli* plus clostridia together, and remained at these levels thereafter. However, the formation of coprostanol and also that of sitostanol were somewhat delayed compared with that of deoxycholic acid, especially in clostridia monoassociated rats. *Escherichia coli* did not form coprostanol or sitostanol by itself, but previous inoculation of *E. coli* enhanced the formation of these sterols by clostridia.

TABLE 2
Changes in the Fecal Bile Acid Composition in Male Germ-Free Rats Inoculated with Clostridia

	Days after inoculation with clostridia				
	Germ-free	3 d	1 wk	2 wk	4 wk
			(%)		
T–3α7α12α	29.6 ± 0.4[a]	0.8 ± 0.1	0.2 ± 0.1	0.2 ± 0.0	0.1 ± 0.0
U–3α7α12α		16.2 ± 2.2	0.7 ± 0.3	0.3 ± 0.1	0.3 ± 0.1
U–3α12α		12.0 ± 2.4	16.2 ± 1.6	14.7 ± 1.8	14.1 ± 2.0
U–3α12α7-oxo		8.6 ± 2.6	0.5 ± 0.2	0.3 ± 0.1	0.2 ± 0.0
U–3α12-oxo		2.9 ± 0.8	8.1 ± 2.2	5.2 ± 0.6	5.9 ± 0.1
T–3α7α	1.8 ± 0.3				
U–3α7α		0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.2 ± 0.0
U–3α		0.4 ± 0.2	2.9 ± 0.3	2.6 ± 0.2	2.5 ± 0.4
T–3α7β	0.9 ± 0.1				
U–3α7β		0.5 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.5 ± 0.1
T–3α6β7α	2.4 ± 0.1				
U–3α6β7α		3.0 ± 0.2	3.2 ± 0.2	2.9 ± 0.2	3.1 ± 0.3
T–3α6β7β	65.2 ± 0.7	2.0 ± 0.3			
U–3α6β7β		44.1 ± 1.2	47.8 ± 3.1	48.8 ± 2.8	54.5 ± 2.2
U–3α6α7β		8.5 ± 0.2	18.3 ± 2.2	21.2 ± 2.6	17.7 ± 4.4
U–3α6α		0.2 ± 0.1	1.0 ± 0.1	0.7 ± 0.1	0.8 ± 0.1

[a]Mean ± SE (*n* = 4 rats). T, taurine conjugated; U, unconjugated. Compound designation: substituted 5β-cholan-24-oic acids. Greek letters denote configuration of hydroxyl groups at C-3, C-6, C-7, or C-12.

Coprostanol was not found in the diet, but sitostanol was present. A considerable amount of sitostanol was detected in the feces of germ-free rats (Fig. 1C).

DISCUSSION

The present data showed that clostridia, or fusiform bacteria, are capable of biotransforming bile acids and cholesterol in rats as do the usual intestinal microflora in conventional rats. The bacterial counts were 10^{10} per g wet weight feces in ex-germ-free rats after inoculation of clostridia, and this level was comparable to that in conventional rats.

Clostridia are found in rodents and comprise over 50 biovars (16). They grow in the cecum and are believed to require highly anaerobic conditions for growth. Since no *in vitro* culture systems have yet been fully established, we examined the

TABLE 3
Fecal Bile Acid Composition in Germ-Free Rats Inoculated with Clostridia and with Conventional Rat Feces for 6 wk

	Male			Female		
	Germ-free	Clostridia	Rat feces	Germ-free	Clostridia	Rat feces
		(%)			(%)	
T–3α7α12α	29.6 ± 0.4[a]	0.2 ± 0.0[a]	0.3 ± 0.1[b]	57.8 ± 1.0[a]	0.4 ± 0.1[a]	0.4 ± 0.1[b]
U–3α7α12α		0.8 ± 0.4	0.6 ± 0.1		1.1 ± 0.1	2.6 ± 0.3
U–3α12α		22.9 ± 2.2	28.1 ± 0.7		32.7 ± 3.5	37.6 ± 5.5
U–3α12α7-oxo		0.8 ± 0.2	1.7 ± 0.7		0.9 ± 0.3	1.2 ± 0.0
U–3α12-oxo		5.6 ± 0.5	5.0 ± 1.5		10.7 ± 1.5	11.4 ± 1.6
T–3α7α	1.8 ± 0.3			3.4 ± 0.1		
U–3α7α		0.2 ± 0.0	0.2 ± 0.0		0.4 ± 0.1	1.5 ± 0.0
U–3α		3.4 ± 0.2	3.7 ± 0.4		9.7 ± 2.2	9.0 ± 2.2
T–3α7β	0.9 ± 0.1					
U–3α7β		0.7 ± 0.2	0.4 ± 0.1		0.2 ± 0.1	0.3 ± 0.1
T–3α6β7α	2.4 ± 0.1			2.4 ± 0.1		
U–3α6β7α		2.1 ± 0.7	2.3 ± 0.0		3.6 ± 0.6	5.9 ± 0.5
T–3α6β7β	65.2 ± 0.7			38.4 ± 1.9		
U–3α6β7β		49.0 ± 3.0	36.7 ± 1.5		25.2 ± 0.4	12.5 ± 0.7
U–3α6α7β		12.5 ± 0.3	20.6 ± 1.0		15.3 ± 1.0	16.7 ± 4.9
U–3α6α		0.7 ± 0.2	0.5 ± 0.1		1.0 ± 0.1	0.5 ± 0.2

[a]Mean ± SE (*n* = 4 rats).
[b]Mean ± SE (*n* = 2 rats. For abbreviations see Table 2.

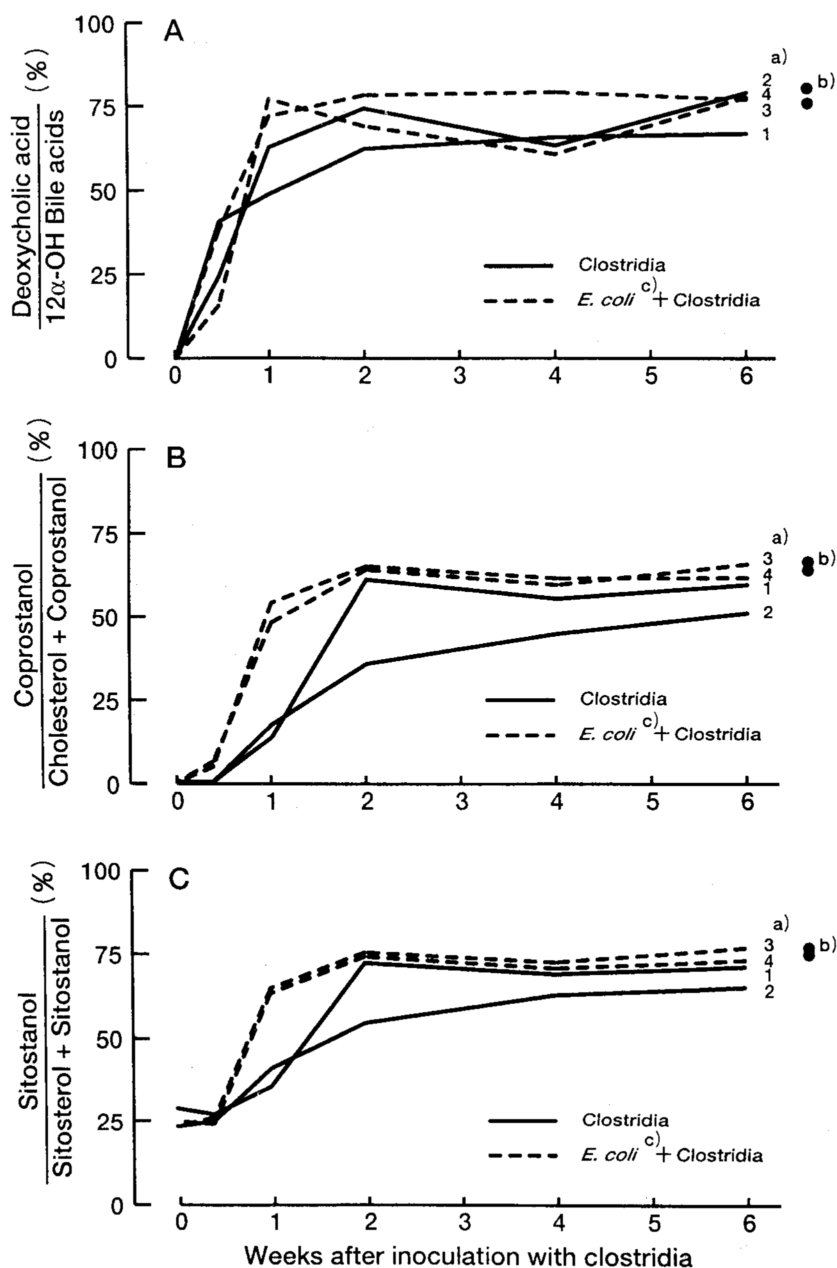

FIG. 1. Changes in the composition ratios of deoxycholic acid in 12α-hydroxyl bile acids (A), coprostanol in C-27 sterols (B), and sitostanol in C-29 sterols (C) in the feces of male germ-free rats inoculated with clostridia with and without preinoculation of *Escherichia coli.* [a]Rat #; [b]rats given conventional rat feces; [c]*E. coli* preinoculated 1 wk before the inoculation of clostridia.

effect of clostridia in germ-free rats and in *E. coli* preinoculated rats because *E. coli* does not biotransform bile acids (12) and its presence was expected to increase the anaerobic conditions in the intestinal lumen.

Escherichia coli did not affect the fecal bile acid or sterol composition in these experiments, and no differences were found for the effect of clostridia on fecal bile acids between germ-free and *E. coli* preinoculated rats, suggesting that clostridia grow in germ-free and *E. coli*-monoassociated rats. However, the formation of coprostanol and sitostanol after clostridia inoculation was delayed in germ-free rats compared with *E. coli* preinoculated rats (Fig. 1). These observations suggest that the growth of clostridia biovars responsible for coprostanol formation is enhanced by *E. coli* pre-inoculation, and these biovars may be different from those responsible for deoxycholic acid formation.

Major bile acids found in germ-free rats were taurine-conjugated cholic and β-muricholic acids. Both bile acids were deconjugated after clostridia inoculation, and most cholic acid was further transformed to secondary bile acids, but

β-muricholic acid was rather resistant to biotransformation by clostridia. Low conversion of β-muricholic acid was also noted in germ-free rats after implantation of human microflora (21).

Which bacteria in human intestinal microflora correspond to the present clostridia in rats is not yet known but some species of human intestinal bacteria have been reported to have 7α-dehydroxylation activity (4–11), although the counts of 7α-dehydroxylating positive bacteria were as low as 10^5–10^6 counts per g wet weight feces. Stellwag and Hylemon (7) reported that the 7α-dehydroxylation activity of mixed fecal bacteria was 0.06–0.012 μmol/h·mg protein for cholic acid in an *in vitro* system. Most cholic acid is 7α-dehydroxylated in human and also in rats. This formation of deoxycholic acid *in vivo* seems not to be achieved by the activity reported for cholic acid *in vitro*. On the other hand, 7α-dehydroxylation activity and also 7β-dehydroxylation activity of *Eubacterium* were reported to be enhanced by the presence of *Bacteroides,* which exhibits no activity by itself (10). These data suggest that a mechanism as yet unknown enhances 7α- and 7β-dehydroxylation activities.

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White Adipose Tissue Fatty Acids of Alpine Marmots During Their Yearly Cycle

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ABSTRACT: Alpine marmots (*Marmota marmota*) were maintained on a laboratory diet, and the fatty acid composition of gonadal and subcutaneous white adipose tissues (WAT) was studied during a yearly cycle. Fatty acids (FA) released from isolated adipocytes were also identified after stimulation of *in vitro* lipolysis. Analysis of the FA composition of WAT depots showed that marmot WAT mainly contained monounsaturated FA (65%, mostly oleic acid, 18:1n-9) although laboratory food contained 45% of linoleic acid (18:2n-6) and only 21% of 18:1n-9. During stimulated lipolysis, saturated FA were preferentially released from isolated adipocytes whereas unsaturated FAs were retained. Despite this selective release of FA from isolated WAT cells *in vitro*, and despite the FA composition of the food, marmots maintained a constant FA composition in both WAT depots throughout the year. Six months of hibernation and fasting as well as an intense feeding period did not affect this composition. The potential adaptive benefit of such regulation of WAT composition, based on a high level of monounsaturated FA, might be to maintain fat with appropriate physical properties allowing animals to accommodate to and survive the wide range of body temperatures experienced during hibernation.

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Alpine marmots (*Marmota marmota*) exhibit a marked yearly rhythm of food intake, with a feeding period during summer and a fasting period during hibernation, which can last 5 to 6 mon depending on geographic and climatic conditions (1,2). During hibernation, marmots alternate long torpor bouts [lasting 10–15 d with a body temperature (Tb) regulated a few degrees above 0°C] with short euthermic phases [lasting 1–2 d with a Tb of 37°C (3)]. Rewarming during arousal mainly depends on brown and white fat lipid oxidation (4–6). Large hibernating mammals such as marmots therefore rely mainly on their large fat deposits, which can reach 50% of their body mass prior to hibernation (reviewed in Ref. 7). Nearly 80% of this stored fat can be used during hibernation (8).

In small rodents, such drastic depletion in fat mass may affect the lipid composition of adipose tissue because of the known differential mobilization of fatty acids (FA) during fasting (9). Therefore, after several days of starvation in the rat, the relative distribution of the remaining FA in white adipose tissue (WAT) is profoundly altered (9). If such change in lipid composition occurs in long-term fasting hibernators, it may in turn have marked effects on hibernation. Indeed, it was shown that diet-induced changes in lipid composition of WAT affect hibernation characteristics. Deficiency in essential FA (linoleic acid, 18:2n-6; and α-linolenic acid,18:3n-3) led to marked changes in the hibernation pattern of marmots (10,11). Similarly, feeding a high polyunsaturated acid diet to chipmunks (*Tamias amoenus)* altered their hibernation bout lengths and the minimum Tb reached during deep hibernation (12). These physiological effects may be due to changes in the FA composition of cell membranes and fat stores, which would alter physical properties such as fluidity at low Tb. Although of likely functional importance, WAT lipid composition during an annual cycle with regard to preferential mobilization of FA released by lipolysis has not been investigated in marmots.

The aim of the present study was to investigate the FA composition of two WAT depots (subcutaneous and abdominal) throughout an entire annual cycle in captive marmots maintained on identical laboratory diets. Furthermore, isolated adipocytes from these two depots were stimulated *in vitro* so that we could analyze the composition of the FA released during the annual cycle.

MATERIAL AND METHODS

Animals. Marmots were trapped in the French Alps (Bonneval-sur-Arcs, Savoie). The study was performed on 12 adults (6 males and 6 females). Marmots were cared for under the French Code of Practice for the Care and Use of Animals for Scientific Purpose, and the experimental protocols were approved by the French Ministry of Agriculture Ethics Committee (Animals). They were caged in pairs under outdoor conditions of photoperiod and ambient temperature (Ta) during the summer (Ta ranged from 15 to 25°C) and during the hibernation season in a cold room (Ta = 6 ± 1°C, light/dark 0:24). Standard food (rodent chow pellet, Genthon, Cheyssieu,

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Abbreviations: FA, fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; Ta, ambient temperature; Tb, body temperature; WAT, white adipose tissue.

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France) was given to the animals. Food and water were provided *ad libitum* after the emergence from hibernation (April) and were removed when the animals ceased to feed in late fall. The mean body mass loss during hibernation was 29.4% for males and 34.0% for females (6.8 kg *vs.* 4.8 kg and 5.8 *vs.* 3.8 kg, respectively) and was not significantly different between genders.

Sequencing of the cycle. The marmot cycle was studied for 15 mon, beginning in October and finishing in late December of the following season (see Fig. 1). In the first part of the cycle, the period of hibernation occurred within the fasting period. During the second part of the study, marmots reduced their food consumption in mid-September, somewhat earlier than in the previous year. This drop in food consumption coincided with a sudden drop in the outdoor temperature, which was a few degrees above 0°C during the night. During the study, animals were kept alive and sampled at least twice during the experimental cycle (except for marmot #4 which was sampled once, see Fig. 1). The sampling frequency was approximately one animal every 15 d. Because marmots did not start fasting at the same time prior to the first and second hibernation season, we decided to consider only the periods in which all the animals were both hibernating and fasting (Fasting period) or both active and eating (Food Intake period). Among the 12 marmots we used in this study, 7 were sampled once at both periods (marmots #1, 5, 6, 7, 8, 9, 11) and then data on these 7 were used to perform the statistical analysis.

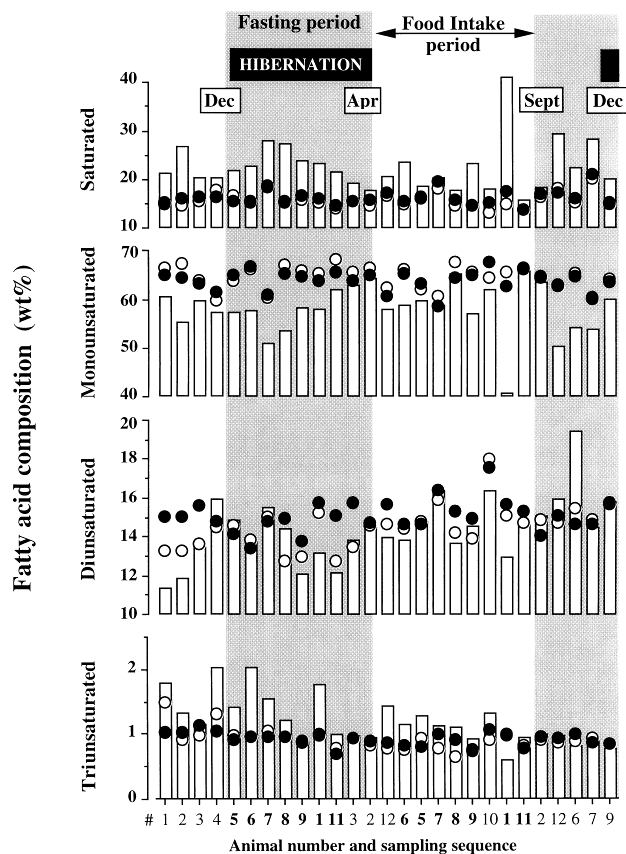

FIG. 1. Fatty acid composition of gonadal (open circles) and subcutaneous white adipose tissue (closed circles) compared to the composition of the fatty acids released *in vitro* (open bars) from gonadal isolated adipocytes over the annual cycle of marmots. The sampling sequence was approximately one animal every 15 d. The identification number of the marmots is reported on the abscissa and the numbers in bold indicate marmots used for statistics; for comments see the paragraph on "Sequencing of the cycle" in the Materials and Methods section.

Adipose tissue samplings. Gonadal and inguinal subcutaneous WAT was surgically removed from marmots during the active and hibernating periods. In summer, nonhibernating marmots were fasted overnight before being anesthetized the next morning with zoletil 100 (Virbac, Carros, France; 100 mg/kg) and a fat biopsy performed (4–5 g). In winter, fasting hibernating marmots were removed from the cold room 24 h before surgery and allowed to arouse at room temperature (Ta = 21°C) without any food being available. Marmots were then anesthetized and biopsies were performed as described above. Fat samples were either immediately frozen at −30°C until lipid extraction and analysis or else minced in a collagenase buffer and incubated at 37°C for adipocyte isolation. Rodent pellet samples were collected four times over the food intake period to analyze the lipid composition of the diet.

To investigate whether the diet could similarly influence the WAT composition in another species, we also analyzed the FA composition of gonadal WAT from 6-mon-old rats fed the same diet. Gonadal WAT samples from rats were treated as described above for marmot WAT samples.

Composition of fatty acids released after in vitro *stimulated lipolysis.* At different times during the yearly cycle, the FA composition found in the fat depots was compared with the composition of the FA released *in vitro* after stimulated lipolysis of gonadal and subcutaneous WAT (feeding period only). This was achieved by preparing a suspension of 30–35 mg of isolated adipose cells per milliliter of buffer as described previously (13) with slight modifications. The pieces of gonadal and subcutaneous WAT were subjected to collagenase P digestion (0.3 mg/mL, Boehringer-Mannheim Chemicals, Meylan, France) in a Krebs-Hepes buffer (10 mM, pH = 7.4) containing 5% (wt/vol) of FA free bovine serum albumin and 6 mM glucose. An aliquot of the cell suspension was incubated at 37°C (shaking water bath) in polypropylene microcentrifuge tubes, and lipolysis was stimulated with adrenocorticotropic hormone (ACTH, from Sigma Chemicals, Saint Quentin Fallavier, France). ACTH was as effective as norepinephrine in stimulating FA release from isolated marmot adipocytes as noted in rats (14) and in rabbits (15,16). After 1 h incubation, samples were centrifuged and cells were removed by vacuum suction. Fatty acids in the medium were recovered with chloro-form/methanol (2:1, vol/vol) containing 50 mg/L of butylated hydroxytoluene (BHT) and stored immediately at −30°C under nitrogen prior to lipid extraction and analysis.

FA analysis. WAT samples were homogenized in chloroform/methanol (2:1, vol/vol) containing 50 mg/L of BHT, extracted, and purified according to the procedure of Folch *et al.* (17). Lipids were evaporated to dryness under a stream of

nitrogen, taken up in an appropriate volume of chloroform/methanol, and stored in glass tubes at −30°C under nitrogen. The methylation was performed according to Slover and Lanza (18) with minor modifications. A known amount of heptadecanoic acid (17:0) was added as an internal standard, transmethylation was achieved by heating at 80°C for 150 min in methanol/sulfuric acid (98:2, vol/vol). After neutralization with a 5% K_2CO_3 solution, methyl esters were extracted in hexane and dried on anhydrous sodium sulfate. Samples were analyzed by gas–liquid chromatography at constant oven temperature (180°C) over the whole run, using a Chrompack CP-9001 (Chrompack, Middelburg, The Netherlands) equipped with a silica 50 m × 0.25 mm i.d. capillary column coated with CP SIL88, using nitrogen as the carrier gas. FA standards were run periodically to determine correct retention times for the FA identification. Peaks were measured using Maestro Chromatography data system software (Chrompack).

Statistics. The composition of FA from both depots (gonadal and subcutaneous) and of FA released from gonadal WAT were compared over the same period and between food intake and fasting periods using a one repeated factor two-way-analysis of variance with Bonferoni/Dunnett *post-hoc* tests ($P < 0.03$). Before calculations, percentage values were transformed into angular values [arcsin root of (value/100)].

RESULTS

Marmot gonadal and subcutaneous WAT composition did not change significantly over the year (Fig. 1). It is clear from this figure that no profound variation in the saturated FA (SFA), monounsaturated FA (MUFA), and polyunsaturated FA (PUFA) composition occurred in either of the depots in either the feeding or fasting period. In addition, no significant difference between depots was observed between the food intake/fasting periods (Table 1). Within depots, small though significant differences in MUFA and PUFA (mainly diunsaturated and triunsaturated) composition were observed between the food intake and fasting periods. The unsaturation index was similar in both depots and did not change during the year.

Marmot WAT FA composition differed markedly from that

TABLE 1
Major Fatty Acid Species Identified in Gonadal and Subcutaneous WAT of The Alpine Marmot.

	Food Intake period		Fasting period and hibernation	
	Gonadal	Subcutaneous	Gonadal	Subcutaneous
Saturated (wt%)				
14:0	1.10 ± 0.1	1.32 ± 0.1	1.29 ± 0.04	1.28 ± 0.07
16:0	12.31 ± 0.3	12.93 ± 0.5	12.91 ± 0.4	13.07 ± 0.3
18:0	1.59 ± 0.2	1.70 ± 0.2	1.45 ± 0.1	1.38 ± 0.1
20:0	0.25 ± 0.04	0.29 ± 0.03	0.20 ± 0.01	0.23 ± 0.02
22:0	0.06 ± 0.01	0.07 ± 0.01	0.07 ± 0.01	0.07 ± 0.01
Total	15.30 ± 0.6	16.12 ± 0.7	15.85 ± 0.6	15.96 ± 0.5
Monounsaturated (wt%)				
14:1n-7	0.52 ± 0.1	0.51 ± 0.05	0.61 ± 0.06	0.58 ± 0.06
16:1n-9	0.79 ± 0.1	0.90 ± 0.1	0.98 ± 0.05	0.98 ± 0.06
16:1n-7	4.25 ± 0.2	4.30 ± 0.2	5.80 ± 0.2[b]	5.18 ± 0.2
18:1n-9	56.85 ± 0.8	55.16 ± 0.9	55.18 ± 0.9	55.49 ± 0.8
18:1n-7	1.93 ± 0.1	2.03 ± 0.1	2.12 ± 0.1[b]	1.99 ± 0.09
20:1n-9	0.58 ± 0.1	0.66 ± 0.04	0.51 ± 0.02	0.57 ± 0.02
22:1n-9	0.05 ± 0.01	0.06 ± 0.01	0.03 ± 0.01	0.05 ± 0.01
24:1n-9	0.09 ± 0.02	0.11 ± 0.01	0.13 ± 0.03	0.11 ± 0.01
Total	65.01 ± 0.9	63.67 ± 0.9	65.33 ± 1.0	64.61 ± 0.7
Diunsaturated (wt%)				
18:2n-6	14.48 ± 0.3	15.04 ± 0.2	13.68 ± 0.4	14.32 ± 0.3
20:2n-6	0.27 ± 0.01	0.28 ± 0.02	0.23 ± 0.02	0.25 ± 0.02[b]
Total	14.75 ± 0.2	15.31 ± 0.2	13.91 ± 0.4	14.57 ± 0.3
Triunsaturated (wt%):				
18:3n-6	0.02 ± 0.01	0.02 ± 0.00	0.05 ± 0.01	0.04 ± 0.01
18:3n-3	0.80 ± 0.04	0.83 ± 0.04	0.84 ± 0.04	0.84 ± 0.03
20:3n-6	0.05 ± 0.00	0.05 ± 0.00	0.05 ± 0.01	0.04 ± 0.01
Total	0.82 ± 0.05	0.87 ± 0.04	0.95 ± 0.03	0.91 ± 0.04[b]
Tetra- and hexaunsaturated (wt%)				
20:4n-6	0.16 ± 0.01	0.19 ± 0.01	0.17 ± 0.02	0.16 ± 0.01
22:6n-3	0.45 ± 0.03	0.52 ± 0.03	0.46 ± 0.06	0.44 ± 0.04
Total	0.61 ± 0.04	0.71 ± 0.03	0.63 ± 0.06	0.60 ± 0.05
Others	3.51 ± 0.4	3.32 ± 0.3	3.33 ± 0.2	3.35 ± 0.2
UI	1.01 ± 0.01	1.01 ± 0.01	1.00 ± 0.01	1.01 ± 0.01

[a]Fatty acids (mean ± SEM; gonadal, $n = 7$; subcutaneous, $n = 7$) are expressed in % of the total fatty acids identified in each tissues. [b]Significant difference between Food intake and Fasting periods within the same depot ($P < 0.05$). No difference was found between the gonadal and the subcutaneous depots. UI, unsaturation index.

TABLE 2
Fatty Acid Composition of Marmot Laboratory Diet and Epididymal WAT of 6-month-old Rats Fed with the Same Diet as Marmots[a]

	Laboratory diet (*n* = 4)	Rat WAT epididymal depot (*n* = 4)
Saturated (wt%)		
14:0	3.1 ± 0.6	1.5 ± 0.0
16:0	15.6 ± 0.3	22.1 ± 0.3
18:0	2.9 ± 0.7	3.6 ± 0.1
20:0	1.0 ± 0.1	0.8 ± 0.1
Total	23.3 ± 0.2	28.2 ± 0.0
Monounsaturated (wt%)		
16:1n-7	1.3 ± 0.3	3.3 ± 0.2
18:1n-9	21.5 ± 0.2	26.7 ± 0.1
Total	24.5 ± 0.8	32.5 ± 0.3
Diunsaturated (wt%)		
18:2n-6	45.4 ± 3.1	35.5 ± 0.5
Triunsaturated (wt%)		
18:3n-3	3.0 ± 0.2	1.7 ± 0.0
UI	1.2 ± 0.0	1.1 ± 0.0

[a]Fatty acids (mean ± SEM) are expressed in wt% of the total fatty acids identified. WAT, white adipose tissue.

of the diet (Table 2). The linoleic acid (18:2n-6) content of marmot gonadal and subcutaneous WAT depots (13–15%) was three times lower than that found in the rodent diet (45%, Tables 1 and 2), whereas oleic acid (18:1n-9) content (55–57%) was three times higher in both marmot fat depots as compared with the diet (21%, Tables 1 and 2). The total SFA content in marmot WAT was decreased by 7% in comparison with the diet, and the linolenic content by 2%.

In rats, the WAT composition reflected more closely the laboratory diet composition than in marmots. In rat WAT (Table 2), the 18:2n-6 content was 35.5% (diet was 45%). This decrease in the percentage of 18:2n-6 was balanced by high SFA and MUFA content, and the relative percentage of α-linolenic acid (18:3n-3, 1.7%) was nearly 50% of that ingested and presumably absorbed from the diet (3%).

The composition of FA released into the medium was quite different from that found in WAT, as indicated by the lowering of the UI of the total FA identified in the medium after *in vitro* lipolysis (compare Tables 1 and 3). The main differences observed were for 14:0, 16:0, and 18:0. Bars in Figure 1 also

TABLE 3
Major Fatty Acids Released *in vitro* from Gonadal WAT and Subcutaneous WAT Isolated Adipocytes[a]

	Food Intake period		Fasting period and hibernation[b]
	Gonadal	Subcutaneous	Gonadal
Saturated (wt%)			
14:0	2.64 ± 0.6[d]	5.00 ± 1.6	1.43 ± 0.05[c]
16:0	15.78 ± 1.6[d]	15.42 ± 0.5	16.89 ± 0.4[d]
18:0	4.27 ± 1.1[d]	4.64 ± 1.1	5.50 ± 0.6[d]
20:0	0.05 ± 0.0	—	0.06 ± 0.04
22:0	—[e]	—	—
Total	22.75 ± 3.2[d]	25.06 ± 2.8	23.88 ± 0.9[d]
Monounsaturated (wt%)			
14:1n-7	0.82 ± 0.1[d]	1.20 ± 0.2	0.88 ± 0.03[d]
16:1n-9	1.44 ± 0.4[d]	1.48 ± 0.3	1.62 ± 0.2[d]
16:1n-7	4.95 ± 0.5	5.28 ± 0.5	5.60 ± 0.4[c]
18:1n-9	48.28 ± 3.0[d]	45.08 ± 2.4	46.00 ± 1.0[d]
18:1n-7	2.00 ± 0.1[d]	2.08 ± 0.4	2.29 ± 0.1[c]
20:1n-9	0.37 ± 0.1[d]	0.47 ± 0.3	0.24 ± 0.07[c,d]
Total	57.86 ± 3.1[d]	55.60 ± 2.8	56.63 ± 1.3[d]
Diunsaturated (wt%):			
18:2n-6	14.11 ± 0.4	14.31 ± 0.7	13.68 ± 0.5
20:2n-6	0.26 ± 0.1[d]	—	—
Total	14.37 ± 0.4	14.31 ± 0.7	13.68 ± 0.5
Triunsaturated (wt%)			
18:3n-6	0.10 ± 0.04[d]	0.03 ± 0.01	0.26 ± 0.1
18:3n-3	0.87 ± 0.09	1.04 ± 0.06	1.05 ± 0.1
20:3n-6	0.05 ± 0.05	—	0.09 ± 0.09
Total	1.01 ± 0.08[d]	1.07 ± 0.08	1.40 ± 0.2[c,d]
Tetra- and hexaunsaturated (wt%)			
20:4n-6	0.68 ± 0.2[d]	0.83 ± 0.5	0.60 ± 0.2[d]
22:6n-3	—	—	0.05 ± 0.03[d]
Total	0.68 ± 0.2	0.83 ± 0.53	0.65 ± 0.2
Others	3.33 ± 0.4	3.13 ± 0.5	3.76 ± 0.2
UI	0.93 ± 0.03	0.92 ± 0.03	0.92 ± 0.01

[a]Fatty acids (mean ± SEM; gonadal, *n* = 7; subcutaneous, *n* = 4) are expressed in wt% of the total.
[b]Release of fatty acids from subcutaneous WAT isolated adipocytes taken from animals during the fasting period was not determined.
[c]Significant difference between Food Intake and Fasting periods within a same depot ($P < 0.05$).
[d]Significant difference between gonadal WAT composition and fatty acids released from gonadal WAT after an *in vitro* lipolysis.
[e]—, below the detection limit. For abbreviations see Tables 1 and 2.

show the clear difference between the FA released from gonadal adipocytes *in vitro* and gonadal WAT composition. This suggests a preferential release of SFA throughout the whole cycle whereas MUFA appeared to be retained. However, we do not know how the absolute concentration of each FA changes but only how each FA changes as a percentage of the total. It follows that the increased release of one FA species will result in an apparent decreased release of other FA species.

In the case of fasting, the same general results were observed in gonadal WAT as seen during the feeding period (Tables 1 and 3), although slight differences emerged between the relative mobilization rates of FA in gonadal WAT for the two periods.

DISCUSSION

The main finding of the present study is that, despite fasting and refeeding periods and a differential mobilization of FA during *in vitro* lipolysis, alpine marmots maintain a surprisingly stable WAT FA composition throughout the yearly cycle.

Numerous studies have reported that the FA composition of WAT is largely influenced by the dietary FA intake, both in nonhibernating mammals (19,20) and hibernators (10,21–24). However, the FA profile of captive alpine marmot WAT (Table 1) exhibited greater differences from the diet than that of the rat (Table 2), indicating that the pattern of the FA storage may be species-specific. Present results indicate that, in marmots, a preferential accumulation of MUFA occurs at the expense of PUFA, although the latter are known to have beneficial effects on hibernation (10–12,25). Surprisingly, this FA composition is maintained throughout the annual cycle, during the intense feeding period, when marmots rapidly reconstitute their fat stores, as well as during the fasting period, when marmots metabolize most of these fat stores. In the rat, by contrast, several days of starvation profoundly alter the distribution of the FA remaining in WAT (9).

As previously observed in rats (9,26,27), the composition of the FA mobilized from marmot white adipocytes (bars in Fig. 1) did not exactly reflect the composition of WAT. However, the pattern appeared to differ somewhat between rats and marmots. The conclusions drawn from rat studies are that short-chain and unsaturated FA, with a double bond close to the methyl end of the chain, are preferentially mobilized during *in vitro* lipolysis. In marmots, however, FA mobilization seems directed toward a preferential release of SFA rather than PUFA, whereas MUFA are retained within the tissue (Fig. 1 and Table 3). Previous results with marmots *in vivo* correlate well with the present *in vitro* data, *i.e.*, SFA amount to 60% of the FA found in plasma during deep hibernation, rewarming, and arousals (28). Moreover, contrary to rats, this preferential mobilization of FA does not lead to changes in WAT composition after several months of fasting.

The mechanism(s) underlying this process may involve hormone-sensitive lipase specificity and/or differential reesterification by analogy with the situation in rats. In mammals, hormone-sensitive lipase preferentially hydrolyses the first and the third ester bonds of triacylglycerols (29) where SFA are frequently found. However, in spite of this specificity, lipolysis in the rat (26,30) is directed toward the preferential release of PUFA. Wilson *et al.* (31) reported the presence of high amounts of messenger RNA for hormone-sensitive lipase in marmot WAT during fall and winter, but the activity and the specificity of this enzyme on triacylglycerols were not determined. Another mechanism could be FA reesterification which has been shown to occur *in vitro* in rats, even under stimulated lipolysis (32,33), and could explain the unchanged FA composition in marmot in spite of a nonramdom FA mobilization. However, during hibernation of *M. flaviventris*, under nonstimulated conditions the venous blood of gonadal fat shows a molar ratio FA/glycerol of 3:1 (10), which is not in favor of reesterification within WAT. Whatever the mechanism, it appears that, owing to a marked repletion/depletion of fat stores during the yearly cycle of marmots and because of the preferential release of FA by adipose tissue (as shown by *in vitro* lipolysis), the FA composition should markedly change throughout the year. Because we did not observe such changes, it is likely that the composition of fat depots is actively regulated in marmots. Such regulation has never been described before in either hibernators or nonhibernators.

Apart from the unresolved problem of the mechanisms allowing the constancy of FA composition throughout the yearly cycle of marmots, the question arises as to what could be the role of such regulation of FA composition. It is tempting to suggest that a particular FA composition may play a major role during hibernation and particularly during periodic arousals. When body temperature has to change rapidly within a 35°C range, i.e., from a few degrees above zero to +37°C and back in a period of few days, some physical properties of fat may be necessary to maintain the optimal functions of the tissue. Because such dramatic change in tissue temperature is likely to affect fluidity of membranes and triglyceride stores, a given FA composition may ensure a low WAT melting point. Supporting this interpretation, WAT melting temperature is −4.3°C in the adult California ground squirrel (*Spermophilus beldingi*) before hibernation (34). Similarly, differential fluidity of outer and inner sides of subcutaneous fat depots in cattle (19) and polar bear (35) is also achieved through regulation of FA composition.

In this way, a high UI, an estimate of the amount of unsaturated FA in a tissue (36), is usually associated with high fluidity at low temperature. Present results in marmots suggest that the apparent regulation of the FA composition of WAT, and possibly fluidity, is primarily achieved through the regulation of the MUFA content. This is in accord with the well-established fact that the introduction of the first double bond in SFA chains has the maximal effect on the decrease in melting point and thus fluidity of WAT, whereas the introduction of further double bonds affects physical properties less (36–38). In agreement with this point is the observation that the decrease in WAT melting point in the ground squirrel (*S. beldingi*) before hibernation is more highly correlated with the content of MUFA, which increases from 25 to 44%, than with the content of PUFA, which decreases from 54 to 38%

(34). Similarly, physiological differences were reported in chipmunks fed with a diet supplemented in either 18:0, 18:1, or 18:2 (22). Geiser *et al*. (22) demonstrated that the addition of 18:1 to the food caused the greatest change in the pattern of torpor (minimum Tb reached during torpor and duration of torpor bouts), and these observations were highly correlated with the increase of MUFA in the WAT of chipmunks (22). In captive yellow-bellied marmots, it was also observed that, relative to free-ranging animals, the drastic lowering of 18:3n-3 was compensated by an increase in 18:1n-9 rather than 18:2n-6, despite the high availability of the latter in the lab food (10,11).

It therefore appears that the preferential storage of MUFA may be a general feature of hibernating animals, enabling the constitution of fat with appropriate physical properties for deep hibernation and periodic arousals. The synthesis of MUFA, which does not depend on the diet, may be an efficient and simple adaptive mechanism allowing marmots and possibly other hibernators to survive rapid and frequent large changes in body temperature without further FA chain restructuration. In captive marmots (present study) fed a laboratory diet low in n-3 PUFA, it appears that the regulation of WAT optimal fluidity leads to a steady FA composition during the yearly cycle. The importance of MUFA in hibernating marmots may be in relation with the preferential release of SFA by isolated adipocytes (present study), contrary to what is observed in nonhibernating rodents such as the rat (26,30). The precise physical characteristics and thus biological activity which are to be preserved in hibernating animals by such precise regulation now needs to be determined.

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Fatty Acid Composition of the Adipose Tissue and Yolk Lipids of a Bird with a Marine-Based Diet, the Emperor Penguin (*Aptenodytes forsteri*)

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ABSTRACT: The emperor penguin (*Aptenodytes forsteri*) is an Antarctic seabird feeding mainly on fish and therefore has a high dietary intake of n-3 polyunsaturated fatty acids. The yolk is accumulated in the developing oocyte while the females are fasting, and a large proportion of the fatty acid components of the yolk lipids are derived by mobilization from the female's adipose tissue. The fatty acid composition of the total lipid of the yolk was characterized by high levels of n-3 polyunsaturated fatty acids. However, it differed in several respects from that of the maternal adipose tissue. For example, the proportions of 14:0, 16:1n-7, 20:1n-9, 22:1n-9, 20:5n-3, and 22:6n-3 were significantly greater in adipose tissue than in yolk. Thus adipose tissue lipids contained 7.6 ± 0.3% and 8.0 ± 0.3% (wt% of total fatty acids; mean ± SE; *n* = 5) of 20:5n-3 and 22:6n-3, respectively, whereas the yolk total lipid contained 1.6 ± 0.1 and 5.5 ± 0.3% of these respective fatty acids. The proportions of 16:0, 18:0, 18:1n-9, 18:2n-6, and 20:4n-6 were significantly lower in the adipose tissue than in the yolk lipids. The proportions of triacylglycerol, phospholipid, free cholesterol, and cholesteryl ester in the yolk lipid were, respectively, 67.0 ± 0.2, 25.4 ± 0.3, 5.3 ± 0.2, and 1.8 ± 0.2% (wt% of total yolk lipid). The proportions of 20:4n-6, 20:5n-3, 22:5n-3, and 22:6n-3 were, respectively, 5.7 ± 0.3, 2.8 ± 0.2, 1.4 ± 0.1, and 11.7 ± 0.5% in phospholipid and 0.4 ± 0.0, 1.2 ± 0.1, 0.8 ± 0.1 and 3.6 ± 0.3% in triacylglycerol. About 95% of the total vitamin E in the yolks was in the form of α-tocopherol with γ-tocopherol forming the remainder. Two species of carotenoids, one identified as lutein, were present.

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The prime importance of yolk lipid as a nutrient source for the avian embryo is illustrated by the fact that over 90% of the energy required for development is generated by β-oxidation of yolk-derived fatty acids (1). In addition, the rapid rate of cell proliferation during development is dependent on the provision of yolk-derived lipid components for the synthesis of membrane phospholipids (1). A feature of particular interest is the role of C_{20-22} polyunsaturated fatty acids of both the n-3 and n-6 series in the functional differentiation of certain embryonic tissues (2). Most notably, the phospholipids of the developing brain (3) and retina (4) of the avian embryo are characterized by very high proportions of docosahexaenoic acid (22:6n-3). Studies on mammals, including humans, have indicated that inadequate provision of 22:6n-3 to the neural tissues during fetal/neonatal life can result in a range of behavioral, cognitive, and visual impairments (2,5–8). With regard to birds, the importance of 22:6n-3 for neurological development is emphasized by the expression of a series of mechanisms in the embryonic chicken which selectively promote the transfer of this n-3 polyunsaturate from the lipids of the yolk to the target tissues of the embryo (9). For example, 22:6n-3 is preferentially taken up from the yolk (10) and is also selectively mobilized from the adipose tissue of the chicken embryo (11). The n-6 polyunsaturate, arachidonic acid (20:4n-6), also has important functions in the developing tissues of the chick embryo, with roles in signal transduction and eicosanoid synthesis (9). Thus it may be envisaged that the successful development of the avian embryo may be promoted by the availability in the yolk of an appropriate balance of n-3 and n-6 polyunsaturates. The profile of polyunsaturated fatty acids in the yolk lipids is profoundly influenced by the fatty acid composition of the diet of the parent hen (9). In particular, supplementation of chicken diets with fish oil produces major elevations in the 22:6n-3 content of the yolk lipid (4,12–15). The yolk fatty acid composition, particularly with regard to the proportions of 22:6n-3 and 20:4n-6, is also markedly affected by genetic differences between avian species (9).

The purpose of the present work was to determine the fatty acid composition of the yolk lipids of a bird with a very high natural intake of C_{20-22} n-3 fatty acids. The emperor penguin (*Aptenodytes forsteri*) is an Antarctic seabird feeding on fish, krill, and squid (16) and thus ingests large amounts of n-3 polyunsaturates and also of very long chain monounsaturates, as can be inferred from its adipose tissue fatty acid composition (17). The defining feature of the life cycle of this species is that feeding only occurs while at sea whereas activities as-

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Abbreviations: CE, cholesteryl ester; FC, free cholesterol; FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PS, phosphatidylserine; Sph, sphingomyelin; TAG, triacylglycerol; VLDL, very low density lipoprotein.

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sociated with breeding (courtship, egg laying, incubation of eggs, chick brooding) take place ashore at colony sites which may be many kilometers from the sea (18). Thus breeding activities are associated with prolonged periods of fasting during which the birds are sustained mainly by extensive stores of subcutaneous adipose tissue which were accumulated during the previous sojourn at sea (19). The most extreme period of fasting (90–120 d) is endured by the male, which assumes responsibility for incubating the eggs throughout the winter, losing 40% of its initial body mass and 80% or more of its lipid reserves (17). In the case of the female, egg laying is preceded by about 6 wk of total fasting during courtship and pairing (17,20). On arrival at the breeding colony, the ovary is relatively undeveloped and weighs only a few grams compared with about 100 g 4 wk later and about 200 g just prior to laying (18,20). The egg yolk is therefore almost entirely formed while the female is fasting, and consequently the yolk fatty acids are derived largely from the mobilization of adipose tissue lipid (20).

These exceptional circumstances relating to the diet, metabolism, and reproductive cycle of the emperor penguin provided the opportunity for: (i) determining the fatty acid composition of the yolk lipids of a high n-3 consumer; (ii) relating this composition to that of the potential precursor lipids, i.e. the maternal adipose tissue; and (iii) evaluating if a high degree of unsaturation of yolk lipids is associated with a high content of yolk antioxidants.

MATERIALS AND METHODS

Egg collection and adipose tissue sampling. Biological samples were obtained from emperor penguins breeding within 1 km of the Dumont d'Urville Station (Adelie Land, Antarctica, 66°40′S, 140°01′E) in 1996. About 3000 pairs breed there each year. Five females were caught in early April on arrival at the breeding colony and after the pre-breeding fattening period at sea. Their body weights averaged 29.5 kg. A subcutaneous adipose tissue biopsy (about 1 g) was immediately taken under local anesthesia (xylocain, 1%), and the birds were then released into the colony. Adipose samples were rinsed with saline, blotted dry, and extracted with chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (21). The extracts were stored at −20°C in the presence of butylated hydroxytoluene (0.5% wt/vol) prior to analysis.

The emperor penguin is a protected species and for ethical reasons we collected only eggs which had been abandoned. By checking the colony daily in May, we were able to collect 6 abandoned eggs within 1 d of laying. Since the ambient temperature was around −20°C at this time of year, the eggs were rapidly frozen on being abandoned and were therefore well-preserved. The mass of these eggs averaged 460 g, which is close to the mean mass of 470 g in this species (17). The eggs were partly thawed and the yolk was collected. The wet mass of the yolks was 115 ± 3 g, which is similar to the reported 120-g average yolk mass (17). Approximately 3 g of each yolk was extracted for lipid analysis as described for the adipose tissue. The preparation of the lipid extracts of adipose tissue and yolk was performed at the Antarctic Field Station. The extracts were maintained at −20°C during transport to the authors' laboratories in France and Scotland.

Lipid analysis. The fatty acid compositions of the total lipid of the yolks and of the adipose tissue were determined following the transmethylation of aliquots of the extracts to form the fatty acid methyl esters (22). Analysis of the fatty acid methyl esters was performed by gas–liquid chromatography using a capillary column (Carbowax, 30 m × 0.25 mm, film thickness 0.25 μm; Alltech, Carnforth, United Kingdom) in a CP9001 Instrument (Chrompack, Middleburg, The Netherlands) connected to an EZ Chrom Data System (Scientific Software Inc., San Ramon, CA). The Data System enabled the expression of the fatty acid compositions in terms of wt%. The peaks were quantified by comparison with a 19:0 standard. The identification of the peaks was confirmed by comparison with the retention times of standard fatty acid methyl ester mixtures (Sigma, Poole, United Kingdom).

Portions of the extracts of the yolks were subjected to thin-layer chromatography on silica gel G using a solvent system of hexane/diethyl ether/formic acid (80:20:1, by vol) in order to isolate the major lipid classes [triacylglycerols (TAG), phospholipids (PL), free fatty acids (FFA), cholesteryl ester (CE), and free cholesterol (FC)]. Visualization of the bands and elution of the lipid classes from the silica were performed as described previously (22). The isolated PL fraction was further fractionated by thin-layer chromatography on silica gel D using a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, by vol). The major PL classes present [phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (Sph)] were visualized and eluted from the silica (22). The isolated acyl-containing lipid and PL classes were transmethylated, and the fatty acid methyl esters were analyzed by gas–liquid chromatography as described above. The amounts of each lipid and PL class present in the yolk extracts were calculated from the amount and composition of the fatty acyl groups derived from each class, together with the acyl group contribution to the molecular weights of these compounds. FC was determined using an enzymatic-colorimetric assay kit (Boehringer Mannheim, Lewes, United Kingdom).

Analysis of vitamin E and carotenoids. Vitamin E was measured in the yolk extracts by high-pressure liquid chromatography using a Spherisorb S30DS2 3μ C_{18} reversed-phase column, 150 mm × 4.6 mm (Phase Separations Ltd., Clwyd, United Kingdom) with a mobile phase of methanol/water (97:3, vol/vol) and fluorescence detection as previously described (23). Calibration was performed using standard solutions of the vitamin E forms (α-, β-, δ-, and γ-tocopherols and tocotrienols; Sigma, and Merck, Darmstadt, Germany). Carotenoids were analyzed by high-pressure liquid chromatography using a Spherisorb S30DS2 3μ C_{18} reversed-phase column, 250 mm × 4.6 mm (Phase Separations Ltd.) with a mobile phase of acetonitrile/dichloromethane/methanol (7:2:1, by

vol) and detection by absorbance at 445 nm as described previously (24). Carotenoid standards (lutein, zeaxanthine, canthaxanthin, β-cryptoxanthin, lycopene, α-carotene, β-carotene, and citranaxanthin obtained from Fluka, Gillingham, United Kingdom; Apin, Abingdon, United Kingdom; and Hoffman-La Roche, Basel, Switzerland) were used for calibration and identification of the peaks.

Statistical analysis. Statistical significance of differences in fatty acid composition between yolk and adipose tissue was assessed by unpaired Student's t-test after arcsin transformation of the percentage values in order to make the variance independent of the mean (25).

RESULTS

Fatty acid composition of total lipids of yolk and of maternal adipose tissue. There were significant differences in detail between the fatty acid profiles of yolk and adipose tissue (Table 1). The total lipid from both sources contained oleic (18:1n-9) and palmitic (16:0) acids as the major fatty acids with substantial levels of 22:6n-3 also present. However, of the 17 fatty acids listed in Table 1, 14 differed significantly in relative content between the two sources. In several cases, the magnitude of these differences was considerable. For example, the proportions of myristic (14:0), gondoic (20:1n-9) and eicosapentaenoic (20:5n-3) acids were, respectively, 6.9, 4.0, and 4.8 times higher in adipose tissue than in yolk. The proportion of 22:6n-3 was 1.5 times higher (8.0% vs. 5.5%, $P < 0.001$) in adipose tissue compared with yolk. Also, palmitoleic (16:1n-7) and docosapentaenoic (22:5n-3) acids were represented to a greater extent (1.8 and 1.4 times, respectively) in adipose tissue than in yolk. Erucic (22:1n-9) acid was a significant constituent of adipose tissue lipid but was not detected in the yolk samples. On the other hand, the proportions of stearic acid (18:0) and 20:4n-6 were, respectively, 3.2 and 4.3 times greater in yolk than in adipose tissue. The yolk lipids also exhibited greater proportions of 16:0, 18:1n-9, and linoleic acid (18:2n-6) than were present in adipose tissue lipids. As a consequence of the enhanced proportions of 18:2n-6 and 20:4n-6 and the reduced proportions of 20:5n-3, 22:5n-3, and 22:6n-3 in the yolk relative to the adipose tissue, the n-3/n-6 ratio was far higher in adipose tissue than in yolk. Similarly, the 22:6n-3/20:4n-6 ratio was very high in adipose tissue but much lower in yolk (Table 1).

TABLE 1
Fatty Acid Composition of Total Lipid of Yolk and Maternal Adipose Tissue[a]

Fatty acid[b]	Yolk (n = 6)	Adipose tissue (n = 5)
14:0	1.3 ± 0.2	8.9 ± 0.2**
16:0	23.7 ± 0.3	16.9 ± 0.2**
16:1n-7	4.9 ± 0.2	8.9 ± 0.2**
18:0	10.4 ± 0.4	3.3 ± 0.1**
18:1n-9	36.4 ± 1.3	22.3 ± 0.6**
18:1n-7	6.4 ± 0.4	6.5 ± 0.2
18:1n-5[c]	0.6 ± 0.0	0.6 ± 0.0
18:2n-6	3.3 ± 0.2	1.8 ± 0.1**
18:3n-3	0.5 ± 0.0	0.5 ± 0.0
18:4n-3	0.2 ± 0.0	1.0 ± 0.0**
20:1n-9[d]	1.4 ± 0.2	5.6 ± 0.3**
20:4n-6	1.7 ± 0.1	0.4 ± 0.0**
20:5n-3	1.6 ± 0.1	7.6 ± 0.3**
22:1n-9	Trace[e]	4.3 ± 0.4**
22:5n-3	1.0 ± 0.1	1.4 ± 0.1*
22:6n-3	5.5 ± 0.3	8.0 ± 0.3**
24:1n-9	Trace	0.5 ± 0.0**
Ratio n-3/n-6	1.76	8.41
Ratio 22:6n-3/20:4n-6	3.24	20.00

[a]wt% of total fatty acids (means ± SE).
[b]Not shown are 16:1n-9, 17:0, 17:1n-7, 20:2n-6, and 20:3n-6, which were present in yolk and adipose tissue at less than 0.5%.
[c]Identification provisional.
[d]Also includes some 20:1n-11 and 20:1n-7 in adipose samples.
[e]Trace, less than 0.1%. Comparison with yolk values: $*P < 0.02$; $**P < 0.001$.

Lipid and PL classes of the yolk. TAG was by far the major lipid class present in the yolk (67.0 ± 0.2 wt% of total yolk lipid; mean ± SE, n = 6 yolks) with PL also making a substantial contribution (25.4 ± 0.3 wt%). Much lower proportions of FC, CE, and FFA were present (5.3 ± 0.2, 1.8 ± 0.2, and 0.5 ± 0.0 wt%, respectively). The proportions of these lipid classes showed little variation among the six replicate eggs that were analyzed. The very low and constant proportion of FFA suggests that little or no hydrolysis of complex lipids had occurred in the eggs prior to collection or in the lipid extracts prior to analysis. PC (45.9 ± 8.0 wt% of total PL; mean ± SE, n = 6 yolks) and PE (34.3 ± 4.8 wt%) were the main constituents of the total PL although relatively high proportions of PS and Sph were also present (9.3 ± 1.8 and 10.5 ± 1.8 wt%, respectively). However, there was a considerable degree of inter-egg variation in the proportions of these PL classes.

Fatty acid composition of the major lipid and phospholipid classes of the yolk. The two major fatty acyl constituents of the TAG, PL, CE, and FFA were 18:1n-9 and 16:0 (Table 2). A key feature was the presence of high proportions of 22:6n-3 in all of these classes. Although PL displayed the highest proportions of 22:6n-3, a substantial level of this polyunsaturate was also present in the TAG fraction. The minor lipids, CE and FFA, also contained high proportions of 22:6n-3. The four lipid classes contained 20:5n-3 at lower levels than 22:6n-3. The highest proportion of 20:4n-6 was found in the PL fraction. The C_{18} polyunsaturate, 18:3n-3, was a very minor component of all the lipid classes.

Very high proportions of 22:6n-3 were present in the PE and PS fractions of the yolk PL, forming the major acyl component of these two fractions (Table 3). PE and PS were also rich in 20:4n-6, 20:5n-3, and 22:5n-3, whereas lower proportions of these C_{20-22} polyunsaturates were found in PC and Sph. With regard to the saturated and monounsaturated fatty acids, 16:0 and 18:1n-9 were the major components of PC whereas 18:0 predominated in PE and PS. Sph consisted mainly of saturated and monounsaturated fatty acids although 22:6n-3 also made a significant contribution.

Vitamin E and carotenoids of the yolk. There was considerable inter-egg variation in the yolk content of vitamin E and carotenoids. The major form of vitamin E in the yolk was α-tocopherol (206.9 ± 46.6 μg/g lipids mean ± SE, n = 6 yolks)

TABLE 2
Fatty Acid Composition of the Yolk Lipid Classes[a]

Fatty acid	TAG	PL	CE	FFA
14:0	1.6 ± 0.2	0.9 ± 0.1	0.7 ± 0.1	Trace
16:0	24.0 ± 0.3	25.1 ± 0.4	10.8 ± 0.8	14.1 ± 0.6
16:1n-7	6.1 ± 0.2	1.7 ± 0.1	3.2 ± 0.2	2.0 ± 0.2
18:0	8.7 ± 0.5	15.5 ± 0.4	4.2 ± 0.4	19.3 ± 0.6
18:1n-9	38.7 ± 1.3	28.8 ± 0.7	53.3 ± 1.6	29.8 ± 1.1
18:1n-7	7.3 ± 0.4	2.8 ± 0.2	4.8 ± 0.2	5.2 ± 0.3
18:1n-5	0.6 ± 0.0	0.2 ± 0.0	Trace	Trace
18:2n-6	3.7 ± 0.2	1.8 ± 0.1	4.4 ± 0.2	3.0 ± 0.1
18:3n-3	0.6 ± 0.1	Trace	Trace	1.0 ± 0.2
18:4n-3	0.2 ± 0.0	Trace	Trace	Trace
20:1n-9	1.1 ± 0.4	0.2 ± 0.0	0.9 ± 0.1	4.9 ± 0.5
20:4n-6	0.4 ± 0.0	5.7 ± 0.3	2.0 ± 0.1	2.2 ± 0.1
20:5n-3	1.2 ± 0.1	2.8 ± 0.2	3.7 ± 0.4	3.3 ± 0.3
22:5n-3	0.8 ± 0.1	1.4 ± 0.1	Trace	1.4 ± 0.2
22:6n-3	3.6 ± 0.3	11.7 ± 0.5	9.1 ± 0.7	9.4 ± 0.5
Ratio n-3/n-6	1.56	2.12	2.00	2.90
Ratio 22:6n-3/20:4n-6	9.00	2.05	4.55	4.27

[a]wt% of fatty acids in the lipid class (means ± SE, *n* = 6 yolks). TAG, triacylglycerols; PL, phospholipids; CE, cholesteryl ester; FFA, free fatty acids; Trace, less than 0.1%.

TABLE 3
Fatty Acid Composition of the Phospholipid Classes[a]

Fatty acid	PC	PE	PS	Sph
14:0	1.3 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.8 ± 0.1
16:0	27.8 ± 0.3	11.4 ± 0.6	12.0 ± 0.5	28.6 ± 0.8
16:1n-7	1.9 ± 0.1	0.7 ± 0.1	0.9 ± 0.1	1.2 ± 0.1
18:0	14.3 ± 0.4	26.3 ± 1.5	18.9 ± 0.8	20.3 ± 0.5
18:1n-9	30.7 ± 0.8	14.0 ± 0.8	9.7 ± 0.4	29.4 ± 1.2
18:1n-7	3.0 ± 0.1	1.4 ± 0.1	1.5 ± 0.1	3.3 ± 0.1
18:1n-5	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
18:2n-6	1.9 ± 0.1	1.2 ± 0.1	1.2 ± 0.2	1.4 ± 0.1
20:1n-9	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.1
20:4n-6	5.2 ± 0.3	9.7 ± 0.9	9.8 ± 0.6	2.3 ± 0.2
20:5n-3	2.4 ± 0.2	5.6 ± 0.4	4.6 ± 0.3	1.2 ± 0.2
22:5n-3	1.0 ± 0.1	3.8 ± 0.4	3.9 ± 0.4	0.8 ± 0.1
22:6n-3	8.8 ± 0.4	27.3 ± 0.8	34.6 ± 1.6	7.3 ± 0.6
Ratio n-3/n-6	1.72	3.37	3.92	2.51
Ratio 22:6n-3/20:4n-6	1.69	2.81	3.53	3.17

[a]wt% of fatty acids in the phospholipid class (means ± SE, *n* = 6 yolks). PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin.

with much smaller amounts of γ-tocopherol also present (11.3 ± 2.0 μg/g lipid). No tocotrienols were detected. The total carotenoid content was almost entirely accounted for by two compounds, lutein (3.9 ± 0.3 μg/g lipid; mean ± SE, *n* = 6 yolks) and an unidentified component (4.7 ± 0.6 μg/g lipid), with retention times of 2.3 and 17.8 min, respectively, at a flow rate of 1.5 mL/min. The unidentified carotenoid did not correspond to any of the standards used (see the Materials and Methods section), which all had retention times of less than 10 min.

DISCUSSION

The fatty acid composition of the subcutaneous adipose tissue of the female penguins at the time of arrival at the breeding colony is consistent with the prior consumption of a diet based on fish and other marine organisms during the prebreeding fattening period at sea (16). This is typified by the presence of high proportions of the long-chain n-3 polyunsaturates, 20:5n-3 and 22:6n-3, and of the long-chain monounsaturates, 20:1n-9, 22:1n-9 and 24:1n-9, in the adipose tissue lipids. The results from previous studies comparing the fatty acid composition in penguins of the adipose depots with that of the diet suggest that the stored fatty acids are mainly of dietary origin and are probably incorporated into adipose TAG without major transformation (26).

In the emperor penguin, the processes of yolk formation and the deposition of lipids in the developing oocyte are almost entirely restricted to the period following arrival at the breeding colony, a time of stringent fasting and extensive mo-

bilization of the maternal adipose tissue reserves (17,18,20). It is therefore very likely that fatty acids released from adipose tissue TAG are the predominant precursors for yolk lipid assembly. The yolk components of the avian egg are initially assembled in the maternal liver and then secreted into the bloodstream, mainly in the form of a specialized type of very low density lipoprotein (VLDL) which is then delivered *via* the circulation to the ovary and taken up by the oocyte (9). As a result, the lipid composition of the yolk is almost identical to that of the precursor VLDL and is therefore dependent on metabolic events in the maternal liver (9). It may be envisaged that, in the specialized circumstances encountered by the female emperor penguin, yolk lipid formation results from an interplay between the adipose tissue and liver, with the former tissue releasing large amounts of FFA into the circulation for delivery to the hepatocytes where the fatty acids are esterified to form TAG and PL which are incorporated into the yolk-precursor VLDL.

The fatty acid composition of the total lipid of the yolk shared certain general features with that of the maternal adipose tissue, as befits the idea of a precursor–product relationship. In particular, the proportions of $C_{20–22}$ n-3 polyunsaturated fatty acids in the yolk lipids of the emperor penguin were far higher than the values reported for the eggs of terrestrial birds (1,9). By contrast, the C_{18} polyunsaturates were very minor components of both the yolk and the adipose tissue of the penguin. This observation represents a major contrast with birds such as the chicken, duck, goose, and ostrich, where 18:2n-6 or 18:3n-3 are the major polyunsaturated fatty acids of yolk lipid as a result of maternal consumption of seeds (rich in 18:2n-6) or of green plant material (rich in 18:3n-3) (9,27).

There were, however, extensive differences, both qualitative and quantitative, between the fatty acid profiles of the yolk and the maternal adipose tissue of the emperor penguin. We suggest these differences arise from a combination of three factors. The first factor is that a proportion of the yolk fatty acids possibly may be derived from *de novo* lipogenesis in the liver, using glucose as the precursor. A previous study using radioactively labeled precursors indicated that the liver of the emperor penguin has the ability to synthesize fatty acids, mainly 16:0 and 18:0 and to a lesser extent 16:1n-7 and 18:1n-9 (17). This could partly explain the higher proportions of 16:0, 18:0, and 18:1n-9 in the yolk compared with the adipose lipids. On the other hand, the possibility that the liver could synthesize significant amounts of fatty acid from glucose during an extensive period of fasting is questionable and *de novo* lipogenesis may make only a minor contribution to the yolk fatty acid content.

The second factor that could affect the fatty acid profile of the yolk is the differential mobilization of the various fatty acids from the maternal adipose TAG. Studies using rat (28,29) and human (30) adipocytes incubated *in vitro* in the presence of lipolytic agonists have shown that certain fatty acids are preferentially released into the incubation medium whereas others are selectively retained as components of the TAG of the cells. These investigations established the general rule that the relative mobilization of a fatty acid was positively correlated with the number of double bonds and negatively correlated with the chain length (28). Thus, highly polyunsaturated C_{16}, C_{18}, and C_{20} fatty acids including 20:4n-6 and 20:5n-3 were preferentially mobilized whereas long-chain saturated and monounsaturated fatty acids such as 22:0, 20:1n-9, 22:1n-9 and 24:1n-9 displayed a high degree of resistance to mobilization. In the case of 22:6n-3, the promobilization effect of the six double bonds was effectively counterbalanced by the long chain length, resulting in an intermediate rate of release (28). These principles of differential mobilization were subsequently demonstrated to apply to mammals *in vivo* during fasting (31) and following hormonal stimulation of lipolysis (32). It was proposed that TAG molecular species that contain fatty acids such as 20:4n-6 and 20:5n-3 are excellent substrates for the hormone-sensitive lipase of the adipocytes, whereas those TAG species that contain fatty acids such as 24:1n-9 are very poor substrates for this enzyme (28). These differences in susceptibility to hydrolysis by hormone-sensitive lipase are not due to differences in the positional distribution of highly and weakly mobilized fatty acids on the glycerol backbone of the TAG (33). Rather, it seems that the more polar TAG species (i.e., those that contain the highly polyunsaturated shorter-chain fatty acids) partition to a greater extent at the lipid–water interface at the surface of the lipid droplet and are thus more accessible to the enzyme (28,34).

Preliminary results suggest that the emperor penguin is no exception to these features of differential mobilization of fatty acids (19). The fatty acid composition of subcutaneous adipose tissue of the male emperor changes markedly during the course of the breeding fast, consistent with the preferential release of 18:4n-3, 20:4n-6, and 20:5n-3 and the selective retention of long-chain monounsaturates. The *in vitro* release of fatty acids from adipose tissue slices also suggests that the relative mobilization of different fatty acids occurs in accordance with the same dependence on chain length and unsaturation exhibited by mammalian adipocytes (19). In the present work, the low relative mobilization of 20:1n-9, 22:1n-9, and 24:1n-9 from the maternal adipose tissue could at least partly explain the low levels of these monounsaturates in the lipids of the yolks. Also, the preferential mobilization of 20:4n-6 from the adipose tissue may contribute to the enhanced proportion of this fatty acid in the yolk lipid. However, several prominent features of the yolk fatty acid profile cannot be explained solely on the basis of the differential mobilization of fatty acids from the maternal fat stores. In particular, the proportion of 20:5n-3 in the yolk was about five times lower than in the adipose tissue despite the very high relative mobilization of this fatty acid.

A third determinant of the yolk fatty acid profile is the acyl-substrate specificity of the acyltransferases responsible for TAG and PL synthesis in the maternal liver. Presumably, such specificity would represent a mechanism to ensure that the fatty acid composition of the yolk is suited to the needs of

the embryo and is not simply a passive reflection of the pattern of fatty acids released from the maternal fat stores. In the case of the domestic chicken, feeding the laying hen with a fish oil-supplemented diet in which the ratio of 20:5n-3/22:6n-3 was 1.5 resulted in eggs in which this ratio was only 0.3 (4). Thus, there appears to be a relative discrimination against the incorporation of 20:5n-3 into egg lipids which could, in the case of the penguin, explain the low proportion of this fatty acid in the yolk compared with that in the maternal adipose tissue.

Species of birds that exhibit the precocial developmental mode produce well-developed hatchlings capable of independent feeding and thermoregulation whereas hatchlings of altricial species are relatively helpless and dependent on the parents for food provision and warmth (35). The proportions of the different lipid classes in the yolk also differ markedly between the two developmental modes. For example, the egg lipids of the chicken, a precocial species, consist of 70% TAG, 21% PL and 1% CE (3) whereas in an altricial species such as the pigeon these proportions are 58, 31, and 5%, respectively (36). The higher proportion of TAG in the eggs of precocial birds will provide the embryo with a greater amount of energy in order to achieve the more advanced state of development before hatching. Since, by most criteria, the hatchlings of the emperor penguin are altricial (for example, they depend on the parents for warmth for 2 mon posthatching), it may appear surprising that the lipid class profile of the eggs is typical of the precocial pattern. However, studies on eggs of the king penguin and the gentoo penguin have revealed that the changes in embryonic oxygen consumption during development follow the precocial rather than the altricial pattern (37). Thus, the developmental energy needs of these penguin embryos may require an energy-rich yolk with a high proportion of TAG.

As a proportion of total yolk PL, the penguin egg contained relatively less PC and relatively more PE and PS than the chicken egg (1). Because PE and PS are the main repositories for 22:6n-3 in eggs of both these avian species (1), the higher proportions of these two PL classes in the penguin egg may represent a means of facilitating the accommodation of the high levels of 22:6n-3 within the yolk lipid. It should also be noted that very high proportions of 22:6n-3 are present in the PE and PS fractions of the avian embryo brain (3). The large inter-egg variation in the proportions of the PL classes was surprising given that there was little inter-egg variation in the proportion of total PL in the yolk lipid. This degree of variation cannot be explained by the analytical methodology since replicate analyses ($n = 5$) of a single yolk sample indicated coefficients of variation of 4–5% in the estimation of the proportions of the PL classes. Future work, using measurements on a larger number of eggs, would help to resolve this point.

The very high proportion of 22:6n-3 in the yolk lipid of the emperor penguin in comparison with the levels of this polyunsaturate in the eggs of birds such as the chicken has already been commented upon. A further finding relates to the distribution of 22:6n-3 between the main acyl-containing lipid classes, TAG and PL. In the yolk of the domestic chicken, and of many other species of captive and wild birds that have been studied (3,9,27), 22:6n-3 is almost entirely confined to the PL fraction, with only trace levels present in TAG. The relative amount (mg of 22:6n-3 in PL per g total yolk lipid) of this n-3 polyunsaturate in the PL fraction is three times greater in the penguin yolk than in that of the chicken; however, an equivalent amount of 22:6n-3 was also present in the TAG fraction of the penguin yolk (Fig. 1). Thus, in the penguin eggs, 22:6n-3 is almost equally distributed between PL and TAG. This arises because, although the proportion (wt% of fatty acids) of 22:6n-3 is higher in PL than in TAG, the amount of TAG in the yolk exceeds that of PL. It may be suggested that, during the assembly of yolk lipid in the maternal liver, the supply of 22:6n-3 in the form of FFA mobilized from the maternal adipose tissue far exceeds the capacity for incorporation into PL, resulting in a "spillover" into TAG. In other words, there may be a limit on the proportion of this fatty acid that can be accommodated in PL, possibly to avoid compromising the role of PL in stabilizing the yolk–precursor VLDL structure (9).

It has been suggested that the commercial grain-based diets provided to herbivorous domestic or captive birds (e.g., duck, pheasant, goose, ostrich) may contain an imbalance in the proportions of n-6 vs. n-3 polyunsaturates with the result that the eggs and possibly the embryonic neural tissues may exhibit a degree of deficiency in 22:6n-3 (9). Clearly, the penguin embryo in the natural state is at no such risk. On the contrary, the penguin embryo may face the opposite problem: a relative limitation in the availability of 20:4n-6. The concentrations of 20:4n-6 in the PL and TAG of the penguin eggs are, however, very similar to those present in eggs of the chicken (Fig. 1). Possibly the preferential mobilization of 20:4n-6 from the maternal adipose tissue may help to achieve adequate levels of this fatty acid in the yolk lipid, although

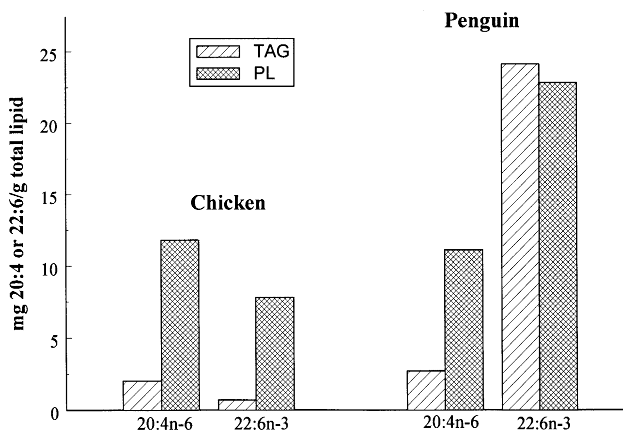

FIG. 1. Distribution of arachidonic (20:4n-6) and docosahexaenoic (22:6n-3) acids between triacylglycerol (TAG) and phospholipid (PL) of the yolk: comparison between chicken and penguin eggs. The mg of each acid present in each lipid class per g total yolk lipid is shown. Chicken data calculated from Reference 9. Penguin data calculated from the means in the Results section and in Table 2.

other mechanisms such as the acyl substrate specificity of acyltransferases in the maternal liver could also be involved.

It may be envisaged that the exceptional content of highly polyunsaturated n-3 fatty acids in the penguin eggs will require a considerable degree of antioxidant protection in order to prevent peroxidative damage during the development of the embryo (24). Thus, the concentrations of two major types of lipid-soluble antioxidants, vitamin E and carotenoids, were determined. The concentration of total vitamin E in the penguin yolks was very similar to that measured in free-range chicken eggs, which were found to contain 200–300 µg vitamin E/g lipid (Surai, P., unpublished data) and in eggs of the feral Canada goose, which contained 180–280 µg vitamin E/g lipid (38). It has been estimated that the amount of vitamin E in the chicken's egg is twice the steady-state level of this vitamin in the maternal liver, emphasizing the importance of continuous dietary provision to support egg production (39). In the emperor penguin, the fact that the yolk contents are deposited in the oocytes during a period of fasting may therefore place limitations on the amount of vitamin E that can be accumulated in the yolk. The concentration of carotenoids in the penguin yolks was very low, approximately a tenth of the concentration measured in the free-range chicken egg (Surai, P., unpublished data) and in eggs of the feral Canada goose (38). The unidentified carotenoid peak would appear from its retention time to be a very hydrophobic molecule and is presumably derived ultimately from phytoplankton at the base of the penguins' food chain. Vitamin E and carotenoids, together with the trace element selenium, are the main antioxidant components present in the avian yolk (23,24). It would appear that the yolk of the emperor penguin is only moderately provisioned with antioxidants, despite the very high levels of polyunsaturated fatty acids.

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Fatty Acid Composition of 19 Species of Fish from the Black Sea and the Marmara Sea

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ABSTRACT: Evidence suggests that differences in fatty acid composition among various fish species may be due to differences in diet or to environmental factors such as temperature, salinity, and depth at which the fish are caught. The beneficial effects of a diet containing fish on cardiovascular or other diseases have been associated with their high content of eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids. In this study we analyzed the fatty acid composition of the flesh of 18 different species of marine fish and of cultured rainbow trout. The fish were obtained from the Black and the Marmara Seas, both of which have unique biological and ecological systems as well as eutrophication and pollution. The contents of 20:5n-3 and 22:6n-3 in the marine fish ranged from 4.2 to 13.3 wt% of total fatty acids, and from 6.6 to 40.8 wt%, respectively. The most important differences from other studies on oceanic fish were the tendencies toward higher percentages of 16:0 and 22:6n-3. The n-3 series of polyunsaturated fatty acids were present as 32.4 ± 1.9% of the total fatty acids. The present study suggests that mature and immature *Pomatomus saltator* as well as *Engraulis encrasicolus, Mullus surmuletus, Sardina pilchardus, Mugil cephalus,* and *Sarda sarda* may be preferred for the Turkish diet as a result of their high 20:5 n-3 and 22:6 n-3 contents. The cultured rainbow trout *Oncorhynchus mykiss* is not as good a source of n-3 fatty acids as are the marine fish.

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Consumption of fish oil has beneficial effects on growth and development, coronary heart disease, blood lipids and lipoprotein levels, platelet aggregability, hypertension, inflammation, Crohn's disease, arthritis, autoimmune disorders, and cancer (1–10). Consumption of modest amounts of n-3 fatty acids (FA), as little as one meal of fatty fish per week, is associated with reduced incidence of fatal coronary heart disease and decreased vulnerability to ventricular fibrillation, perhaps because the fish provide an essential amount of long-chain n-3 polyunsaturated fatty acids (PUFA), or some still-unidentified nutrient (11–14). The evidence of reduced incidence of cardiovascular disease in Eskimos, a population with a high dietary intake of n-3 FA derived from fish, suggests that dietary supplementation with n-3 FA may be beneficial in the reduction of cardiovascular risk (15).

Proximate fat composition of fish varies considerably from species to species and also for individual fish or lots of fish of the same species taken at different times or under different conditions. The FA composition of fish lipids, especially PUFA, varies according to the FA profile of the lipids consumed by the fish. The FA composition also varies in response to water temperature changes, salinity, and depth through adaptive mechanisms. Most studies related to the proximate composition of fish have been carried out on fish obtained from oceanic waters. There is little information on the FA composition of fish species commonly consumed in Turkey. As a basis for possible nutritional and health benefits, we have determined the FA composition of some fish commonly available for consumption.

MATERIALS AND METHODS

All the fish, which were caught from the Black Sea and the Marmara Sea, were collected from a local fish market between September and November, 1994, when the fat content of wild fish tended to be at its maximum. The mean total weight and standard length showed that the fish were mature at the time of capture (Table 1). Only *Pomatomus saltator* was immature and did not have its maximal fat content at that time. Rainbow trout was purchased from a trout farm.

The freshly caught fish were scaled, and 2–3 g samples were taken from the edible portion of the dorsal muscle between the dorsal fin and the head. Sampling was always done by one of the authors (E. Şener) from the Faculty of Aquatic Products. The number of fish examined is included in Table 1. Tissue samples were stored at −70°C until analyzed.

FA analysis. Tissue FA profiles were prepared by a slight modification of a method previously described by Yazıcı *et al.* (16). The frozen tissue sample was thawed, weighed (200–300 mg taken from the whole sample), and homogenized in cold 154 mM NaCl. Total lipids, added internal standard (heptadecanoic acid), and butylated hydroxytoluene (an antioxidant, 20 gr L^{-1}) were extracted with chloroform/methanol (2:1). The extracted lipids were then saponified with 2% KOH in

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; PUFA, polyunsaturated fatty acids.

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TABLE 1
Biometric and Biologic Characteristics of Fish Species from the Black Sea and the Marmara Sea

Fish species[a] (*n*)	Mean total weight (g)	Mean standard length (cm)	Reproduction	Where captured	Habitat		Food habits		
					Pelagic	Demersal	Planktivorous	Omnivorous	Carnivorous
Engraulis encrasicolus (12)	14.1 ± 0.4	12.6 ± 0.2	Summer–autumn	BS	x		x		
Pomatomus saltator (young) (11)	68 ± 3.0	19.4 ± 0.3		MS					x
P. saltator (mature) (2)	266 ± 4.0	31.5 ± 2.3	Spring–summer	MS					
Mullus surmuletus (10)	26.5 ± 3.5	13.3 ± 0.9	April–July	BS		x			x
Sardina pilchardus (10)	20.5 ± 0.7	14.8 ± 0.2	June–August	MS	x		x		
Scomber scrombrus (6)	113.5 ± 5.0	24.7 ± 0.2	Winter–spring	MS	x				x
Pagellus erythrinus (5)	86.1 ± 4.9	17.4 ± 1.1	Spring–autumn	MS		x		x	
Atherina boyeri (4)	11.3 ± 0.2	12.7 ± 0.3	April–July	MS	x				x
Belone belone (4)	225 ± 46	56.4 ± 4.2	April to Sept.	BS	x				x
Solea vulgaris (3)	108 ± 25	23.9 ± 2.2	Jan.–Feb.	MS		x			x
Mugil cephalus (3)	254.3 ± 13.8	30 ± 0.3	July–October	BS		x		x	
Serranus cabrilla (2)	40 ± 1.0	15.3 ± 1.0	April–July	MS		x			x
Merlanguis merlangus euxinus (2)	88 ± 40	22 ± 3.3	Jan.–spring (summer in BS)	BS		x			x
Neogobius melanostomus (2)	65 ± 0.0	19.9 ± 0.4	May–June	MS		x			x
Trachurus trachurus (2)	91 ± 4.6	22.6 ± 0.4	Jan.–April	MS	x				x
Scorpaena scrofa	125 ± 21	18.3 ± 1.3	May–August	MS		x			x
Trigla lucerna (1)	57.9	20	Dec.–April	MS		x			x
Scomber japonicus (1)	260	27	June–Aug.	MS	x		x		
Sarda sarda	571	38	May–July	MS	x				x
Onchorrhynchus mykiss (2)	267.5 ± 7.5	29.5 ± 0.0	Sept.–Oct.	F	x				x

[a]*n*, number of specimens analyzed. BS, Black Sea; MS, Marmara Sea; F, freshwater. Weight and length data presented as mean ± SEM.

methanol, and finally the FA were methylated with 20% BF_3 in methanol. The FA methyl esters were extracted with hexane and analyzed by capillary gas chromatography (column: 50 × 0.25 mm WCOT fused silica, CP-Sil 88; flame-ionization detection temperature 300°C; carrier gas, N_2, 16 mL min^{-1}; splitter injector temperature 290°C; oven temperature programmed from 150 to 240°C at 2°C min^{-1}); Perkin-Elmer 8420 capillary gas chromatograph (Gouda, The Netherlands).

A mixture of FA standards (Sigma Chemical Co., St. Louis, MO), containing the following methyl esters of all-*cis* or of saturated FA, was used to determine the total FA profiles: lauric, 12:0; myristic, 14:0; palmitic, 16:0; palmitoleic, 16:1n-7; stearic, 18:0; oleic, 18:1n-9; *cis*-vaccenic, 18:1n-7; linoleic, 18:2; linolenic, 18:3; 6,9,12,15-octadecatetraenoic, 18:4; arachidic, 20:0; eicosenoic, 20:1n-9; 1,14-eicosadienoic, 20:2; 11,14,17-eicosatrienoic, 20:3; arachidonic, 20:4n-6; 5,8,11,14,17-eicosapentaenoic (EPA), 20:5; behenic, 22:0; erucic, 22:1n-9; 13,16-docosadienoic, 22:2; 7,10,13,16-docosatetraenoic, 22:4; 7,10,13,16,19-docosapentaenoic, 22:5; 4,7,10,13,16,19-docosahexaenoic (OHA), 22:6; lignoceric, 24:0; and nervonic, 24:1n-9.

The values of FA are presented as percentage of total FA mass.

Data are presented as mean ± SEM, unless otherwise stated. Analysis of variance test was used to determine the significance of levels of FA in various fish species. The Student-Newman-Keuls test was used to compare FA of each fish with the FA of other fish species.

RESULTS

The total lipid contents of the edible muscle in different species of fish were compared. When the fish were grouped by fat levels, the high-fat (>8%) species were blue fish (*Pomatomus saltator,* mature and immature), red mullet (*Mullus surmuletus*), sardine (*Sardina pilchardus)*, striped mullet (*Mugil cephalus),* horse mackerel (*Trachurus trachurus),* and bonito (*Sarda sarda)* (17). The fish with medium fat content (4–8%) were anchovy (*Engraulis encrasicolus),* Atlantic mackerel (*Scomber scombrus), garfish (Belone belone),* common sole (*Solea vulgaris),* comber (*Serranus cabrilla),* Spanish mackerel (*Scomber japonicus),* and rainbow trout (*Oncorhynchus mykiss).* On the other hand, sea bream (*Pagellus erythrinus),* sand smelt (*Atherina boyeri),* whiting (*Merlangius merlangus euxinus),* scorpion fish (*Scorpaena scrofa)*, gurnard (*Trigla lucerna*), and round goby (*Neogobius melanostomus)* could be classified as low-fat (2–4%) fish.

Analysis of the compositions of FA from different species of fish revealed significant differences (Tables 2 and 3). The most abundant individual FA in our study were palmitic, oleic, eicosapentaenoic, and docosahexaenoic acid. Palmitic and stearic acids were the most abundant saturated FA, and oleic acid was the dominant monounsaturate. Of the PUFA EPA and DHA were the major n-3 FA.

There was some variation in the FA composition of different fish species. The levels of each FA in individual fish that were significantly different compared to those of other fish

TABLE 2
Fatty Acid Composition of Muscle Lipids of the Fish^{a} from the Black Sea and the Marmara Sea

Fatty acid	*Engraulis encrasicolus* (*n* = 12) BS	*Mullus surmuletus* (*n* = 10) BS	*Belone belone* (*n* = 4) BS	*Mugil cephalus* (*n* = 3) BS	*Pomatomus saltator* (young) (*n* = 11) MS	*Sardina pilchardus* (*n* = 10) MS	*Scomber scombrus* (*n* = 6) MS	*Pagelius erythrinus* (*n* = 5) MS	*Atherina boyeri* (*n* = 4) MS	*Solea vulgaris* (*n* = 3) MS
14:0	7.1 ± 0.3^{a}	3.2 ± 0.1^{b}	3.2 ± 0.6^{b}	5.3 ± 0.7^{a}	6.2 ± 0.1^{a}	7.4 ± 0.7^{a}	2.1 ± 0.4^{b}	1.8 ± 0.3^{b}	2.6 ± 0.3^{b}	5.6 ± 1.2^{a}
16:0	29.0 ± 1.1^{a}	25.2 ± 0.3^{a}	26.7 ± 1.2^{a,b}	25.9 ± 0.8	23.9 ± 0.2^{b}	28.2 ± 0.9^{a}	27.7 ± 1.5^{a,b}	31.6 ± 2.8^{a}	27.1 ± 3.2^{a,b}	23.3 ± 0.6^{b}
16:1n-7	5.9 ± 0.2^{a}	7.8 ± 0.3^{c}	6.0 ± 0.8^{a}	10.0 ± 0.4^{d}	6.9 ± 0.2^{a}	6.1 ± 0.4^{a}	4.3 ± 1.0^{b}	4.1 ± 0.6^{b}	2.9 ± 0.4^{b}	8.7 ± 1.7^{c,d}
18:0	4.8 ± 0.2^{a,e}	5.3 ± 0.2^{a,e}	4.8 ± 0.3^{a,e}	4.2 ± 0.6^{a,b}	3.5 ± 0.1^{b}	5.2 ± 0.2^{a,e}	6.7 ± 0.2^{c}	7.8 ± 0.3^{d}	7.0 ± 0.8^{c,d}	6.0 ± 0.3^{c,e}
18:1n-9	10.7 ± 0.9^{a}	11.8 ± 0.4^{a}	11.0 ± 1.4^{a,c}	10.9 ± 0.6^{a,c}	21.3 ± 0.7^{b}	12.6 ± 0.8^{a}	12.2 ± 1.9^{a}	7.3 ± 1.2^{c,d}	4.6 ± 0.3^{d}	9.9 ± 0.1^{a,c}
18:1n-7	4.0 ± 0.2^{a,c}	4.7 ± 0.3^{c}	2.6 ± 0.1^{b,d}	3.6 ± 0.6^{a,b,c,d}	2.0 ± 0.3^{b}	3.1 ± 0.2^{a,d}	4.2 ± 0.6^{a,c}	2.6 ± 0.4^{b,d}	2.3 ± 0.3^{b,d}	3.23 ± 0.6^{a,b,c,d}
18:2n-6	2.8 ± 0.3^{a}	1.8 ± 0.2^{b}	2.3 ± 0.3^{a,b}	2.7 ± 0.7^{a,b}	2.3 ± 0.0^{a,b}	2.2 ± 0.1^{a,b}	2.1 ± 0.2^{a,b}	1.6 ± 0.0^{a,b}	4.2 ± 0.3c	2.2 ± 0.2^{a,b}
18:3n-3	1.4 ± 0.1^{a}	1.1 ± 0.1^{b}	N.D.	0.73 ± 0.0^{c}	N.D.	1.4 ± 0.1^{a}	0.68 ± 0.1^{c}	0.52 ± 0.0^{c}	N.D.	N.D.
18:4n-3	1.5 ± 0.1^{a}	0.64 ± 0.1^{c}	0.72 ± 0.2^{c,d}	1.0 ± 0.6^{a,c,d}	1.96 ± 0.1^{b}	1.61 ± 0.1^{a,b}	0.45 ± 0.1^{c}	0.30 ± 0.0^{c}	1.0 ± 0.1^{a,c}	1.5 ± 0.7^{a,b,d}
20:0	1.1 ± 0.1^{a}	0.46 ± 0.0^{b}	N.D.	0.37 ± 0.1^{b}	0.60 ± 0.0^{b}	1.2 ± 0.1^{a}	0.47 ± 0.0^{b}	0.44 ± 0.0^{b}	0.65 ± 0.0^{b}	0.67 ± 0.1^{b}
20:1n-9	1.2 ± 0.2^{a,c}	1.2 ± 0.1^{a,c}	5.6 ± 1.8^{b}	0.77 ± 0.4^{a,c}	6.5 ± 0.3^{b}	2.6 ± 0.2^{c}	1.8 ± 0.5^{a,c}	0.74 ± 0.1^{a,c}	2.5 ± 0.5^{a,c}	1.6 ± 0.1^{a,c}
20:2n-6	0.40 ± 0.1^{a}	0.68 ± 0.1^{a}	0.82 ± 0.1^{a}	1.7 ± 0.7^{b}	0.46 ± 0.0^{a}	0.33 ± 0.1^{a}	0.32 ± 0.1^{a}	0.44 ± 0.1^{a}	0.30 ± 0.0^{a}	N.D.
20:4n-6 + 22:1	2.4 ± 0.1^{a,d}	4.6 ± 0.2^{c}	4.6 ± 0.1^{a,b,d}	3.1 ± 0.4^{a,c}	6.1 ± 0.5^{b,d}	2.6 ± 0.2^{b,d}	2.8 ± 0.3^{a}	7.9 ± 0.1	4.3 ± 0.1^{c}	5.4 ± 0.7^{c}
20:5n-3	9.3 ± 0.5^{a}	10.8 ± 0.4^{c}	4.3 ± 0.7^{b,e}	13.3 ± 0.4^{f}	4.2 ± 0.2^{b}	8.6 ± 0.3^{a,d}	5.9 ± 0.7^{e}	6.8 ± 0.9^{d,e}	12.8 ± 0.8^{f}	6.8 ± 0.5^{d,e}
22:0	0.23 ± 0.0	0.35 ± 0.1	N.D.	0.17 ± 0.1	0.23 ± 0.1	0.19 ± 0.0	0.22 ± 0.0	0.18 ± 0.0	N.D.	N.D.
22:4n-6	0.64 ± 0.1^{a}	0.77 ± 0.1^{a}	0.73 ± 0.1^{a,b}	1.1 ± 0.3^{a}	0.30 ± 0.0^{b}	0.59 ± 0.1^{a}	0.87 ± 0.1^{a}	2.0 ± 0.2	1.1 ± 0.1^{a}	1.5 ± 0.2
22:5n-3	0.76 ± 0.1^{a}	1.7 ± 0.1^{a,b}	2.1 ± 0.3^{a,b}	3.2 ± 0.3^{b}	1.5 ± 0.7^{a,b}	0.99 ± 0.1^{a}	1.0 ± 0.2^{a}	2.1 ± 0.3^{a,b}	1.4 ± 0.1^{a,b}	6.3 ± 0.2
22:6n-3	16.2 ± 1.8^{a,b,c}	17.3 ± 0.7^{a,b,c}	24.3 ± 5.3^{a,c}	11.7 ± 1.1^{a,b,c}	12.1 ± 0.5^{b}	14.7 ± 1.5^{a,b}	25.3 ± 4.5^{c}	21.5 ± 3.6^{a,b,c}	24.8 ± 3.9^{a,c}	16.4 ± 2.4^{a,b,c}
24:1n-9	0.68 ± 0.1^{a}	0.54 ± 0.1^{a}	0.6 ± 0.1^{a}	0.40 ± 0.0^{a}	1.2 ± 0.1^{b}	-N.D.	0.87 ± 0.2^{a,b}	0.60 ± 0.1^{a}	0.75 ± 0.2^{a,b}	1.1 ± 0.3^{a,b}
n-3	29.1 ± 2.2^{a}	31.6 ± 0.1^{a}	31.3 ± 5.9^{a}	29.9 ± 1^{a,b}	19.0 ± 0.8^{b}	27.3 ± 1.5^{a}	33.3 ± 4.7^{a}	31.2 ± 4.5^{a}	39.9 ± 4.6^{a}	31.0 ± 1.9^{a,b}
n-6	5.49 ± 0.4^{a}	7.3 ± 0.3^{c}	5.4 ± 0.2^{a,b}	8.4 ± 1.4^{d}	3.5 ± 0.1^{b,d}	4.4 ± 0.4^{a,b}	5.7 ± 0.3^{a}	11.5 ± 1	9.4 ± 0.3^{d}	8.6 ± 0.8^{d}
Saturated	42.2 ± 1.4^{a}	34.6 ± 0.4^{b}	34.7 ± 1.5^{b,c}	35.9 ± 0.9	34.4 ± 0.3^{b}	42.0 ± 1.3^{a}	37.2 ± 1.7^{a,b}	41.7 ± 3.2^{a,c}	37.3 ± 3.9^{a,b}	35.4 ± 0.5^{a,b}
Unsaturated	57.8 ± 1.4^{a}	65.5 ± 0.4^{b}	65.4 ± 1.5^{b,c}	64.1 ± 0.9^{a,b}	65.7 ± 0.3^{b}	58.0 ± 1.4^{a,c}	63.5 ± 2.1^{a,b}	58.3 ± 3.2^{a}	62.7 ± 3.9^{a,b}	64.6 ± 0.5^{a,b}
Sat./unsat.	0.74 ± 0.0^{a}	0.53 ± 0.0^{b}	0.53 ± 0.0^{a,b}	0.56 ± 0.0^{a,b}	0.52 ± 0.0^{b}	0.73 ± 0.0^{a}	0.60 ± 0.1^{a,b}	0.75 ± 0.1^{a}	0.62 ± 0.1^{a,b}	0.55 ± 0.0^{a,b}
Total FA^{b}	7.1 ± 0.7^{a,c}	8.8 ± 0.7^{a,c}	4.2 ± 1.3^{a,c}	11.0 ± 2.0^{a,c}	42.9 ± 2.4^{b}	11.3 ± 0.8^{a}	6.3 ± 1.0^{a,c}	3.2 ± 0.3^{c}	3.8 ± 0.6^{c}	4.7 ± 1.0^{a,c}

[a]Figures are given as percentage of the total amount of fatty acids (FA). Data presented as mean ± SEM. Values across rows not sharing a common superscript letter are significantly different from each other, $P < 0.05$. Sat./unsat., ratio of contents of saturated to unsaturated FA. N.D., not detected. For other abbreviations see Table 1.
[b]g/100 g tissue (wet weight basis).

species are given in Table 2. The content of EPA, a precursor of the third series of eicosanoids varied in marine fish between 4.2 and 13.3% (mean: 7.8 ± 0.6%), with the highest content of 20:5n-3 found in striped mullet. On the other hand, 20:5n-3 contents in mature and immature blue fish, garfish, and Atlantic mackerel were relatively low, but immature and mature blue fish had the highest calculated amount of EPA (1.8 g and 1.5 g/l00 g of fish muscle, respectively) among other fish. Whiting had the lowest saturated/unsaturated FA ratio. Whiting also had the highest percentage content of DHA; whereas, amounts of DHA were highest in the young of the blue fish (5.1 g /100 g). The following fish all had DHA levels >15% of total FA: comber, round goby, gurnard, Spanish mackerel, scorpion fish, Atlantic mackerel, sea bream, common sole, anchovy, red mullet, sand smelt, garfish, and rainbow trout. The highest percentage of myristic acid was found in sardine and immature and mature blue fish as well as anchovy. Palmitic acid content was very high in all the fish examined and was highest in sea bream. The stearic acid content was high in comber, round goby, horse mackerel, scorpion fish, Spanish mackerel, red mullet, sardine, Atlantic mackerel, sea bream, sand smelt, and common sole. Horse mackerel, blue fish, red mullet, and common sole had high 16:1n-7 percentage levels, and horse mackerel had the highest 18:1n-9 percentage content. With regard to the ratio of saturated to unsaturated FA content, the highest values were found in anchovy, sea bream, and sardine. The percentage contents of 18:0 and 24:1n-9 were significantly higher in fish caught from the Marmara Sea as compared to those from the Black Sea (6.8 ± 0.5 vs. 4.6 ± 0.1%, $P = 0.002$; 0.9 ± 0.1 vs. 0.66 ± 0.1%, $P = 0.05$, respectively). When pelagic fish species caught from the Black Sea were compared with pelagic species from the Marmara Sea, none of the FA differed significantly. However, when demersal fish from either sea were compared, the percentage content of 20:5n-3 and the calculated amount of 22:6n-3 were higher in fish from the Black Sea (11.2 ± 1.9 vs. 7.5 ± 2%, $P = 0.05$, and 1.26 ± 0.1 vs. 0.74 ± 0.08 g, $P = 0.02$, respectively). On the other hand, 18:0 and 24:1n-9 were higher in demersal fish species caught from the Marmara Sea (6.8 ± 1 vs. 4.8 ± 0.3%, $P = 0.006$; 0.87 ± 0.10 vs. 0.49 ± 0.0%, $P = 0.03$). Demersal fish species from the Black Sea had higher percentages of 20:5n-3 than pelagic species from the Marmara Sea (11.2 ± 1.1 vs. 7.4 ± 1.1%, $P = 0.05$).

Fish classified as carnivorous had significantly higher proportions of 20:0, 20:1n-9, and 24:1n-9 than omnivorous fish (0.5 ± 0.0 vs. 0.4 ± 0.0%, $P = 0.03$; 2.8 ± 1.8 vs. 0.7 ± 0.0%, $P = 0.001$; 0.9 ± 0.3 vs. 0.5 ± 0.1%, $P = 0.04$, respectively). Percentages of 18:3n-3 and 20:0 were lower in omnivorous than in planktivorous fish (0.6 ± 0.1 vs. 1.5 ± 0.1%, $P = 0.01$; 0.4 ± 0.0 vs. 1.1 ± 0.0%, $P = 0.001$, respectively).

TABLE 3
Fatty Acid Composition of the Flesh Lipid of the Fish[a] from the Black Sea, the Marmara Sea, and Freshwater

Fatty acid	*Merangius merlangus euxinus*[b] (BS)	*Serranus cabrilla*[b] (MS)	*Neogobius melanostomus*[b] (MS)	*Trachurus trachurus*[b] (MS)	*Pomatomus saltator*[b] (mature) (MS)	*Scorpaena scrofa*[b] (MS)	*Trigla lucema*[c] (MS)	*Scomber japonicus*[c] (MS)	*Sarda sarda*[c] (MS)	*Oncorhynchus mykiss*[b] (freshwater)
14:0	1.2 ± 0.1	3.1 ± 0.10	1.3 ± 0.2	2.7 ± 0.4	5.4 ± 0.3	0.90 ± 0.1	0.50	1.8	5.9	2.0 ± 0.0
16:0	19.5 ± 0.4	23.5 ± 0.5	22.2 ± 1.1	24.5 ± 1.2	25.1 ± 0.1	23.1 ± 0.7	21.5	22.1	23.3	23.2 ± 0.8
16:1n-7	2.2 ± 0.4	5.5 ± 1.2	3.2 ± 0.4	7.0 ± 1.3	5.9 ± 0.5	3.2 ± 0.3	2.1	2.4	5.6	4.0 ± 0.0
18:0	4.9 ± 0.3	6.0 ± 0.1	6.5 ± 0.1	10.2 ± 0.2	4.1 ± 0.5	8.5 ± 0.0	6.4	10.3	4.9	4.5 ± 0.3
18:1n-9	8.3 ± 0.0	9.9 ± 0.9	8.2 ± 0.2	26.1 ± 0.9	21.0 ± 1.4	8.7 ± 0.5	8.5	14.7	13.9	13.2 ± 0.7
18:1n-7	3.3 ± 0.4	3.0 ± 0.3	3.4 ± 0.1	3.1 ± 0.2	2.2 ± 0.2	2.3 ± 0.5	3.0	4.3	2.2	2.0 ± 0.4
18:2n-6	1.8 ± 0.1	1.7 ± 0.0	2.2 ± 0.1	1.1 ± 0.0	2.2 ± 0.0	2.4 ± 0.1	2.1	1.9	2.5	20.2 ± 0.5
18:3n-3	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	1.8	N.D.	0.75 ± 0.1
18:4n-3	0.25 ± 0.1	0.35 ± 0.1	1.1 ± 0.1	0.50 ± 0.1	2.1 ± 0.4	0.35 ± 0.1	1.2	0.6	1.8	0.35 ± 0.1
20:0	N.D.	0.50 ± 0.1	N.D.	0.30 ± 0.1	0.65 ± 0.1	N.D.	N.D.	1.1	0.8	0.60 ± 0.2
20:1n-9	2.1 ± 0.1	0.85 ± 0.1	1.8 ± 0.3	1.4 ± 0.3	5.7 ± 0.1	2.4 ± 0.8	1.9	3.8	3.9	1.8 ± 0.5
20:2n-6	0.45 ± 0.1	0.35 ± 0.2	N.D.	0.55 ± 0.3	0.50 ± 0.0	N.D.	N.D.	1.2	N.D.	1.6 ± 0.3
20:3n-3	N.D.	N.D.	N.D.	0.15 ± 0.1	N.D.	N.D.	N.D.	N.D.	N.D.	1.4 ± 0.2
20:4n-6 + 22:1	3.5 ± 0.5	6.0 ± 0.8	5.5 ± 1.4	1.4 ± 0.1	4.2 ± 0.2	10.3 ± 1.1	9.3	6.9	3.5	3.2 ± 0.2
20:5n-3	9.5 ± 0.6	8.3 ± 0.9	11.6 ± 0.6	8.4 ± 0.4	4.8 ± 0.4	6.9 ± 0.1	5.0	4.4	7.7	2.4 ± 0.0
22:0	N.D.	0.10 ± 0.0	N.D.	0.10 ± 0.0	0.20 ± 0.0	N.D.	N.D.	0.2	0.3	0.20 ± 0.0
22:4n-6	0.55 ± 0.3	1.3 ± 0.2	0.70 ± 0.0	0.60 ± 0.1	0.30 ± 0.1	0.70 ± 0.1	2.1	1.1	0.7	1.1 ± 0.0
22:5n-3	1.9 ± 0.3	2.2 ± 0.2	2.6 ± 0.0	3.9 ± 0.2	1.1 ± 0.1	2.2 ± 0.0	2.4	2.1	0.9	0.70 ± 0.0
22:6n-3	40.8 ± 0.1	27.0 ± 4.1	28.8 ± 2.7	6.6 ± 0.8	13.8 ± 1.4	26.3 ± 1.3	33.2	19.4	21.1	17.1 ± 1.5
24:1n-9	0.55 ± 0.1	0.80 ± 0.0	0.65 ± 0.2	1.8 ± 0.2	1.2 ± 0.2	1.4 ± 1	0.7	0.9	1.0	0.30 ± 0.0
n-3	52.4 ± 0.8	37.8 ± 3.3	44.0 ± 3.2	19.5 ± 0.5	21.6 ± 2.0	35.6 ± 1.2	41.8	28.3	31.5	22.9 ± 1.7
n-6	5.6 ± 0.4	9.0 ± 0.5	8.6 ± 1.4	3.4 ± 0.4	3.9 ± 0.0	14.1 ± 1.2	13.6	8.0	4.4	25.5 ± 0.3
Saturated	25.4 ± 0.2	33.1 ± 0.4	29.9 ± 1.2	37.8 ± 0.2	35.5 ± 0.3	32.5 ± 0.6	28.5	35.5	35.0	30.4 ± 0.7
Unsaturated	74.6 ± 0.2	66.9 ± 0.4	70.2 ± 1.2	62.2 ± 0.2	64.6 ± 0.3	67.5 ± 0.6	71.5	64.6	65.0	69.7 ± 0.7
Sat./unsat.	0.34 ± 0.0	0.50 ± 0.0	0.43 ± 0.0	0.61 ± 0.0	0.55 ± 0.0	0.48 ± 0.0	0.40	0.55	0.54	0.44 ± 0.0
Total FA[d]	2.7 ± 0.5	4.2 ± 0.1	2.7 ± 0.1	12.8 ± 2.0	31.3 ± 5.7	2.1 ± 0.0	2.05	6.3	19.0	6.7 ± 0.6

[a]Figures are given as percentage of the total amount of FA.
[b]*n* = 2.
[c]Single specimens. For abbreviations see Table 2.
[d]g/100 g tissue (wet weight basis).

DISCUSSION

The FA compositions of 18 commonly eaten fish species caught from the Black Sea and the Marmara Sea and of cultured rainbow trout (freshwater fish) were determined. In our study, marine fish, such as comber, whiting, round goby, horse mackerel, anchovy, red mullet, sardine, sand smelt, and striped mullet contained levels of 20:5n-3 at a mean of 7.8 ± 0.6% (range: 8.3–13.3%). Levels of 20:5n-3 were found to average 10.1% in marine fish by Ackman (18) , 5.3% by van Vliet and Katan (19), 8.07% by Castell (20), 4.3% by Belling *et al*. (21), and 11.7% in marine and freshwater fish by Puustinen *et al*. (22). Despite the presence of a relatively lower percentage of 20:5n-3 in our study, EPA was abundant in most of the fish, especially in immature and mature blue fish and in bonito *(Sarda sarda)*. Compared to other food, the lipids of fish are particularly rich in long-chain n-3 PUFA, e.g., EPA and DHA. Tissue FA of fish are derived from two sources, namely, the diet and *de novo* biosynthesis. Furthermore, fish can incorporate 18:2n-6 or 18:3n-3 acids, which may be in their diet, into PUFA, such as 20:4n-6, 20:5n-3, 22:5n-3 and 22:6n-3 (23). However, this ability differs from one species to another. The biosynthesis of 22:6n-3 and 22:5n-3 has recently been evaluated. Voss *et al*. (24) and Aveldaño *et al*. (25) showed that 24:5n-3, 24:6n-3, 24:4n-6, and 24:5n-6 are intermediates in the conversion of the C_{18} FA to 22:6n-3 and 22:5n-6 in the rat, which also may represent what occurs in fish.

Most of the fish analyzed in our study had high levels of 22:6n-3, averaging 21.1 ± 1.9% of the total FA. A survey of North European fish showed that several species also had high levels of 22:6n-3, for example, 11.2% in seven marine fish and 8.6% in six freshwater fish (20); 3.9% in Atlantic herring, 12.7% in Atlantic cod (18); 15% in wild trout, 4% in wild eel, 10% in wild salmon (19); and 15.6% in 11 species of Australian fish (21). Taking into consideration that the Turish fish examined in this study had low 20:1 contents and high DHA levels, one may speculate that the proportion of phospholipids was higher than that of triglycerides in muscle lipids of their fish (26).

The variation in FA composition of different fish species in our study may be explained by the unique features of the seas from which our fish were obtained. Studies concerning the composition of FA of fish living in these two seas are very few and limited. Among European semienclosed and coastal seas, the Black Sea is the most isolated from the deep oceans (Fig. 1). The biological consequence of the excess nutrient runoff from incoming rivers is the most intense eutrophication in the world, thus transforming the Black Sea ecosystem (27). The recent consequence of this phenomenon has been an increase of some small phytoplanktonic species, and of pi-

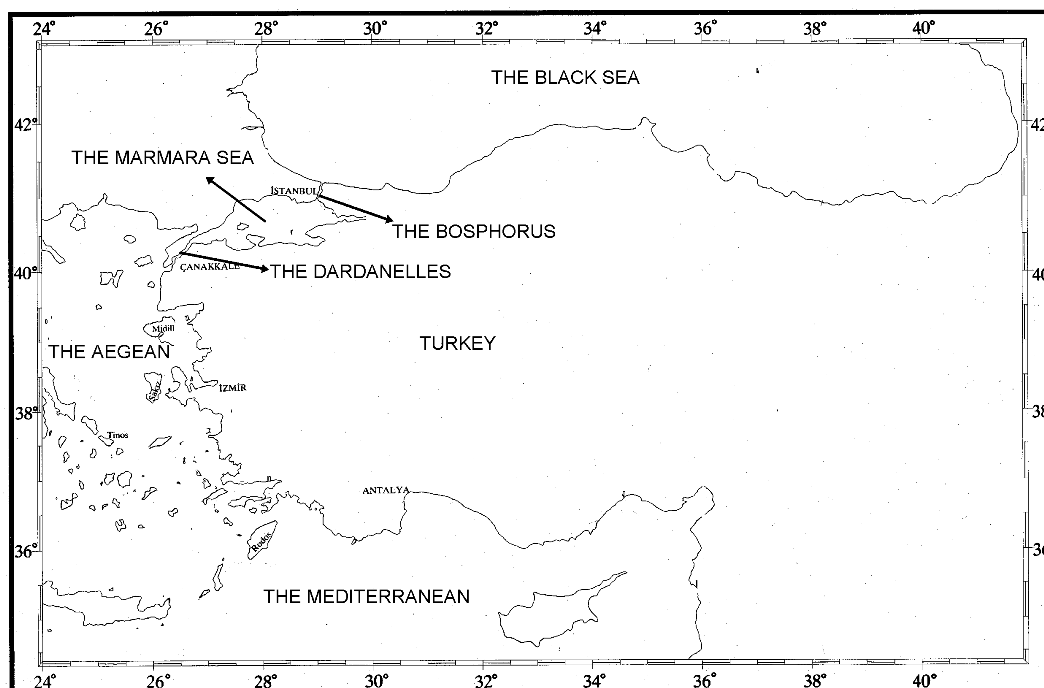

FIG. 1. Map of Turkey showing fishing areas discussed in text.

coplankton and zooplankton species. In addition, populations of some large planktonic species, especially *Phyllaphora* fauna, large pelagic carnivorous crustaceans, and mixotrophs have declined (28,29). Other important aspects of eutrophication have been the increase in water turbidity, which diminishes photosynthesis by bottom-dwelling algae, and the sedimentation of large amounts of dead and decomposing phytoplankton, causing hypoxia in the near-bottom layers of water and limiting the vertical range of different species of fish (30). In addition, a bloom or *Mnemiopsis leidyi* in the late 1980s reduced the biomass of copepods and other zooplankton almost tenfold, diminishing these important food species in the Black Sea (30). Because of the decline in populations of zooplankton and the increase of phytoplankton mass, especially of the dinoflagellates, as a response to the immense eutrophication in the Black Sea, the fish in our study (excluding *P. saltator*) had low levels of 20:1n-9 (mean, 2.5 ± 0.4% by wt) and high levels of n-3 PUFA (32.4 ± 1.9% of the total FA). This situation leads to FA compositions like those of marine fish found elsewhere in lower latitudes (31). Although the levels of 20:5n-3. In contrast, the low EPA to DHA ratio of 0.4 ± 0.07 was due to the higher 22:6n-3 content.

Another source of our marine fish was the Marmara Sea, which also has special biological and ecological features because of its enclosed configuration and estuarine characteristics. The importance of the Marmara Sea is emphasized by its location on the migratory path of large numbers of fish. While the surface waters of the Marmara Sea are affected by the Black Sea, deeper waters remain under the influence of the Mediterranean (32). In winter, nutrient-rich waters of the Black Sea enter the Marmara Sea from the surface, but in the spring and summer, the nutrient flow decreases. The temperature and the salinity in the Marmara Sea vary with depth and the distance from both seas and affect the phytoplankton biomass (33). The currents from the Black Sea bring in nutrients, leading to significant eutrophication that affects fish populations migrating into the system (34). Pelagic fish species enter the Marmara Sea during the spring for feeding, then migrate back to the Black Sea, then return to the Marmara Sea toward winter. The proportions of 18:0 and 24:1n-9 in total FA were significantly higher in those demersal and pelagic fish species caught from the Marmara Sea. Pelagic species of fish from either the Black Sea or the Marmara Sea had significantly higher percentage contents of 14:0, 18:1n-9, 20:0, 20:1n-9, saturated FA, total FA, and DHA compared to demersal species, whereas the proportions of 22:5n-3 and other minor unsaturated FA were considerably lower than those of demersal fish species. However, more importantly, there was no significant difference in the FA compositions between the pelagic species of fish either from the Black Sea or the Marmara Sea, which showed that the upper layer of the Marmara Sea was greatly influenced by the nutrients gained from the Black Sea. However, when demersal fish species from these seas were compared, 20:5n-3 as percentages of FA and the approximate amounts of 22:6n-3 (g/100 g) were significantly higher in fish from the Black Sea (11.2 vs. 7.5% and 1.2 vs. 0.7 g, respectively). Whiting, which belongs to the demersal group, is an exceptional fish since it usually spends the winter in the Black Sea although it may occasionally be caught in the Marmara Sea all year around. We found that this fish had the lowest percentage contents of 14:0, 16:0 (second-lowest), 16:1n-7, and 18:4n-3 FA and the highest percentages of

22:6n-3 and n-3 PUFA among the 18 fish species. These findings suggest that there is a need for further studies to determine how the ecosystem of the Black Sea affects the FA composition of fish that are endemic inhabitants to the Black Sea.

The mean level of 18:3n-3 was 1.09 ± 0.17% in Turkish marine fish, which, while low, was similar to other food fish such as Chinook salmon (1% by wt), mackerel (1.3% by wt), and wild salmon (1% by wt) (19,20). In our study, the contents of 18:2n-6 and 18:3n-3 FA in Turkish marine fish were low as expected. Although the levels of 20:4n-6 would be expected to be low, similar to 18:2n-6 and 18:3n-3 in marine fish, the levels of arachidonic acid were spuriously higher than expected, probably due to technical error. However, 22:1n-(13+11) isomers may be eluted in exactly the same position as 20:4n-6 on the capillary columns that were employed in our study (Ackman, R.G., personal communication). This source of error may be responsible for spuriously elevated levels of arachidonic acid, which are therefore not discussed. It should also be mentioned that palmitic acid levels, with a mean content of 24.9 ± 0.6%, was one of the highest among the FA examined in the present study, generally differing from other studies (17,18,22) although with some exceptions (20). Stearic acid content in marine fish in the present study was higher compared to North European fish species (mean: 6.1 vs. 3.1% by wt), possibly owing to moderately higher water temperatures. The levels of 16:1n-7 and 18:1n-9 in Turkish marine fish were not high, probably because they are confined to relatively shallow waters owing to bottom hypoxia or anoxia.

The only freshwater fish in our study was cultured rainbow trout. Although its saturated/unsaturated FA ratio was moderate, it had the lowest percentage of EPA and relatively low percentage content of DHA, but a very high percentage of 18:2n-6, which was up to almost 10 times that of marine fish.

Epidemiological data suggest that a decrease in coronary heart disease mortality occurs in people consuming relatively small amounts of fish and/or fish oils, as little as 0.4 g n-3 FA per day (14). The beneficial effect of a fish diet has been suggested to be mediated by the amount of EPA in fish (35,36). In this respect, the present study suggests that mature or immature blue fish, sardine, anchovy, horse mackerel, red mullet, sand smelt, striped mullet, and bonito may be preferred in the diet because of their relatively high EPA contents (>0.4 g/100 g) compared to other Turkish marine fish. Some studies have shown that an adequate DHA supply is essential for normal eye and brain development in infants and in children (37,38). In this regard, our study showed that DHA was present in measurable high amounts (>1 g/100 g) in blue fish, anchovy, red mullet, sardine, whiting, Atlantic mackerel, striped mullet, Spanish mackerel, bonito, garfish, comber, and rainbow trout.

In conclusion, there is a need for prospective epidemiological studies to determine whether the beneficial effects of fish consumption might be due to some specific nutrient or to the FA composition of each species of fish, before the reduction in cardiovascular diseases is attributed solely to high levels of EPA and DHA. In this respect, fish from Turkish waters, with their unique FA compositions, may be interesting to study.

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Rapid Complexing of Oxoacylglycerols with Amino Acids, Peptides and Aminophospholipids

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ABSTRACT: We prepared model Schiff bases from 2-[9-oxo]nonanoyl glycerol (2-MAG-ALD) and various amino compounds. 2-MAG-ALD was obtained by pancreatic lipase hydrolysis of trioleoyl glycerol and reductive ozonolysis of the resulting 2-monooleoyl glycerol. The reaction products were purified by thin-layer chromatography. Schiff bases were synthesized in greater than 50% yield by reacting 2-MAG-ALD with twofold molar excess of valine, *N*α-acetyl-L-lysine methyl ester and the tripeptides glycyl-glycyl-glycine, glycyl-glycyl-histidine, and glycyl-histidyl-lysine in aqueous methanol and with 1-palmitoyl-2-stearoyl glycerophosphoethanolamine (PE) in chloroform/methanol for 16 h at room temperature. Prior to analysis the bases were reduced with sodium cyanoborohydride in methanol for 30 min at 4°C. Reaction products were analyzed by high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS). Reduced Schiff bases of 2-MAG-ALD with PE and amino acids were analyzed by normal-phase HPLC/ESI/MS and those with peptides by reversed-phase HPLC/ESI/MS. Single adducts were obtained in all cases and both the α-amino group of valine and the ε-amino group of *N*α-acetyl-L-lysine methyl ester were reactive. Molecular ions of reaction products were the only detected ions in the negative ionization mode, whereas in the positive ion mode sodiated molecular ions were also detected. The present study suggests that 2-MAG-ALD may form Schiff base adducts with amino compounds in other aqueous media, such as the intestinal lumen and in the hydrophobic environment of cell membranes.

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Oxidation of unsaturated triacylglycerols leads to the formation of low- and high-molecular-weight aldehydes as secondary products (1). Low-molecular-weight aldehydes have been studied extensively whereas investigation of high-molecular weight aldehydes is fairly recent. It has been shown that α,β-unsaturated aldehydes originating from oxidation of polyunsaturated fatty acids are absorbed from the gut and excreted in urine as C-3 mercapturate conjugates in rats (2). Reactions of low-molecular-weight aldehydes with various amino compounds have been described, with the most reactive aldehydes being the bifunctional compounds, malonaldehyde and 4-hydroxynonenal. Malonaldehyde forms eneamines (3), *N*-substituted 3-iminopropenals (4), and *N*,*N*′-disubstituted 1-amino-3-iminopropenes (5) with amino acids and also reacts with amino groups of proteins (5,6) and deoxynucleosides (7) *in vitro* forming various adducts. A glycerophosphoethanolamine-malonaldehyde-glycerophosphoserine Schiff base conjugate was identified in rabbit lens oxidized *in vitro*, and similar reactions are believed to occur in human cataract (8). Schiff base formation between peptide- mimicking the hemoglobin β–chain N-terminus and acetaldehyde has been reported (9). Acetaldehyde is the major oxidation product of ethanol *in vivo,* and its adducts with hemoglobin can serve as markers of ethanol intake (10). High-molecular-weight aldehydes of cholesteryl esters have been prepared (11) and isolated from oxidized human plasma lipoproteins (12). These aldehydes form complexes with proteins (13). Schiff bases of glycerophosphocholine core aldehydes and aminophospholipids, amino acids, and myoglobin have been prepared (14) as well as the Schiff base adducts of triacylglycerol core aldehydes and aminophospholipids (15). The formation and metabolic reactivity of acylglycerol core aldehydes are of potential interest to studies of digestion and absorption of food. Food systems contain complex mixtures of lipids, amino compounds, and other nutrients. Edible oils are mainly unsaturated triacylglycerols, the oxidation of which produces triacylglycerol core aldehydes and low-molecular-weight aldehydes. Triacylglycerols are hydrolyzed to free fatty acids and 2-monoacylglycerol by pancreatic lipase. Pancreatic lipase hydrolysis of oxidized unsaturated triacylglycerols would be anticipated to produce 2-[9-oxo]nonanoyl glycerol (2-MAG-ALD). The objective of the present study was to investigate if 2-MAG-ALD forms Schiff bases with model amino compounds and to determine their chromatographic and mass spectrometric characteristics in anticipation of the isolation of such compounds during digestion and absorption of oxidized dietary oils.

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Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; GGG, glycyl-glycyl-glycine (triglycine); GGH, glycyl-glycyl-histidine; GHK, glycyl-histidyl-lysine; HPLC, high-performance liquid chromatography; HPLC/ESI/MS, liquid chromatography/electrospray ionization/mass spectrometry; 2-MAG-ALD, 2-[9-oxo]nonanoyl glycerol; PE, phosphatidylethanolamine.

MATERIALS AND METHODS

Solvents and reagents. Trioleoyl glycerol, 1-palmitoyl-2-oleoyl glycerophosphoethanolamine (PE), valine, *N*α-acetyl-

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L-lysine methyl ester, glycyl-glycyl-glycine (GGG), glycyl-glycyl-histidine (GGH), glycyl-histidyl-lysine (GHK), and sodium cyanoborohydride were purchased from Sigma Chemical Co. (St. Louis, MO). 2,4-Dinitrophenylhydrazine (DNPH) and triphenylphosphine were obtained from Aldrich Chemical Company Inc. (Milwaukee, WI). Pancreatic lipase was a crude preparation (Steapsin), obtained from Nutritional Biochemicals Corporation (Cleveland, OH). All solvents were high-performance liquid chromatography (HPLC) grade, when available, otherwise reagent grade and were purchased from Caledon Chemicals (Toronto, Canada). All chemicals used were of reagent grade or better quality.

Preparation of 2-MAG-ALD. Trioleoyl glycerol was hydrolyzed by pancreatic lipase in borate–gum arabic buffer to obtain 2-oleoyl glycerol. Buffer (0.1 M, $[Ca^{2+}] = 5 \times 10^{-3}$ M) contained 1 g gum arabic/mL and pH was adjusted to 8 by adding 0.1 N NaOH solution. Hydrolysis was carried out in a mechanical shaker (38°C), and the mixture contained 10 mg substrate/mL buffer, whereas the amount of pancreatic lipase preparation used in hydrolysis was 20% of the amount of the substrate. After 1 h reaction time the hydrolysis products were extracted with diethyl ether and dried by anhydrous Na_2SO_4. 2-Oleoylglycerol was isolated by thin-layer chromatography using boric acid-impregnated silica gel G plates. A slurry of silica gel (0.5 g/mL) was prepared in 2.4% (wt/vol) boric acid solution and a layer of 500 μm spread on 20 × 20 cm glass plates. Before use the plates were air-dried and activated at 120°C for 1 h. Plates were developed in chloroform/acetone (88:12, vol/vol), and lipid bands were visualized by spraying with 2′,7-dichlorofluorescein. The 2-oleoyl glycerol-containing band was scraped off the plate, and lipids were extracted with diethyl ether. 2-MAG-ALD was prepared by ozonization of the 2-oleoyl glycerol and reduction of the resulting ozonide by triphenylphosphine as described previously (16,17). The method was modified slightly by using acetone as ozonization solvent instead of hexane to obtain better solubility. 2-MAG-ALD was purified by thin-layer chromatography as described above, except the aldehyde-containing bands were visualized by spraying part of the band with Schiff base reagent (17), which gave a purple color in the presence of aldehydes. Two bands corresponding to *sn*-1/3 (R_f 0.07) and *sn*-2 (R_f 0.12) isomers of 9-oxononanoyl glycerol were detected as a result of isomerization during ozonization. The *sn*-2 isomer was recovered, and no further isomerization was observed. The structure of 2-MAG-ALD was further confirmed by preparing its dinitrophenylhydrazone (2-MAG-ALD DNPH) derivative as described for aldehydes (18) and analyzing the free aldehyde and the DNPH derivative by HPLC-electrospray ionization-mass spectrometry (HPLC/ESI/MS).

Preparation of reduced Schiff bases of PE and 2-MAG-ALD. Reduced Schiff base of 2-MAG-ALD and PE was prepared as previously described for aminophospholipids (14). 2-MAG-ALD (1 mg) and a twofold molar excess of PE (6 mg) were dissolved in 4 mL chloroform/methanol (2:1, vol/vol) and allowed to react at room temperature for 16 h. After reaction, freshly prepared $NaCNBH_3$ in methanol was added to a final concentration of 70 mM and the mixture kept at 4°C for 30 min to allow reduction of the imine bond of Schiff base. Excess reducing reagent was removed by washing the organic phase with water and passing it through anhydrous Na_2SO_4. The reduced Schiff base was identified by HPLC/ESI/MS.

Preparation of reduced Schiff bases of amino acids and peptides with 2-MAG-ALD. Reduced Schiff bases of valine, *N*α-acetyl-L-lysine methyl ester, and tripeptides (GGG, GGH, and GHK) with 2-MAG-ALD were obtained using a modification of the method previously described for preparation of reduced Schiff bases of amino acids with phosphatidylcholine core aldehydes (14). 2-MAG-ALD (1 mg) was dissolved in 2 mL methanol, and a twofold molar excess of amino acid or peptide dissolved in 2 mL water was added. The reaction and reduction were done as described above except no washing was performed. After reduction 5 mL of $CHCl_3$ was added and the phases were allowed to separate. Schiff base adducts of amino acids were recovered from the organic phase and Schiff bases of peptides from the aqueous phase.

HPLC/ESI/MS. The HPLC/ESI/MS system consisted of a Hewlett-Packard (Palo Alto, CA) 1090 Liquid Chromatograph connected to a Hewlett-Packard 5989A quadrupole mass spectrometer equipped with a nebulizer-assisted electrospray interface. HPLC effluent was split 1:50 using a commercial splitter (LC Packings) to deliver suitable flow to the electrospray interface. The capillary exit voltage was +160 V in positive and −160 V in negative ion mode. The capillary, end plate, and cylinder voltages were 3500, 3000, and 3500 V, respectively, in negative and −4000, −3500 and −5000 V, respectively, in positive mode. The mass range scanned as total ion current was *m/z* 200–1100. A minimum of 5 μg of total sample was injected. 2-MAG-ALD and its reduced Schiff bases with PE and amino acids were analyzed on a silica column (Supelcosil LC-Si, 5 μm, 250 × 4.6 i.d., Supelco Inc., Bellefonte, PA). The solvent system consisted of solvent mixture A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) and B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by vol) (19). The column was eluted at a flow rate of 1 mL/min with a linear gradient starting from 100% solvent A and changing to 100% solvent B in 14 min after holding the starting composition for 3 min. The final composition was held for 10 min. Reduced Schiff bases of 2-MAG-ALD and peptides were analyzed using a reversed-phase column (ODS Hypersil, 5 μm, 100 × 2.1 i.d., Hewlett-Packard) with a flow rate of 0.5 mL/min. The mobile phase composition changed from 0.5% ammonium hydroxide in water/methanol/hexane (12:88:0, by vol) to (0:88:12, by vol) in 17 min after holding the starting composition for 3 min (20). The final composition was held for 5 min.

RESULTS

2-MAG-ALD. Figure 1 shows the total negative ion current chromatogram (A) of 2-MAG-ALD and single-ion chromatograms of ions *m/z* 281 (B) and *m/z* 261 (C). Table 1 lists

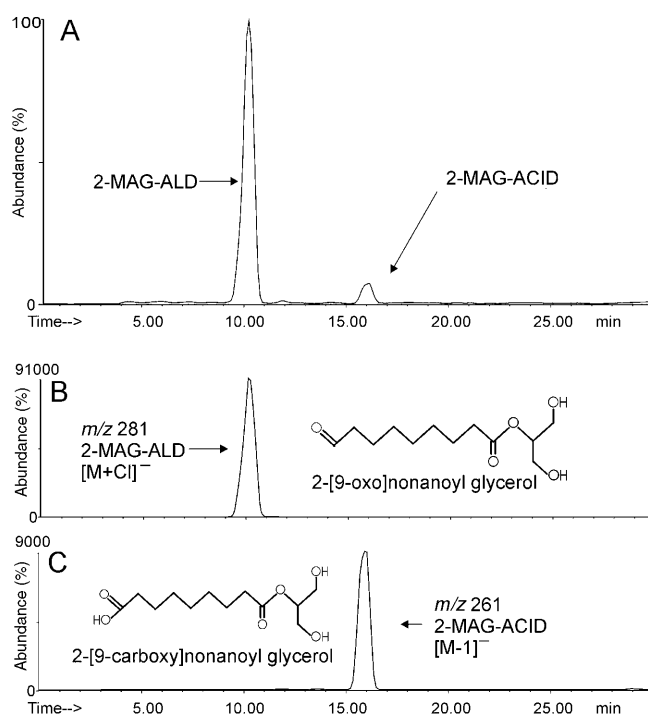

FIG. 1. Normal-phase high-performance liquid chromatography/electrospray ionization/mass spectrometry (HPLC/ESI/MS) of 2-[9-oxo]nonanoyl glycerol (2-MAG-ALD). Total negative ion current chromatogram (A) and single-ion chromatograms of ions *m/z* 281 (B) and *m/z* 261 (C). Ions are identified in figures and discussed further in text. The HPLC/ESI/MS instrumentation is described in Materials and Methods section. The solvent system consisted of solvent mixture A (chloroform/methanol/30% ammonium hydroxide, 80:19.5:0.5, by vol) and B (chloroform/methanol/water/30% ammonium hydroxide, 60:34:5.5:0.5, by vol) with a linear gradient starting from 100% solvent A and changing to 100% solvent B in 14 min after holding the starting composition for 3 min.

TABLE 1
Retention Times of Oxoacylglycerols and Reduced Schiff Bases and Yields of the Bases As Calculated by Using Ion Abundance in HPLC/ESI/MS

Compound	Retention time (min)	Yield (%)
2-MAG-ALD	10.2[a]	—
2-MAG-ALD DNPH	4.1[a]	—
2-MAG-ACID	15.9[a]	—
2-MAG-ALD + PE	13.1[a]	60
2-MAG-ALD + Lysine	11.2[a]	>50[c]
2-MAG-ALD + Valine	10.9[a]	>50[c]
2-MAG-ALD + GGG	2.2[b]	76
2-MAG-ALD + GHK	2.1[b]	74
2-MAG-ALD + GGH	2.0[b]	71

[a]Normal-phase HPLC/ESI/MS. See Figure 1 for conditions.
[b]Reversed phase HPLC/ESI/MS. See Figure 3 for conditions.
[c]Yield approximated by comparing with ion abundance of Schiff bases of peptides since the unreacted amino acids were lost in aqueous phase before analysis. Abbreviations: 2-MAG-ALD, 2-[9-oxo]nonanoyl glycerol; 2-MAG-ACID, 2-[9-carboxy]nonanoyl glycerol; DNPH, dinitrophenylhydrazine; GGG, glycyl-glycyl-glycine; GHK, glycyl-histidyl-lysine; GGH, glycyl-glycyl-histidine; PE, phosphatidylethanolamine; HPLC/ESI/MS, high-performance liquid chromatography/electrospray ionization/mass spectrometry.

the normal-phase HPLC retention times for these compounds. The ion at *m/z* 281 eluting at 10.2 min corresponds to the $[M + Cl]^-$ ion of 2-MAG-ALD (MW 246.3). The peak at 15.9 min (*m/z* 261) is due to the $[M - 1]^-$ ion of 2-[9-carboxy]nonanoyl glycerol. As shown in the single-ion chromatograms the abundance of 2-[9-carboxy]nonanoyl glycerol is about 10% of the abundance of 2-MAG-ALD. In comparison to the flame-ionization response in the gas chromatograph, the acid gave about four times higher response than the aldehyde. Thus, the amount of the 2-[9-carboxy]nonanoyl glycerol in the 2-MAG-ALD preparation was 2–3%. The DNPH derivative of 2-MAG-ALD eluted at 4.1 min (Table 1), and the total mass spectrum averaged over the peak showed no other ions than the $[M - 1]^-$ ion of the derivative at *m/z* 425 (mass chromatogram not shown). The alternative method for preparing 2-MAG-ALD, where trioleoyl glycerol or sunflower oil was ozonized before pancreatic lipase hydrolysis, was also tested. The final products of lipase hydrolysis of the triacylglycerol core aldehydes were 2-MAG-ALD for trioleoyl glycerol and 2-MAG-ALD and 2-monoacylglycerols for sunflower oil. Diacylglycerols and diacylglycerol core aldehydes were also detected as intermediate hydrolysis products.

Schiff bases of amino acids. Figure 2 shows the total positive ion current chromatogram of the reduced Schiff base of valine and 2-MAG-ALD (A) and the full mass spectrum averaged over the Schiff base peak (B). Figure 2 also shows the corresponding total mass spectrum of Schiff base peak (C) obtained from negative ion HPLC/ESI/MS. Valine Schiff

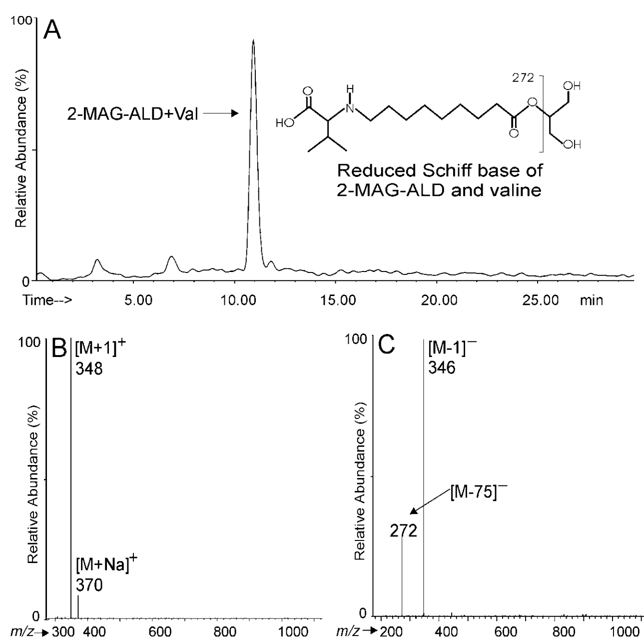

FIG. 2. Normal-phase HPLC/ESI/MS of sodium cyanoborohydride-reduced Schiff base of 2-MAG-ALD and valine. Total positive ion current chromatogram (A) and total mass spectrum averaged over Schiff base peak from positive (B) and negative (C) total ion current chromatogram. Ions are identified in figures and described in text. See Figure 1 for HPLC/ESI/MS conditions and abbreviations.

base eluted at 10.9 min (Table 1) and the $[M + 1]^+$ ion of the adduct (MW 347) at *m/z* 348 was the major ion in the positive ion mode (B). In addition to the molecular ion a sodium adduct of the Schiff base was detected at *m/z* 370. The molecular ion of the adduct also was detected in the negative ionization mode at *m/z* 346 (C), as well as a fragment ion $[M - 75]^-$ at *m/z* 272 which was formed by loss of glycerol as marked in Figures 2A and 2C. The reduced Schiff base of 2-MAG-ALD and *N*α-acetyl-L-lysine methyl ester also was analyzed by HPLC/ESI/MS in negative and positive ionization mode. The elution time of *N*α-acetyl-L-lysine methyl ester adduct was 11.2 min (Table 1), and the $[M - 1]^-$ molecular ion was the only ion detected in the negative ionization mode at *m/z* 467. The total mass spectrum of the reduced Schiff base peak obtained from the positive ion chromatogram showed $[M - 36]^+$ as a major ion (mass chromatogram not shown). This ion was formed by loss of the $[CH_3\text{-CO-}NH_2]$ group from the α-carbon of sodiated Schiff base adduct of *N*α-acetyl-L-lysine methyl ester and 2-MAG-ALD. The fragmentation pattern was confirmed by analyzing the pure *N*α-acetyl-L-lysine methyl ester, which also yielded the $[M + Na - CH_3\text{-CO-}NH_2]^+$ fragment as a major ion.

Schiff bases of peptides. Figure 3 shows the total positive ion current chromatogram obtained by reversed-phase HPLC/ESI/MS for the reduced Schiff base of triglycine (GGG) and 2-MAG-ALD (A) and the full mass spectrum averaged over the Schiff base peak (B). The Schiff base adduct of GGG eluted at the beginning of the run (2.2 min) although not in the solvent front (Table 1). The adducts of GGH and GHK eluted at 2.0 and 2.1 min, respectively (Table 1). The total mass spectrum averaged over the cyanoborohydride-reduced Schiff base peak of GGG and 2-MAG-ALD showed the $[M + 1]^+$ molecular ion to be the major ion of reaction products at *m/z* 420 (B). The monosodium adduct of the reduced Schiff base was detected at *m/z* 442 with approximately half of the intensity of the molecular ion, whereas the intensity of the ion for disodiated adduct at *m/z* 464 was even smaller. Ions representing the unreacted triglycine were *m/z* 190, 212, and 234, corresponding to $[M + 1]^+$, $[M + Na]^+$, and $[M + 2Na]^+$ ions, respectively, the sodiated ion being the most intense (B). For reduced Schiff bases of peptides GHK and GGH with 2-MAG-ALD the $[M + 1]^+$ molecular ion was the main ion in the positive ionization mode. Sodiated ions were also detected as in the case of GGG. For unreacted peptides the main ion was monosodiated adduct ion, but molecular ion and disodiated adducts were also present. Figure 4 shows the total negative ion chromatogram of the reduced Schiff base of GHK and 2-MAG-ALD (A) and the total mass spectrum averaged over the Schiff base peak (B), where deprotonated molecular ions for the reduced Schiff base of GHK and 2-MAG-ALD (*m/z* 569) and unreacted peptide (*m/z* 339) were the only ions detected. The schematic structure in Figure 4 represents the most probable structure for the reduced Schiff

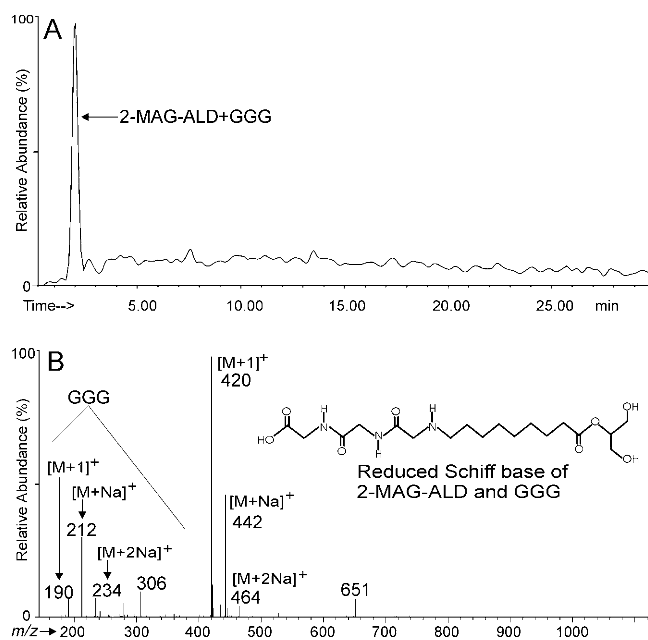

FIG. 3. Reversed-phase HPLC/ESI/MS of sodium cyanoborohydride-reduced Schiff base of 2-MAG-ALD and triglycine (GGG). Total positive ion current chromatogram (A) and total mass spectrum of Schiff base peak (B). Ions are identified in figures and discussed further in text. HPLC/ESI/MS instrumentation is described in Materials and Methods section. The mobile phase composition changed from 0.5% ammonium hydroxide in water/methanol/hexane (12:88:0, by vol) to (0:88:12, by vol) in 17 min after holding the starting composition for 3 min. For abbreviations see Figure 1.

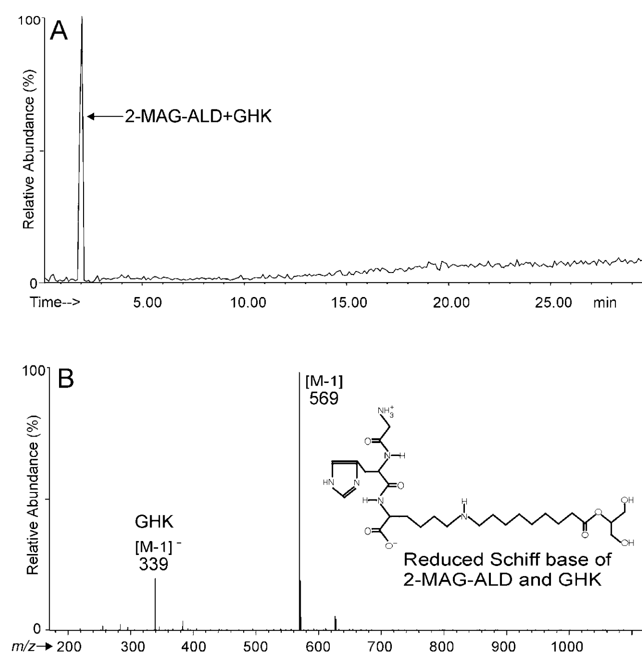

FIG. 4. Reversed-phase HPLC/ESI/MS of sodium cyanoborohydride-reduced Schiff base of 2-MAG-ALD and glycine-histidine-lysine (GHK). Total negative ion current chromatogram (A) and total mass spectrum of Schiff base peak (B). Ions are identified in figures and discussed further in text. See Figure 3 for HPLC/ESI/MS conditions and Figure 1 for abbreviations.

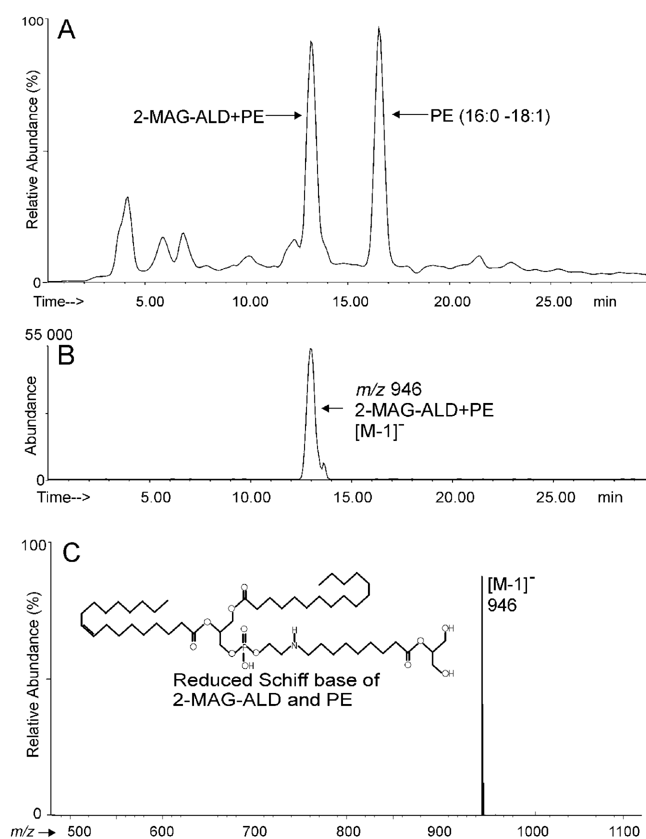

FIG. 5. Normal-phase HPLC/ESI/MS of sodium cyanoborohydride-reduced Schiff base of 2-MAG-ALD and 1-palmitoyl-2-oleoyl glycerophosphoethanolamine (PE). Total negative ion current chromatogram (A), single-ion chromatogram of Schiff base adduct (*m/z* 946) (B), and total mass spectrum averaged over Schiff base peak (C). Ions are identified in Figures. See Figure 1 for HPLC/ESI/MS conditions and abbreviations.

base adduct, although reaction with the α-amino group of glycine is also possible as will be discussed later. Deprotonated molecular ions were also the only detected ions in negative ionization mode for reduced Schiff base adducts of 2-MAG-ALD with GGG (*m/z* 418) and GGH (*m/z* 498) as well as for the unreacted peptides at *m/z* 188 and *m/z* 268, respectively (ion chromatograms not shown).

Schiff bases of PE. Figure 5 shows the total negative ion current chromatogram obtained for the reduced Schiff base of 2-MAG-ALD and PE (16:0–18:1) by normal-phase HPLC/ESI/MS (A), the single-ion chromatogram for Schiff base adduct (*m/z* 946) (B), and the total mass spectrum averaged over the Schiff base peak (C). The Schiff base adduct and PE were clearly resolved in the chromatographic system used, the former eluting at 13.1 min (Table 1) and the latter at 16.4 min. The total mass spectrum of the Schiff base peak averaged from 12.6 to 13.7 min shows no other ions than $[M-1]^-$ ion for the reduced Schiff base of PE and 2-MAG-ALD at *m/z* 946. It was estimated by using the integrated peak areas of PE and its Schiff base that the yield of the adduct is 60% (Table 1), assuming that there are no major differences in ionization efficiency between these compounds.

DISCUSSION

The present study shows that 2-MAG-ALD readily reacts with amino groups in amino acids, peptides, and aminophospholipids in hydrophobic and aqueous media. The reaction products are shown to be of Schiff-base type by reducing the adducts with sodium cyanoborohydride and analyzing the reduced reaction products by HPLC/ESI/MS. The results are consistent with Schiff base formation reported earlier between lipid ester core aldehydes and amino compounds (13–15,21). Bifunctional 4-hydroxy-2-nonenal has been shown to react with proteins preferentially *via* the Michael addition (22–24), and some low-molecular weight aldehydes have been demonstrated to undergo secondary reactions in complexing with amino groups (25,26). However, the main reaction products between 2-MAG-ALD and amino compounds in the present study were Schiff base adducts. The amino acids used to prepare Schiff base adducts in this work were valine and *N*α-acetyl-L-lysine methyl ester. Since the α-amino group of *N*α-acetyl-L-lysine methyl ester is blocked by an acetyl group, the only possible site for Schiff base formation is the ε-amino group in the side chain, whereas valine contains only an α-amino group. Both amino acids investigated formed Schiff bases with 2-MAG-ALD and no clear difference was observed in the reactivity between α- and ε-amino groups. However, the kinetics of the reaction between L-lysine and hexanal has been studied (27), and the ε-amino group of lysine has been shown to be two times more reactive than the α-amino group. A higher reactivity of the primary amino group compared to the secondary group has also been observed for free lysine and the core aldehyde of glycerophosphocholine (14). Two of the three peptides used (GGG and GGH) in the reaction contained only one free α-amino group at the end of the chain and thus possessed only one possible site for Schiff base formation. The ε-amino group of lysine in a third peptide (GHK) gave rise to another possible reaction site. Owing to the greater reactivity of the primary amino group, 2-MAG-ALD is assumed to react mainly with the ε-amino group in the side chain, without excluding the possibility of a reaction with the α-amino group. Theoretically both α- and ε-amino groups of GHK could react with 2-MAG-ALD, resulting in an adduct with two aldehyde groups attached to the peptide. Such double adducts were not detected nor were significant differences observed in reactivity among the peptides.

The reactions with amino acids and peptides were carried out at room temperature in aqueous solutions and at close to neutral pH. Since the reaction products were obtained in high yields under mild reaction conditions it may be suggested that 2-MAG-ALD could also react with amino compounds in foods and during fat absorption. 2-MAG-ALD is the final product of pancreatic digestion of oxidized triacylglycerols (15). The 2-MAG-ALD are formed by oxidation of unsaturated 2-monoacylglycerols present in food or food additives. The oxidation of triacylglycerols produces triacylglycerol core aldehydes as secondary oxidation products (1,28,29),

and these compounds have been identified in oxidized sunflower oil (30) and fish oil (31). Moreover, it has been shown that primary oxidation products, linoleic acid hydroperoxides (32), and trilinoleoylglycerol hydroperoxides (33) decompose to aldehydes in the stomach. During fat absorption 2-MAG-ALD may react with amino acids and peptides in the gastrointestinal tract and be partly responsible for impaired utilization of dietary protein. The structure of 2-MAG-ALD is identical to that of 2-monoacylglycerol, the normal hydrolysis product of triacylglycerols, which is absorbed from the intestine and metabolized further. In addition the α,β-unsaturated aldehydes originating from polyunsaturated fatty acids are absorbed, metabolized, and excreted in experimental animals (2). Therefore, the 2-MAG-ALD would be anticipated to be absorbed by the micro villus cells, where it could become acylated to triacylglycerol, as well as react with the cell protein. The rapid reaction of 2-MAG-ALD with PE in a hydrophobic environment suggests that 2-MAG-ALD might react with the aminophospholipids present in the cell membrane, where hydrophobic conditions would also be expected to prevail. The Schiff base adducts prepared in the present study should serve as reference compounds when investigating the intestinal uptake of 2-MAG-ALD during fat absorption.

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Synthetic Routes to Lipidic Diamines and Amino Alcohols: A Class of Potential Antiinflammatory Agents

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ABSTRACT: Simple and efficient methods for the synthesis of lipidic amino alcohols and diamines are described in this paper. Lipidic 2-amino alcohols and 1,3-diamines can be synthesized starting from synthetic lipidic α-amino acids. Alternatively, commercially available lipidic 1,2-diols may be used as starting material for the synthesis of 2-amino alcohols. Initial experiments on the *in vivo* antiinflammatory activity of the compounds synthesized gave promising results.

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Currently available nonsteroidal antiinflammatory drugs (NSAID) are widely used in the treatment of inflammatory conditions including rheumatoid arthritis (1). However, long-term NSAID use has been associated with gastrointestinal ulceration, bleeding and nephrotoxicity (2). On the other hand, the use of antiinflammatory steroids can cause systematic suppressive effects on adrenal function and the immune system. As a result, the discovery of novel antiinflammatory agents as an alternative to the conventional steroids or nonsteroidal compounds is in demand.

Recently we have been involved in the synthesis of lipidic amino acids and derivatives and the investigation of their biological properties. The lipidic α-amino acids (LAA) are synthetic α-amino acids with saturated or unsaturated long aliphatic side chains (3). A general approach to enantioselective synthesis of saturated LAA is based on the oxidative cleavage of amino diols obtained by the regioselective opening of enantiomerically enriched 2,3-epoxy alcohols (4). We have recently published the synthesis of optically active unsaturated LAA, including α-amino arachidonic acid, through a Wittig reaction on the suitable glutamate semialdehyde (5). LAA amides and esters with long-chain amines and alcohols, respectively, as well as lipidic dipeptide derivatives, inhibit phospholipase A_2 (6,7). On the other hand, the palmitic acid derivative *N*-(2-hydroxyethyl)hexadecanamide has been reported to have antiinflammatory activity (8), correlating with its ability to down-modulate mast cell activities *in vivo* (9). The interaction of this derivative with the peripheral cannabinoid CB_2 receptor has been demonstrated by *in vitro* studies (9). The aim of this paper is to develop synthetic routes to lipidic 2-amino alcohols and 1,3-diamines. These compounds, which can be called lipid mimetics because they resemble lipids either structurally or functionally, may have potentially useful biological activities.

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Abbreviations: Ac, acetyl; Boc, *tert*-butoxycarbonyl; ^{t}Bu, *tert*-butyl; DMF, *N,N*-dimethylformamide; EtOAc, ethyl acetate; FAB, fast atom bombardment; LAA, lipidic α-amino acid; MS, mass spectrometry; NMR, nuclear magnetic resonance; THF, tetrahydrofuran.

EXPERIMENTAL PROCEDURES AND RESULTS

tert-Butoxycarbonyl (Boc)- or acetyl (Ac)-protected LAA were prepared as described elsewhere (3,10) and reduced to amino alcohols in high yield as described in Scheme 1. N-Protected amino acids **1a–d** were converted into their corresponding fluorides **2a–d** by treatment with cyanuric fluoride in the presence of pyridine. Acyl fluorides **2a–d** were reduced in situ to primary alcohols by sodium borohydride with dropwise addition of methanol at room temperature. Boc or Ac protective groups remained inert under the conditions described. The present method for the conversion of N-protected LAA into alcohols gave products in higher yields in comparison with the previously reported mixed anhydride–$NaBH_4$ method (10). 2-Butoxycarbonylamino-hexadecanoic acid (**1a**) was used in both the racemic and the optically active *S* form; all the other derivatives of LAA were racemic mixtures. As indicated by comparison of the specific rotation values of the *S*-enantiomer of **3a** with that reported in the literature (4), the present reductive method proceeded with retention of optical purity. In addition, the preparation of the corresponding (+) and (−) Mosher esters of *S*-**3a** and ^{1}H nuclear magnetic resonance (NMR) analysis confirmed enantiomeric excess >95% due to the absence of any diastereomeric proton signal. The free lipidic amino alcohols **4a,b** were obtained by removal of the Boc group of **3a,c** using HCl in tetrahydrofuran (THF).

N-Protected amino alcohols **3** can be converted into lipidic 1,2-diamines (10) and 1,3-diamines. The hydroxyl group of **3a** was activated as the mesylate, and the methanesulfonate was converted directly into the nitrile **5** by treatment with sodium cyanide in *N,N*-dimethylformamide (DMF) at 60°C

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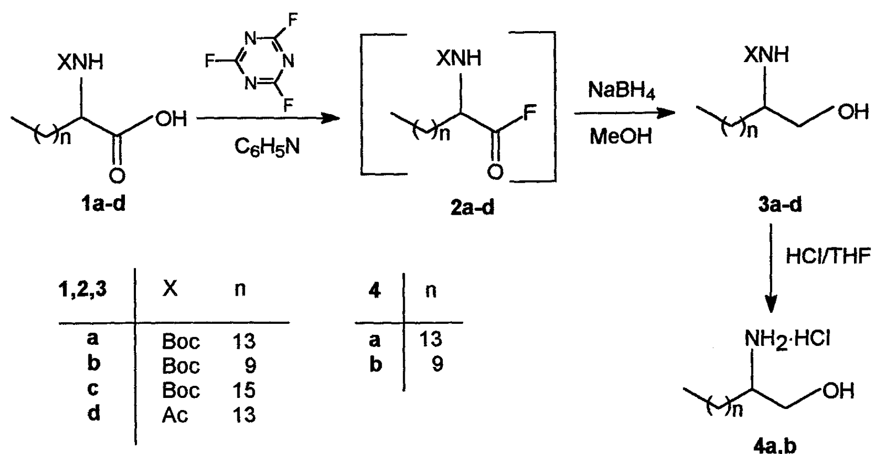


SCHEME 1

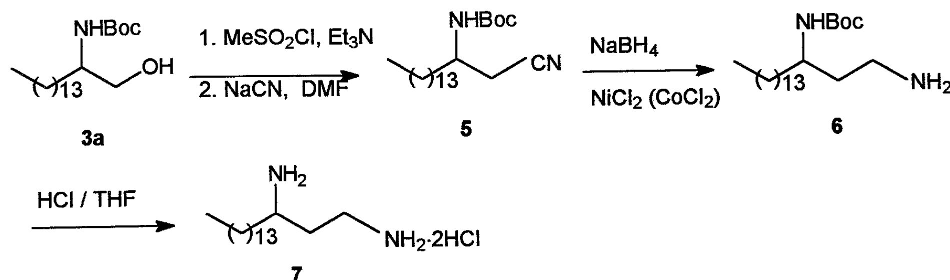
(Scheme 2). Reduction of the cyano group of **5** with sodium borohydride–transition metal system ($NiCl_2$ or $CoCl_2$) gave the N^3-monoprotected diamine **6**. Free 1,3-diamine **7** was obtained by treatment of **6** with HCl in THF.

Lipidic 2-amino alcohols can be also prepared using long-chain 1,2-diols as starting material (Scheme 3). Selective protection of the primary hydroxyl group of 1,2-hexadecanediol (**8**) by the *tert*-butyl (^{t}Bu) group was achieved in good yield by treatment with ^{t}Bu-trichloroacetimidate in the presence of a catalytic amount of boron trifluoride etherate. The secondary hydroxyl group of **9** was activated as the mesylate and converted directly into the azido group by treatment with sodium azide in DMF at 60°C in high yield. The reduction of **10** was carried out using sodium borohydride in the presence of 10% palladium-charcoal at room temperature. The ^{t}Bu group of **11** was removed by treatment with trifluoroacetic acid.

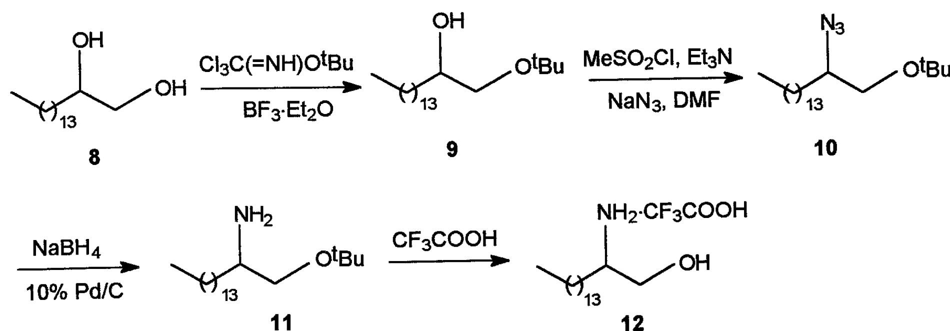
The compounds synthesized were characterized by elemental analysis, mass spectrometry, ^{1}H NMR and ^{13}C NMR spectroscopy. ^{13}C NMR spectra using DEPT pulse sequence were also recorded.

Melting points were determined on a Buchi micro melting point apparatus and are uncorrected. Specific rotations were determined with a Perkin-Elmer 141 polarimeter using a 10-cm cell. ^{1}H NMR and ^{13}C NMR spectra were run on a Bruker AM-200 spectrometer. Fast atom bombardment (FAB) spectra were obtained on a VG Analytical ZAB-SE instrument. THF was passed through a column of aluminum oxide and distilled over CaH_2. All solvents and chemicals were of reagent grade and used without further purification. Silica gel 60 (70–230 mesh, Merck) was used for column chromatography.

General procedure for the preparation of alcohols **3a–d**. Pyridine (80 μL, 1 mmol) and subsequently cyanuric fluoride (180 μL, 2 mmol) were added to a stirred solution of N-protected amino acid **1a–d** (1 mmol) in CH_2Cl_2 (2.5 mL), kept under a N_2 atmosphere, at –20 to –10°C. Precipitation of cyanuric acid occurred and increased gradually as the reaction proceeded. After the mixture was stirred at –20 to –10°C for 1 h, ice-cold water was added along with 15 mL of additional CH_2Cl_2. The organic layer was separated, and the aqueous layer was extracted once with CH_2Cl_2 (5 mL). The combined organic layers were washed with ice-cold water (10 mL), dried (Na_2SO_4), and concentrated under reduced pressure to a small volume (2 mL). $NaBH_4$ (76 mg, 2 mmol) was added in one portion and MeOH (2 mL) was then added dropwise over a period of 10–15 min at room temperature. The reaction mixture was neutralized with 0.5 M H_2SO_4, and the organic solvents were evaporated under reduced pressure. The residue was treated with CH_2Cl_2 (10 mL) and H_2O (5 mL), the organic layer was separated, and the aqueous layer was extracted with CH_2Cl_2 (2 × 8 mL). The combined organic layers were washed consecutively with 0.5 M H_2SO_4 (5 mL) and H_2O (2 × 10 mL), dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The residue was purified by



SCHEME 2



SCHEME 3

column chromatography using EtOAc/petroleum ether (1:1, vol/vol) as eluent.

*(2S)-2-(*tert*-Butoxycarbonylamino)hexadecanol* **3a**. Yield 88%; m.p. 54–55°C [lit. (10): 55°C]; $[\alpha]_D^{25}$ −8.6 (*c* 1, $CHCl_3$) [lit. (4): −8.5); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-16), 22.7 (C-15), 26.0 (C-4), 28.4 [$(CH_3)_3$], 29.3–29.6 (C-5–C-13), 31.5 (C-3), 31.9 (C-14), 52.9 (C-2), 66.1 (C-1), 79.6 [$C(CH_3)_3$], 156.6 (OCO).

*2-(*tert*-Butoxycarbonylamino)dodecanol* **3b**. Yield 89%; m.p. 41–42°C [lit. (10): 40–41°C).

*2-(*tert*-Butoxycarbonylamino)octadecanol* **3c**. Yield 84%; m.p. 56–57°C; ^1H NMR ($CDCl_3$) δ 0.82 (3H, *t*, *J* = 7 Hz, CH_3), 1.20–1.55 [39H, *m*, 15 × CH_2, $C(CH_3)_3$], 2.42 (1H, *b*, OH), 3.58 (3H, *m*, α-CH, CH_2OH), 4.47 (1H, *b*, OCONH). Calc. for $C_{23}H_{47}NO_3$: C 71.64; H 12.28; N 3.63; MW 385.64. Found: C 71.49; H 12.35; N 3.58.

2-Acetylaminohexadecanol **3d**. Yield 74%; m.p. 84–85°C [lit. (10): 83–85°C].

*3-(*tert*-Butoxycarbonylamino)heptadecane nitrile* **5**. To an ice-cooled solution of N-protected amino alcohol **3a** (0.36 g, 1 mmol) in CH_2Cl_2 (40 mL), triethylamine (0.21 mL, 1.5 mmol) and methanesulfonyl chloride (0.12 mL, 1.5 mmol) were added together dropwise. The reaction mixture was stirred for 30 min at 0°C and for 30 min at room temperature. The organic phase was washed consecutively with brine, 1 M HCl, brine, 5% aqueous $NaHCO_3$ and brine, dried (Na_2SO_4), and the solvent was evaporated under reduced pressure. The crude methanesulfonate ester obtained was dissolved in DMF (2 mL); sodium cyanide (0.12 g, 2.5 mmol) was added and the reaction mixture was heated for 3 h at 60°C. After cooling, water (12 mL) was added and the precipitated product was filtered and dried. The crude product was purified by column chromatography using EtOAc/hexane (2:8, vol/vol) as eluent: Yield 0.27 g (74%); m.p. 55–56°C; ^1H NMR ($CDCl_3$) δ 0.87 (3H, *t*, *J* = 7 Hz, CH_3), 1.20–1.40 (24H, *m*, 12 × CH_2), 1.44 [9H, *s*, $C(CH_3)_3$], 1.59 (2H, *m*, CH_2CHCH_2CN), 2.51 (1H, *dd*, *J* = 4 Hz, *J* = 17 Hz, C*H*HCN), 2.75 (1H, *dd*, *J* = 5 Hz, *J* = 17 Hz, CH*H*CN), 3.78 (1H, *m*, α-CH), 4.60 (1H, *d*, *J* = 8 Hz, OCONH); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-17), 22.7 (C-16), 23.7 and 25.9 (C-4 and C-5), 28.3 [$(CH_3)_3$], 29.3–29.6 (C-6–C-14), 31.9 (C-15), 33.4 (C-2), 47.2 (C-3), 80.1 [$C(CH_3)_3$], 117.4 (CN), 155.0 (OCO); FAB MS: *m/z* 367 ($M + H^+$, 10%), 311 (100), 267 (25), 226 (34). Calc. for $C_{22}H_{42}N_2O_2$: C 72.08; H 11.55; N 7.64; MW 366.59. Found: C 71.91; H 11.51; N 7.32.

*3-(*tert*-Butoxycarbonylamino)heptadecanamine* **6**. To a stirred solution of **5** (0.37 g, 1 mmol) in methanol (8 mL) nickel chloride hexahydrate (1.18 g, 5 mmol) was added at 0°C, followed by $NaBH_4$ (0.30 g, 8 mmol) in small portions. After stirring for 30 min at room temperature, water was added, the mixture was neutralized with 0.5 M H_2SO_4 and the organic solvent was removed under reduced pressure. The aqueous phase was extracted with EtOAc (5 × 10 mL). After drying (Na_2SO_4), the solvent was evaporated to dryness to give **6**. Yield 0.27 g (74%); m.p. 112–114°C; ^1H NMR ($CDCl_3$) δ 0.84 (3H, *t*, *J* = 7 Hz, CH_3), 1.00–1.60 [35H, *m*, $C(CH_3)_3$, 13 × CH_2), 2.10 (2H, *m*, $CH_2CH_2NH_2$), 2.90 (2H, *m*, NH_2), 3.20 (2H, *m*, CH_2NH_2), 3.59 (1H, *m*, α-CH), 4.50 (1H, *d*, *J* = 8 Hz, OCONH); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-17), 22.7 (C-16), 25.9 (C-5), 28.3 [$(CH_3)_3$], 29.3–29.6 (C-6–C-14), 31.9 (C-15), 33.6 (C-4), 35.6 (C-2), 37.1 (C-1), 47.8 (C-3), 80.3 [$C(CH_3)_3$], 157.0 (OCO); FAB MS *m/z* 371 (M + H, 100%), 315 (74), 271 (20), 226 (20). Calc. for $C_{22}H_{46}N_2O_2$: C 71.30; H 12.51; N 7.56; MW 370.62. Found: C 71.21; H 12.32; N 7.52.

1,3-Heptadecanediamine **7**. Compound **6** (0.18 g, 0.5 mmol) was treated with 4 M HCl in THF (8 mL) at room temperature for 30 min. The excess acid and solvent were removed under reduced pressure. Anhydrous THF (4 mL) was added to the residue and the solvent was evaporated. The process was repeated once more. Yield 0.13 g (77%); ^1H NMR (CD_3OD) δ 0.84 (3H, *t*, *J* = 7 Hz, CH_3), 1.00–150 (24H, *m*, 12 × CH_2), 1.62 [2H, *m*, $CH_2(CH_2)_{12}CH_3$], 2.00 (2H, *m*, $CH_2CH_2NH_3^+$), 3.08 (2H, *m*, $CH_2NH_3^+$), 3.35 (1H, *m*, α-CH); ^{13}C NMR (CD_3OD) δ 14.5 (C-17), 23.7 (C-16), 26.0 (C-5), 30.5–30.8 (C-6–C-14), 31.5 (C-15), 33.1 and 33.6 (C-4 and C-2), 37.2 (C-1), 50.6 (C-3); FAB MS *m/z* 271 (M − 2HCl + H^+, 100%), 254 (8), 226 (11). Calc. for $C_{17}H_{38}N_2{\cdot}2HCl{\cdot}0.5H_2O$: C 57.94; H 11.73; N 7.95; MW 352.43. Found: C 57.91; H 11.77; N 7.93.

*1-*tert*-Butoxy-2-hexadecanol* **9**. A solution of *tert*-butyltrichloroacetamidate (0.79 mL, 4.4 mmol) in cyclohexane (20 mL) and $BF_3{\cdot}Et_2O$ (80 μL) were added to a solution of 1,2-hexadecanediol **8** (1.03 g, 4 mmol) in CH_2Cl_2 (15 mL), and the mixture was stirred at room temperature for 36 h. The solid formed was filtered, and the filtrate was washed with

$NaHCO_3$ 5%, then brine, dried (Na_2SO_4), concentrated under vaccum, and purified by column chromatography using EtOAc/petroleum ether (5:95, vol/vol) as eluent. Yield 0.87 g (69%); m.p. 35–36°C; ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, *t*, J = 7 Hz, CH_3), 1.0–1.5 (35H, *m*, $C(CH_3)_3$, 13 × CH_2), 2.44 (1H, *d*, J = 3.5 Hz, OH), 3.12 (1H, *dd*, J = 8 Hz, J = 8 Hz, C*H*HO), 3.35 (1H, *dd*, J = 8 Hz, J = 4 Hz, CH*H*O), 3.62 (1H, *m*, CH); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-16), 22.7 (C-15), 25.6 (C-4), 27.5 [$(CH_3)_3$], 29.3–29.6 (C-5–C-13), 31.9 (C-14), 33.1 (C-3), 66.0 (C-1), 70.6 (C-2), 73.1 [*C*$(CH_3)_3$]; infrared (cm^{-1}) 3585, 3388, 2923, 2856, 1465, 1363, 1085; FAB MS *m/z* 338 (M + Na^+, 100%), 316 (M + H^+, 7), 258 (13), 242 (65). Calc. for $C_{20}H_{42}O_2$: C 76.37; H 13.46; MW 314.55. Found: C 76.30; H 13.26.

*1-*tert*-Butoxy-2-azido-hexadecane* **10**. To a cold solution of **9** (0.75 g, 2.38 mmol) in CH_2Cl_2 (6 mL), triethylamine (0.57 mL, 4.07 mmol) and methanesulfonyl chloride (0.31 mL, 4.07 mmol) were added dropwise. After stirring for 30 min at 0°C and 30 min at room temperature, the reaction mixture was washed with brine, dried (Na_2SO_4), concentrated and the residue was purified by column chromatography using EtOAc/petroleum ether (5:95, vol/vol) as eluent. Yield 0.82 g (88%) of oily product; ^{1}H NMR ($CDCl_3$) δ 0.74 (3H, *t*, J = 7 Hz, CH_3), 1.0–1.4 [33 H, *m*, $C(CH_3)_3$, 12 × CH_2], 1.54 (2H, *m*, C*H*$_2$CHCH$_2$O), 2.93 (3H, *s*, SO_2CH_3), 3.72 (2H, *m*, CH_2OSO_2), 4.58 (1H, *m*, CH); FAB MS *m/z* 416 (M + Na^+, 100%), 338 (16), 320 (8), 242 (30). Calc. for $C_{21}H_{44}O_4S$: C 64.24; H 11.30; MW 329.64. Found: C 63.98; H 11.49.

A mixture of the purified oil (0.51 g, 1.3 mmol) in DMF (2.6 mL) and NaN_3 (0.34 g, 5.20 mmol) was stirred under heating at 60°C for 24 h. The solvent was evaporated, and water (20 mL) and EtOAc (20 mL) were added. The organic phase was washed with brine, dried (Na_2SO_4), concentated, and purified by column chromatography using EtOAc/petroleum ether (4:96, vol/vol). Yield 0.43 g (96%) of colorless oil; ^{1}H NMR ($CDCl_3$) δ 0.82 (3H, *t*, J = 7 Hz, CH_3), 1.0–1.5 (35H, *m*, $C(CH_3)_3$, 13 × CH_2), 3.42 (3H, *m*, CH_2O, CH); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-16), 22.7 (C-15), 26.1 (C-4), 27.3 [$(CH_3)_3$], 29.4–29.6 (C-5–C-13), 30.9 (C-3), 31.9 (C-14), 62.2 (C-2), 65.1 (C-1), 73.3 [*C*$(CH_3)_3$]; infrared (cm^{-1}) 2927, 2856, 2103, 1467, 1364, 1097; FAB MS *m/z* 340 (M + H^+, 5%), 312 (13), 256 (66). Calc. for $C_{20}H_{41}N_3O$: C 70.74; H 12.17; N 12.37; MW 339.57. Found: C 70.67; H 12.22; N 12.19.

*1-*tert*-Butoxy-2-hexadecyl amine* **11**. To a mixture of **10** (0.33 g, 1 mmol), 10% palladium on charcoal (66 mg) and $NaBH_4$ (113 mg, 3 mmol) in THF (4 mL), methanol (8 mL) was added dropwise under cooling at 10°C. After 10 min, the catalyst was filtered off, and the filtrate was neutralized with 0.5 M H_2SO_4 and concentrated to a smaller volume to remove the organic solvents. Water (10 mL) was added and the product was extracted with EtOAc (3 × 10 mL). The organic phase was washed with brine, dried (Na_2SO_4), concentrated, and the product was purified by column chromatography using $CHCl_3$/MeOH (93:7, vol/vol) as eluent. Yield 0.30 g (95%); m.p. 67–69°C; ^{1}H NMR ($CDCl_3$) δ 0.88 (3H, *t*, J = 7 Hz, CH_3), 1.30 [33H, *m*, 12 × CH_2, $C(CH_3)_3$], 1.72 (2H, *m*, C*H*$_2$CHCH$_2$O), 3.18 (1H, *m*, α-CH), 3.40 (1H, *dd*, J = 10 Hz, J = 9 Hz, C*H*HO), 3.55 (1H, *dd*, J = 5 Hz, J = 10 Hz, CH*H*O); ^{13}C NMR ($CDCl_3$) δ 14.1 (C-16), 22.7 (C-15), 25.7 (C-4), 27.4 [$(CH_3)_3$], 29.4–29.7 (C-5–C-13), 30.0 (C-3), 31.9 (C-14), 52.4 (C-2), 61.2 (C-1), 73.7 [*C*$(CH_3)_3$]; FAB MS *m/z* 315 (M + H^+, 100%), 259 (88), 227 (20). Calc. for $C_{20}H_{43}NO \cdot 1.5H_2O$: C 70.53; H 13.61; N 4.11; MW 340.59. Found: C 70.72; H 13.58; N 4.09.

2-Aminohexadecanol trifluoroacetate **12**. A solution of **11** (0.10 g, 0.3 mmol) in trifluoroacetic acid (0.9 mL) was stirred at room temperature for 1 h. The reaction mixture was diluted with CH_2Cl_2 (5 mL), and the excess acid and solvent were removed under reduced pressure. Dichloromethane (5 mL) was added to the residue, and the solvent was evaporated. The process was repeated once more. A white solid was formed upon the addition of dry ether, which was filtered, yielding 96 mg (86%); m.p. 107–109°C; ^{1}H NMR (CD_3OD) δ 0.88 (3H, *t*, J = 7 Hz, CH_3), 1.10–1.40 (24H, *m*, 12 × CH_2), 1.48 [2H, *m*, C*H*$_2$(CH$_2$)$_{12}$CH$_3$], 3.02 (1H, *m*, α-CH), 3.40 (1H, *m*, CH*H*OH), 3.63 (1H, *m*, C*H*HOH); ^{13}C NMR (CD_3OD) δ 14.5 (C-16), 23.8 (C-15), 26.5 (C-4), 30.5–30.8 (C-5–C-14), 33.1 (C-3), 54.6 (C-2), 62.1 (C-1); FAB MS *m/z* 259 (M + H^+ – CF_3COOH, 100%), 227 (12). Calc. for $C_{16}H_{35}NO \cdot CF_3COOH$: C 58.20; H 9.77; N 3.77; MW 371.49. Found: C 57.95; H 9.93; N 3.53.

A sample of 2-aminohexadecanol, isolated as hydrochloride salt, was identical to that prepared as described (10).

DISCUSSION

Initial experiments on the potential *in vivo* antiinflammatory usefulness of these compounds were carried out using the rat carrageenin-induced paw edema assay as a model for acute inflammation. Preliminary results were obtained for lipidic 1,2- and 1,3-diamines, containing at least one free amino group, as well as for 2-amino alcohols. For example, 2-aminohexadecanol (**4a**) and 1,2-hexadecanediamine (10) caused, respectively, 85.6 ± 0.38% and 93.5 ± 0.36% inhibition of paw edema at a high dose of 0.15 mmol/kg, while 1,3-heptadecanediamine (**7**) inhibited the edema by 70.7 ± 2.02% at a dose of 0.04 mmol/kg.

In conclusion, this paper described facile and efficient methods for the synthesis of biologically important lipidic diamines and amino alcohols. *In vivo* studies of the antiinflammatory activity of the compounds presented in this paper are in progress.

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ARTICLE

Docosahexaenoic Acid Ingestion Inhibits Natural Killer Cell Activity and Production of Inflammatory Mediators in Young Healthy Men[1]

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ABSTRACT: The purpose of this study was to examine the effects of feeding docosahexaenoic acid (DHA) as triacylglycerol on the fatty acid composition, eicosanoid production, and select activities of human peripheral blood mononuclear cells (PBMNC). A 120-d study with 11 healthy men was conducted at the Metabolic Research Unit of Western Human Nutrition Reach Center. Four subjects (control group) were fed the stabilization diet throughout the study; the remaining seven subjects were fed the basal diet for the first 30 d, followed by 6 g DHA/d for the next 90 d. DHA replaced an equivalent amount of linoleic acid; the two diets were comparable in their total fat and all other nutrients. Both diets were supplemented with 20 mg D α-tocopherol acetate per day. PBMNC fatty acid composition and eicosanoid production were examined on day 30 and 113; immune cell functions were tested on day 22, 30, 78, 85, 106, and 113. DHA feeding increased its concentration from 2.3 to 7.4 wt% in the PBMNC total lipids, and decreased arachidonic acid concentration from 19.8 to 10.7 wt%. It also lowered prostaglandin E2 (PGE2) and leukotriene B4 (LTB4) production, in response to lipopolysaccharide, by 60–75%. Natural killer cell activity and *in vitro* secretion of interleukin-1β and tumor necrosis factor α were significantly reduced by DHA feeding. These parameters remained unchanged in the subjects fed the control diet. B-cell functions as reported here and T-cell functions that we reported previously were not altered by DHA feeding. Our results show that inhibitory effects of DHA on immune cell functions varied with the cell type, and that the inhibitory effects are not mediated through increased production of PGE2 and LTB4.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HPLC, high-performance liquid chromatography; IL, interleukin; IR, immune response; LTB4, leukotriene B4; LPS, lipopolysaccharide; NK, natural killer; PBMNC, peripheral blood mononuclear cells; PBS, phosphate buffered saline; PGE2, prostaglandin E2; PUFA, polyunsaturated fatty acids; TLC, thin-layer chromatography; TNFα, tumor necrosis factor α.

The low incidence of mortality from coronary heart disease among the Eskimos was initially attributed to the relatively large proportion of n-3 fatty acids from fish in their diet (1). Subsequent human and animal experimental studies also demonstrated the beneficial effects of n-3 fatty acids on cardiovascular health. Fish oils may improve cardiovascular health because of their antiinflammatory effects, lowering of serum triglyceride, and inhibition of platelet aggregation.

The n-3 polyunsaturated fatty acids (PUFA) from marine and plant sources inhibit several aspects of human immune response (IR), including the production of inflammatory cytokines tumor necrosis factor α (TNFα) and interleukin 1β (IL-1β), natural killer (NK) cell activity, and lymphocyte proliferation (2–16). Most of the studies with n-3 PUFA neither held total fat intake constant nor provided extra antioxidant nutrients to meet the need for increased oxidative stress. Both these factors may alter human IR (11,17–19). Thus it is not clear whether the inhibition of IR in previous studies was due to n-3 PUFA, increased total fat intake, increased oxidative stress, or a combination of these factors.

Fish oils contain a variable mixture of eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA), along with many other fatty acids. It is not known whether the decline in IR caused by the fish oils is due to EPA, DHA, both, or other fatty acids. Purified esters of EPA lowered several aspects human IR (9,10,15). *In vitro* addition of DHA to human (20) and rat lymphocytes (21) inhibited their proliferation. Similar results were also obtained in a DHA feeding study conducted with rats (22). Addition of EPA or DHA to human peripheral blood mononuclear cells (PBMNC) in culture indicates that different mechanisms may mediate the alteration of lymphocyte function by these two fatty acids (20,23).

Dietary fatty acids may alter IR by altering eicosanoid production and the fatty acid composition of the participating cells. Prostaglandin E2 (PGE2) and leukotriene B4 (LTB4), both derived from arachidonic acid (20:4n-6), are considered to be some of the most important eicosanoids that alter immune cell functions. PGE2 inhibited a number of lymphocyte

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functions such as proliferation and IL-2 secretion in a dose-dependent manner, while the LTB4 was stimulatory at low concentrations and inhibitory at higher concentrations (24). The n-3 fatty acids, EPA and DHA, may reduce the arachidonic acid concentration within the immune cells and thus reduce the production of inflammatory eicosanoids.

The effect of dietary DHA on production of inflammatory mediators or other aspects of human IR has not been published. This is because until recently, purified DHA was not available for human consumption. Such studies are now possible with the availability of DHASCO™ oil, which contains 40% DHA as natural triacylglycerol. Studies with DHA are important, because it is the major n-3 fatty acid in tissues, and the body tends to conserve it more than EPA. The purpose of this study was to examine the effect of a moderately high intake (6 g/d) of DHA, in the absence of EPA, on select parameters of human IR, including inflammatory cytokine production, NK cell activity, and *in vivo* antibody production. Possible mechanisms by which DHA may alter immune cell functions were determined by examining PBMNC fatty acid composition, *in vitro* eicosanoid production, and the serum concentrations of apoprotein E and antioxidant vitamins. We held total fat intake constant and supplemented diets with D α-tocopherol acetate (20 mg/d) to avoid increased oxidative stress from DHA consumption.

MATERIALS AND METHODS

Subjects and study design. Twelve healthy men between the ages of 20–40 yr were selected from the San Francisco Bay area through local advertisements. They passed a physical examination given by a licensed physician, and had body weights within 20% of ideal body weights based on 1983 Metropolitan Life Insurance tables. They were all nonsmokers and nondrug users. Mean body weights, age, and body mass index (BMI) for the study subjects are given in Table 1. Body weights of the subjects were maintained within 2% of their initial weights throughout the study by adjusting their energy intake when necessary.

The study protocol was approved by the Human use committee of the University of California at Davis and the United States Department of Agriculture committee at Houston, Texas. The study lasted for 120 d (April 1 to July 29, 1996) although no immunological tests were conducted after day 113, because of other scheduled procedures. All subjects were immunized on day 85 with a trivalent 1995–1996 influenza vaccine (Connaught Laboratories Inc., Swiftwater, PA). Subjects lived at the Metabolic Research Unit (MRU) of the Western Human Nutrition Research Center for the duration of the study, except when going for daily walks (2 × 2 miles) or other scheduled outings. They consumed only those foods prepared by the staff of the MRU and were under constant supervision. Subjects were divided into two groups. Four subjects (control group) were fed the control or stabilization diet throughout the study. The remaining eight subjects (DHA group) were fed the control diet for the first 30 d of the study and a DHA-supplemented diet for the last 90 d. One subject from the DHA group did not complete the study, so data from only seven subjects were evaluated. More subjects were included in the DHA group than in the control group, because most of the DHA effects were evaluated by comparing the pre- and post-DHA indices in the DHA group.

TABLE 1
Physical Characteristics of Study Subjects[a]

Index	DHA group (*n* = 7)	Control group (*n* = 4)
Age, yr	33.1 ± 1.8	33.3 ± 3.1
Weight, kg	78.6 ± 4.2	74.7 ± 4.2
BMI, kg/m^2	23.7 ± 0.8	23.1 ± 1.1

[a]DHA, docosahexaenoic acid; BMI, body mass index. Mean ± SEM.

Diets. Diets were made up of natural foods, except DHA and vitamin E, and were fed in a 5-d rotating menu, comprising three meals and a postdinner snack. Dietary composites from each of the 5-d menus for both diets were analyzed for macronutrients. In both diets, protein, fat, and carbohydrates provided approximately 15, 30, and 55% energy, respectively (Table 2). The micronutrient contents of the diets were calculated by using *USDA Handbook 8* (25); all nutrients were at or above the recommended dietary allowances (RDA) and were identical in the two diets. Diets contained about one RDA of vitamin E from natural foods (calculated using values from *USDA Handbook 8*) and were supplemented with an additional 20 mg/d of D α-tocopherol acetate (Bronson Pharmaceutical, St. Louis, MO).

DHA diet contained 15 g of DHASCO™ oil (a gift from Martek Corporation, Columbia, MD), which was incorporated by replacing an isocaloric amount of safflower oil; all other foods were identical in the two diets. The main effect of incorporating DHASCO™ oil into diet was the replacement of 6 g linoleic acid (18:2n-6) with an equivalent amount of DHA. Fatty acid composition of the two diets is given in Table 3. The DHASCO™ oil was kept in sealed containers at –20°C, and all open bottles were flushed with nitrogen before

TABLE 2
Nutrient Composition of Experimental Diets[a]

	Energy %	
Nutrient	DHA diet	Control diet
Protein	15.2 ± 0.6	14.6 ± 0.5
Carbohydrate	53.9 ± 3.0	56.4 ± 3.1
Fat, total	30.9 ± 2.1	29.0 ± 1.8
Saturated	8.9 ± 0.4	8.1 ± 0.4
Monounsaturated	9.2 ± 0.3	9.2 ± 0.3
n-6 Polyunsaturated	6.5 ± 0.3	8.5 ± 0.3
n-3 Polyunsaturated	2.8 ± 0.1	1.1 ± 0.1
trans	1.9 ± 0.1	2.1 ± 0.1
P/S ratio	1.2	1.1
Cholesterol (mg/d)	360	360

[a]Mean ± SEM (*n* = 5). Dietary composites were prepared for each 5-d rotating menu. Both diets were supplemented with D α-tocopherol acetate, 20 mg/d. P/S, polyunsaturated fatty acid/saturated fatty acid. See Table 1 for other abbreviation.

TABLE 3
Fatty Acid (wt%) Composition of Experimental Diets[a]

Fatty acid	DHA diet	Control diet
12:0	1.3 ± 0.1	0.7 ± 0.1[b]
14:0	4.3 ± 0.3	2.2 ± 0.4[b]
16:0	16.6 ± 0.7	16.3 ± 0.7
16:1n-9	0.7 ± 0.2	0.9 ± 0.2
18:0	7.1 ± 0.5	7.5 ± 0.4
18:1*t*, all isomers	6.2 ± 0.5	7.0 ± 0.5
18:1n-9	26.6 ± 0.8	26.0 ± 0.7
18:1n-7	1.7 ± 0.1	2.0 ± 0.1
18:1n-5	1.5 ± 0.2	2.1 ± 0.4
18:2*tt* and 19:0	0.5 ± 0.1	0.6 ± 0.1
18:2n-6	21.6 ± 1.2	28.3 ± 1.0[b]
18:3n-3	2.6 ± 0.2	3.2 ± 0.1
22:0	0.2 ± 0.0	0.2 ± 0.0
20:5n-3	0.4 ± 0.1	0.3 ± 0.1
22:6n-3	6.5 ± 0.22	<0.1 ± 0.1[b]
Total	98.0 ± 0.4	97.2 ± 0.3
Unknowns	2.0 ± 0.2	2.8 ± 0.1

[a]Mean ± SEM (n = 5). See Table 1 for abbreviation.
[b]Significantly different between two diets ($P < 0.05$).

being returned to the refrigerator. It was served only in cold foods such as yogurt, dips, or salads. Thus the chances of DHA oxidation were minimized.

Laboratory procedures. Blood samples were collected between 7: 00 and 8:00 A.M. after an overnight fast on study days 23, 30, 78, 85, 106, and 113. Samples were collected by antecubital venipuncture into evacuated tubes without anticoagulants (for sera preparation) or containing heparin (for cell culture experiments). For both dietary groups two determinations of cytokine production, NK cell activity, and serum antioxidant vitamins were made at the end of stabilization, middle, and end of the intervention periods (days 23, 30, 78, 85, 106, and 113). Means of the two measurements are shown in the results section. PBMNC fatty acid composition and eicosanoid production were determined only on study days 30 and 113. Influenza antibody titers were determined in the sera prepared from blood samples drawn on days 30, 85, 106, and 113.

Isolation and culture of PBMNC for cytokine and eicosanoid secretion. PBMNC were isolated using Histopaque-1077 as previously reported (26) and cultured with or without lipopolysaccharide (LPS, 1.0 mg/L) in 24-well flat-bottom culture plates (5×10^5 PBMNC/mL/well). The culture medium used was RPMI-1640 (Gibco, Grand Island, NY), containing 10% autologous serum and L-glutamine (2 mmol/L), penicillin (100 KU/L), streptomycin (100 mg/L) and gentamicin (20 mg/L). The tissue culture media were collected by centrifugation 24 h after stimulation with LPS and stored frozen at −70°C until the cytokine and eicosanoid concentrations were determined. Enzyme-linked immunosorbent assay (ELISA) kits for cytokine assays were purchased from Immunotech (Miami, FL) and those for LTB4 from Cayman Chemical Company (Ann Arbor, MI).

PGE2 concentration in the media was determined by gas chromatography–mass spectrometry (GC–MS) as the methoxime-pentafluorobenzyl ester-trimethylsilyl ether derivative. Analyte and internal standard were extracted from 200 µL culture media with C_{18} Sep-Pak cartridges after dilution with 10 mL acidified (pH 3.0) water, and addition of 1 ng of (3,3,4,4-$^2H_4$) PGE2 internal standard. They were eluted with methyl formate/petroleum ether (1:1) after sequential rinsing first with acidified water, then with methyl formate/petroleum ether (5:95). The prostaglandins were esterified with pentafluorobenzyl bromide in CH_3CN, then were treated with methoxylamine hydrochloride in pyridine. The PGE2-pentafluorobenzyl ester-methoxime derivative was purified on silica gel thin-layer chromatography plates and finally converted to trimethylsilyl ether derivative with *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA) in pyridine (1:1). Gas chromatography–mass spectrometry in the electron capture chemical ionization (ECCI) mode was carried out with a Finnigan-MAT TSQ-70B instrument operated at 70 eV with methane as the moderating gas. The ions monitored were m/z 524 (M − 181) for the endogenous analyte and m/z 528 for the tetradeuterated internal standard.

Determination of NK cell activity. NK cell activity was determined by using the nonadherent PBMNC and the ^{51}Cr labeled K-562 cells at effector/target cell ratios of 50:1, 25:1, 12.5:1, 6.2:1, and 3.1:1 as previously reported (26). Six wells were used for each effector cell concentration and for the spontaneous and maximal release (caused by 3% centrimide) of ^{51}Cr. After 4 h incubation at 37°C in 5% CO_2, the plates were centrifuged and aliquots of supernatant collected to determine the amount ^{51}Cr released.

Percent lysis was calculated as follows:

$$\%\ \text{lysis} = \frac{(\text{experimental CPM} - \text{spontaneous CPM})}{(\text{maximum CPM} - \text{spontaneous CPM})} \times 100 \quad [1]$$

Serum antibody titers. The antibody titers for the viral strains A/TEXAS/36/91, A/Johannesburg/33/94, and B/Harbin/7/94 (strains included in the vaccine) were determined by using the hemagglutination inhibition assay (27). Results for antibody titers are expressed as the geometric mean (GM) of the antibody titers and 95% confidence intervals.

PBMNC fatty acid composition. PBMNC for fatty acid analysis were isolated using Histopaque-1077 as discussed above. Cells were further washed twice with Dulbecco's phosphate buffered saline (PBS) resuspended in 1 mL PBS and layered over 1.5 mL Histopaque-1077. The tubes were centrifuged for 15 min at 1000 × *g* to remove contaminating erythrocytes. PBMNC were washed with PBS, mixed with 0.8 mL LYMPHO-KWIK (Canoga Park, CA) and incubated for 15 min at 37°C. The PBMNC were then overlaid with 0.2 mL PBS and centrifuged for 2 min at 2000 × *g*. The cell pellet was washed one more time with PBS and stored frozen at −20°C until fatty acid analysis. This isolation procedure removed most of contaminating platelets and erythrocytes, and yielded cells containing 90–95% mononuclear cells as determined by differential cell counting.

PBMNC lipids were extracted by the procedures previously described by Nelson (28,29) using chloroform/

methanol (2:1, vol/vol). The total lipid extract was transmethylated with methanolic HCl (7%, w/w) by the procedures described previously (30,31). The impurities extracted into the hexane phase after termination of the reaction were removed by thin-layer chromatography (29). We have described the conditions of the capillary gas-liquid chromatography (GLC) previously (30). Briefly, the column was a 30-m × 0.025 mm fused-quartz column coated with SP-2340 (Supelco, Inc., Bellefonte, PA). The GLC data were processed with a Hewlett-Packard ChemStation software running on an IBM compatible desktop computer.

Serum antioxidant vitamins. Fasting blood samples were collected from all subjects and sera were separated by centrifugation at 1000 × *g* for 15 min at 4°C. Aliquots of sera were stored under liquid nitrogen until antioxidant analysis. For the analysis of α-tocopherol and retinol in sera, 100 µL of each of the internal standards retinyl acetate and α-tocopherol acetate were transferred into disposable glass tubes and mixed vigorously with 200 µL of sera. For extraction of the antioxidants 400 µL of hexane was added and the contents were mixed vigorously for 1 min. The tubes were centrifuged and the solvent layer was transferred to a centrifuge tube and the contents were evaporated under a stream of nitrogen. The antioxidants in the tube were dissolved in 25 µL of diethyl ether followed by 75 µL of methanol, and 20 µL of the sample solution was injected for high-performance liquid chromatography (HPLC) analysis. The HPLC conditions for α-tocopherol and retinol were as follows: A Waters LC with a stainless steel column packed with microBondapak C18 (10 µm, 300 mm × 3.9 i.d.) as the solid phase was used. The mobile phase was methanol/water (97:3, vol/vol) at a flow rate of 1.0 mL/min. The eluate was monitored with an ultraviolet (UV) detector at 280 nm.

For ascorbic acid, serum proteins were precipitated with 60% methanol in water and 1 mM EDTA. Serum (50 µL) was mixed with 200 µL of 60% methanol/water/ EDTA, incubated for 10 min on ice, centrifuged at 12,000 × *g* at 4°C for 8 min, and then filtered. A 20 µL sample of filtrate was immediately analyzed for L-ascorbic acid. A stainless steel column packed with Inersil-ODS (5 mm, 150 mm × 4.6 mm i.d., Gaskurokogyo Ltd., Japan) was used. The mobile phase was methanol/phosphate buffer containing 0.005 M tetra-*n*-butylammonium bromide (20:80, vol/vol) at a flow rate of 0.5 mL/min. The eluate was monitored at 265 nm with a UV detector.

For serum β-carotene, serum was twice deproteinized with ethanol and then extracted with hexane. The extract was centrifuged, the hexane layer removed, and then evaporated with nitrogen. The dried extracts were dissolved in isopropanol for HPLC with a Nova-pak C_{18}. The mobile phase was acetonitrile/methanol/acetone (40:40:20, by vol). The eluate was monitored with a UV detector at 450 nm.

Data analysis. A repeated measure of analysis of variance model was used to determine the effects of DHA on the indices of IR tested. A univariate, split-plot approach was taken using SAS PROC Mixed (32). Contrasts were constructed for within-group comparisons among the stabilization and intervention periods. Paired *t*-test was used to compare PBMNC fatty acid composition and eicosanoid secretions. Changes in the parameters examined are considered significant for $P < 0.05$, unless otherwise stated.

RESULTS

Effect of DHA feeding on NK cell activity. Figure 1 contains the data for NK cell activity for the DHA group determined at the effector/target cell ratio of 50:1 on study days 30, 85, and 113. NK cell activity was not altered by dietary DHA within the first 55 d (study day 85) of its feeding ($P = 0.82$). However, DHA feeding for 83 d (study day 113) caused a 20% decrease in NK cell activity as compared to the activity found prior to the start of DHA feeding (day 30). This decrease in NK cell activity associated with DHA feeding was statistically significant ($P = 0.004$). NK cell activity for the control group did not change throughout the study. Similar results were obtained at the effector/target cell ratios of 25:1, 12.5:1, 6.2:1, and 3.1:1 (data not shown).

Effect of DHA feeding on the secretion of proinflammatory cytokines. The concentrations of IL-1β and TNFα secreted by PBMNC from the subjects in the DHA group are shown in Figures 2 and 3, respectively. DHA feeding caused a reduction in the secretion of both IL-1β and TNFα within 55 d (study day 85), although it was not statistically significant at that time ($P = 0.093$ for IL-1β and 0.31 for TNFα). With the continued feeding of DHA, the secretion of these cytokines continued to decrease. By the end of the study, their concentrations were reduced by approximately 40–45% as compared to the concentrations before DHA was fed ($P = 0.0004$ for IL-1β and 0.0002 for TNFα). In the control group, the secretion of these two cytokines did not change during the study.

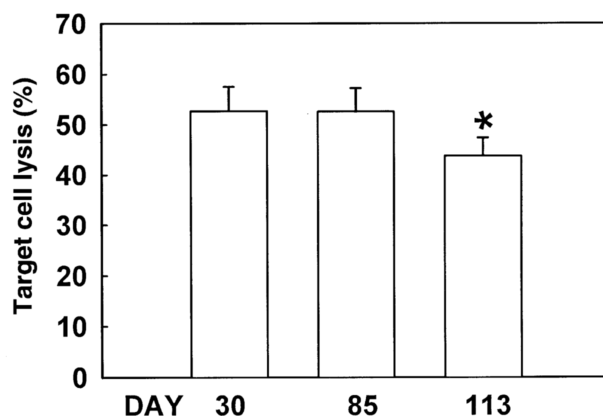

FIG. 1. Docosahexaenoic acid (DHA) feeding decreased natural killer (NK) cell activity. NK cell activity was determined with an effector/target cell ratio of 50:1 and data shown are the mean ± SEM ($n = 7$). NK cell activity was significantly reduced on day 113, compared to the value at day 30 ($P = 0.004$), but not on day 85. The corresponding values for percentage target cell lysis in the control group on days 30, 85, and 113 were 38.5 ± 14.0, 39.0 ± 14.0, and 41.0 ± 18.0, respectively.

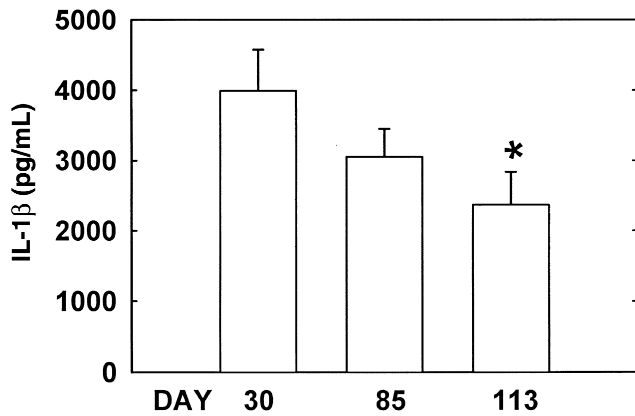

FIG. 2. DHA feeding reduced *in vitro* secretion of interleukin-1β (IL-1β). Peripheral blood mononuclear cells (PBMNC) were cultured with lipopolysaccharide (1.0 mg/L) for 24 h, and the IL-1β secreted was quantified with an enzyme-linked immunosorbent assay (ELISA). Data shown are the mean ± SEM (n = 7). DHA feeding reduced IL-1β secretion, when compared to the value at day 30 (P = 0.093 for day 85 and P = 0.0004 for day 113). The corresponding values for IL-1β secretion in the control group on days 30, 85, 113 were 2912 ± 294, 3104 ± 201 and 2981 ± 678, respectively. See Figure 1 for abbreviation.

DHA feeding and serum influenza antibody titers. The pre- (day 30) and postimmunization (day 113) antibody titers against the three strains of influenza virus for both groups of subjects are shown in Table 4. The preimmunization (day 30) mean titers for A/Johannesburg and B/Harbin strains between the two groups were not different, while the titer for A/Texas strain was about fourfold higher in the DHA group than in the control group. Immunization caused a significant increase (three- to tenfold) in the mean titers for all three strains in both the groups. The postimmunization titers for all three strains were not different between the two dietary groups. However, the differential increase between the two dietary groups after immunization by the A/Texas strain was almost significant (P = 0.059). This difference was most likely due to the higher initial antibody titer for this strain in the DHA group. Antibody titers for all three strains in both dietary groups on day 106 were not significantly different from titers on day 113 (data not shown).

TABLE 4
Effect of DHA Feeding on Serum Influenza Antibody Titers[a]

		DHA group (n = 7)		Control group (n = 4)	
Strain	Day	GM	CI	GM	CI
A/Texas	85	195	98–389	57	23–141
	113	585	278–1234	640	257–1596
A/Johannesburg	85	27	13–56	34	13–88
	113	109	51–234	135	51–354
B/Harbin	85	54	27–109	40	16–101
	113	148	70–311	160	63–405

[a]GM, geometric mean; CI, 95% confidence interval. For other abbreviation see Table 1. All subjects were immunized on day 85. Immunization significantly (P < 0.05) increased the antibody titers for all three strains in both groups. The difference in the increase between the DHA and control group was almost significant (P = 0.059) for A/Texas, but not for the other two strains.

Influence of DHA feeding on the fatty acid composition of PBMNC. Table 5 contains data regarding the major fatty acids of the PBMNC isolated on study days 30 and 113 for both dietary groups. PBMNC fatty acid composition on day 30 was not different between the two dietary groups. Dietary DHA increased the concentration of DHA in the PBMNC from 2.3 wt% on day 30 to 7.4 wt% on day 113. The arachidonic acid content of the PBMNC decreased from 19.8 to 10.7 wt% with DHA feeding. The EPA concentration in PBMNC lipids was 1.3 wt% and it did not change with DHA feeding (not shown). The concentration of major fatty acids in the PBMNC from control group (Table 5), as well as that of other trace fatty acids in both groups (data not shown), did not change during the study.

DHA feeding and eicosanoid secretion. The concentrations of PGE2 and LTB4 secreted into the tissue culture media by the PBMNC stimulated with LPS from both dietary groups are shown in Table 6. DHA feeding caused a 60–75% reduction in the secretion of both these eicosanoids when compared to the concentrations on day 30. Eicosanoids secreted by the PBMNC from control group did not change during the study.

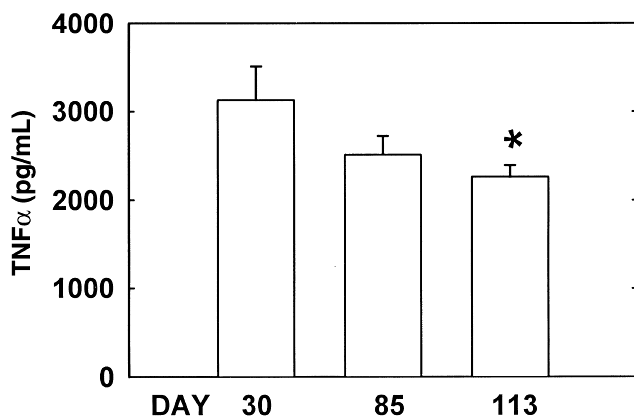

FIG. 3. DHA feeding reduced tumor necrosis factor α (TNFα) secretion. PBMNC culture and TNFα analysis procedures were similar to those given in Figure 2. DHA feeding reduced the secretion of TNFα, with P values of 0.31 and 0.0002, for days 85 and 113, respectively. The corresponding values for TNFα secretion in the control group on days 30, 85, and 113 were 2611 ± 526, 2834 ± 480, and 2648 ± 348, respectively. See Figures 1 and 2 for abbreviations.

TABLE 5
Major Fatty Acids (wt%) of Peripheral Blood Mononuclear Cells[a]

	DHA group (n = 7)		Control group (n = 4)	
Fatty acid	Day 30	Day 113	Day 30	Day 113
16:0	19.2 ± 1.1	18.4 ± 1.2	20.9 ± 1.6	18.9 ± 2.3
18:0	21.2 ± 1.1	18.0 ± 1.1	21.5 ± 0.3	19.1 ± 0.6
18:1n-9	8.0 ± 0.7	8.8 ± 0.8	7.5 ± 0.8	7.5 ± 2.2
18:2n-6	10.2 ± 0.4	12.1 ± 2.7	9.9 ± 0.3	8.7 ± 2.8
20:4n-6	19.8 ± 1.3	10.7 ± 0.8*	20.2 ± 1.4	15.4 ± 0.5
22:6n-3	2.3 ± 0.1	7.4 ± 1.0*	1.5 ± 0.2	1.4 ± 0.1

[a]Mean ± SEM. *P < 0.05. See Table 1 for abbreviation.

TABLE 6
Effect of Dietary DHA on *in vitro* Secretion of Eicosanoids by PBMNC[a]

	DHA group (n = 7)		Control group (n = 4)	
Eicosanoid	Day 30	Day 113	Day 30	Day 113
PGE2, ng/10^6 PBMNC	13.1 ± 2.0	5.0 ± 1.0*	15.1 ± 2.8	17.1 ± 3.0
LTB4, pg/10^6 PBMNC	140 ± 30	34 ± 10*	118 ± 41	92 ± 28

[a]Eicosanoids secreted into the medium (mean ± SEM) in 24 h after LPS (1 mg/L) addition. Abbreviations: PGE2, prostaglandin E2; LTB4, leukotriene, B4; PBMNC, peripheral blood mononuclear cells. For other abbreviation see Table 1. PGE2 was quantified by gas chromatography–mass spectrometry and LTB4 by enzyme-linked immunosorbent assay (ELISA). For each eicosanoid, comparisons were made between day 30 and 113 within each group, and those with the asterisk (*) are significantly different ($P < 0.01$).

TABLE 7
DHA Feeding and Serum Antioxidant Vitamins[a]

	DHA group (n = 7)		Control group (n = 4)	
Vitamin	Day 30	Day 113	Day 30	Day 113
Retinol, μg/mL	0.52 ± 0.06	0.48 ± 0.19	0.40 ± 0.08	0.44 ± 0.06
β-Carotene, μg/mL	78.2 ± 17.1	84.4 ± 17.3	95.3 ± 28.6	113.7 ± 19.9
α-Tocopherol, μg/mL	2.20 ± 0.12	1.82 ± 0.19*	2.37 ± 0.42	1.92 ± 0.44*
Ascorbate, mg/dL	0.48 ± 0.20	0.61 ± 0.28	0.36 ± 0.20	0.52 ± 0.32

[a]Mean ± SEM. *α-Tocopherol concentration on day 113 was lower in both groups than the corresponding values on day 30 ($P < 0.04$); the concentrations of other three vitamins did not change. For abbreviation see Table 1.

In both groups, the concentrations of PGE2 secreted by 1×10^6 PBMNC was about 100-fold higher than that of LTB4.

Effect of DHA feeding on the serum concentration of antioxidant vitamins. The concentrations of retinol, β-carotene, α-tocopherol, and ascorbate in the sera of the subjects in both dietary groups on study days 30 and 113 are shown in Table 7. None of these antioxidant vitamins except α-tocopherol changed during the study in either group. The concentration of α-tocopherol on day 113 was significantly decreased ($P < 0.04$) in both dietary groups when compared to the corresponding values on day 30.

DISCUSSION

This study was conducted to determine whether DHA feeding would inhibit select indices of human IR that have previously been reported to be inhibited with dietary EPA, fish, or flax seed oils. In contrast to most of the previous studies with fish oils, we maintained total fat intake constant, supplemented diets with D α-tocopherol acetate at 20 mg/d, and used diets devoid of EPA. All these factors are important in evaluating the effects of n-3 PUFA on human IR. In an attempt to determine the possible mechanisms underlying the effects of DHA feeding, we examined the fatty acid composition of PBMNC, the production of eicosanoids, the serum concentrations of apoprotein E and antioxidant vitamins.

DHA feeding caused a significant decrease in NK cell activity and the secretion of proinflammatory cytokines, IL-1β, and TNFα by monocytes stimulated with LPS. The time required for the decrease in these functions after DHA feeding was different. After 55 d of DHA feeding, cytokine production was inhibited, but not NK cell activity. However, the NK cell activity was decreased within 83 d of DHA feeding. Our results showing reduction in NK cell activity and the secretion of proinflammatory cytokines by dietary DHA are consistent with the results from earlier human studies involving EPA, fish, or flax seed oils (2–16). Reduction in NK cell activity may lower resistance to cancer and viral infections.

DHA feeding did not alter B-cell functions (Table 4). Nor did it change T-cell functions in this study, as previously reported by us (33). The amount of DHA fed was equivalent to 15–20 g fish oils, which has been found to inhibit T- and B-cell responses within 90 d of its feeding (8–14). Our dietary protocol differed from those used in previous investigations, because we held total fat constant and provided extra vitamin E. These factors may be responsible for our lack of an observed change in T- and B-cell functions.

Dietary DHA increased DHA concentration in PBMNC lipids from 2.3 to 7.4 wt%. It was incorporated into PBMNC lipids primarily by replacing arachidonic acid. Although DHA can be converted back to EPA by humans (34,35), and the serum concentration of EPA in the DHA group of our study subjects increased from 0.38 to 3.39 wt% (36), the concentration of EPA in PBMNC lipids was not altered by DHA feeding (data not shown).

Reduction in PBMNC arachidonic acid concentration with DHA feeding was associated with a concomitant decrease in the production of PGE2 and LTB4. Since PGE2 inhibits NK and macrophage cell functions (24), the decrease in NK cell activity and proinflammatory cytokine production could not be due to reduction in PGE2 production. These results are consistent with the results from other studies using inhibitors of eicosanoid synthesis (37–41). The decrease in immune cell functions was probably not due to increased oxidative stress either, because the serum concentration of retinol, β-carotene, and ascorbic acid were not altered by DHA feeding. The serum concentration of tocopherol decreased in both groups; therefore, it could not be attributed to DHA feeding.

Serum concentration of apoprotein E was not different between the two groups on study day 30 and did not change throughout the study in the control group (not shown). However, its concentration increased from 7.7 ± 1.6 (mean ± SEM) on day 30 to 12.0 ± 1.7 mg/dL on day 113 in the DHA group. Because apoprotein E has been reported to inhibit lymphocyte and monocyte functions (45), it is possible that it contributed to the reduction in NK cell activity and cytokine production in the DHA group. These alterations in immune cell functions may also result from a reduction in LTB4 production, which has been shown to enhance these functions *in vitro* (42,43).

It is possible that some of the effects observed in this study were due to a reduction in linoleic acid intake rather than the increase in DHA intake. The intake of linoleic acid was de-

creased by about 7 g/d in the high-DHA diet compared to the basal diet. The subjects on the high-DHA diet still received more than 20 g/d linoleic acid, an amount of substrate in excess of that required for the fatty acid metabolic acid pathways. It was well above any known requirements for linoleic acid. The plasma linoleic acid content in the basal and high-DHA diets fed subjects was 39 and 36% of total fatty acids, respectively, and was not significantly different (36). The basal diet provided less than 50 mg/d of DHA , and the high-DHA diet provided 6 g/d. Thus it is unlikely that any of the change in immune cell function or eicosanoid production was due to a 20% reduction in linoleic acid intake. These were most likely the result of an increase in DHA intake.

Results from this study show that feeding DHA to humans inhibited select immune cell functions, but it seems to be a less potent and selective inhibitor than that predicted by previous results obtained with fish oils. Although the subjects in the DHA group in our study did not show increased incidence of infections, they may be potentially at a higher risk. The risk for infections may further increase with the increased duration of DHA feeding, increased total fat intake, or increased oxidative stress. The antiinflammatory effects of DHA may be useful in the management of autoimmune disorders; however, such benefits need to be balanced with the increased risk of infections.

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Lipoprotein (a) Metabolism Estimated by Nonsteady-State Kinetics[1]

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ABSTRACT: Lipoprotein (a) [Lp(a)] is a low-density lipoprotein (LDL) particle with an additional apolipoprotein named apo(a). The concentration of Lp(a) in plasma is determined to a large extent by the size of the apo(a) isoform. Because elevated Lp(a) concentrations in plasma are associated with risk for premature coronary heart disease it is important to determine whether variations in production or catabolism mediate differences in Lp(a) concentration. We determined metabolic parameters of Lp(a) in 17 patients with heterozygous familial hypercholesterolemia or severe mixed hyperlipidemia by fitting a monoexponential function to the rebound of Lp(a) plasma concentration following LDL-apheresis. In 8 of those 17 patients this was done twice following two different aphereses. Although this approach allows one to estimate metabolic parameters without the use of a tracer, it requires several major assumptions such as that apheresis itself does not change production or catabolism of Lp(a) and that Lp(a) metabolism can be described by a single compartment. One apheresis decreased Lp(a) concentration by 59.1 ± 8.3%. The fractional catabolic rate (FCR) was 0.16 ± 0.12 d^{-1} and production rate 6.27 ± 5.26 $mg \cdot kg^{-1} \cdot d^{-1}$. However, observed (concentration before first apheresis) and predicted steady-state concentrations differed considerably (more than 20%) in 9 of 17 patients, indicating that not all assumptions were fulfilled in all patients. Production rate but not FCR was correlated with Lp(a) plasma concentration (r^2 = 0.43, P = 0.004) and molecular weight of apo(a) (r^2 = 0.48, P = 0.011), which confirms radiotracer experiments showing that variations in Lp(a) plasma concentrations are due to differences in production not catabolism. When parameters were estimated twice in a subgroup of eight patients, satisfactory reproducibility was observed in six patients. Although parameters determined on two occasions correlated well, only FCR was concordant (intraclass correlation coefficient). Thus, despite the limitations arising from the assumptions implicit to this method, metabolic parameters of Lp(a) can be estimated from the rebound of plasma concentration following apheresis.

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Abbreviations: apo, apolipoprotein; CAD, coronary artery disease; FCR, fractional catabolic rate; LDL, low density lipoprotein; Lp(a), lipoprotein (a); PR, production rate.

Lipoprotein (a) [Lp(a)] is a low density lipoprotein (LDL) particle with an additional apoprotein [apo(a)] linked with a disulfide bridge to apolipoprotein B (apoB) (1,2). An elevated Lp(a) concentration is an independent risk factor for coronary artery disease (CAD), and it has been shown that the Lp(a) concentration in plasma is to a large extent determined genetically with apo(a) polymorphism accounting for more than 50% of the variation observed (3). More than 20 different apo(a) isoforms varying in molecular weight from 300 to over 800 kD have been described, and it has been shown that the smaller isoforms are associated with higher Lp(a) concentrations in plasma (4,5).

Because elevated Lp(a) concentrations in plasma are associated with risk of premature CAD it is important to determine factors that control Lp(a) concentration. Furthermore, knowledge of the underlying metabolic mechanism could indicate possible therapeutic approaches to lower elevated Lp(a) concentrations. Theoretically, an increased concentration in plasma could be the result of an increased production and/or a decreased catabolism.

In studies using radio-iodinated Lp(a) it has been shown that the concentration of Lp(a) in plasma is almost exclusively determined by its production rate (PR) (6,7). Furthermore, it could be shown that the variation of Lp(a) concentrations between subjects characterized by the same apo(a) isoforms is also the result of differences in production and not catabolism (8). However, this method involves the use of radioactive tracers and results may be confounded by the fact that the metabolism of tracer and tracee particles are not identical as it has been shown for other lipoprotein particles (9). An additional disadvantage of radiotracer experiments is that they usually cannot be repeated in the same subjects, thus, intra-individual variability cannot be determined.

Alternatively, Lp(a) metabolism can be determined without the use of a tracer by applying nonsteady-state kinetics (10). This approach is based on the concept that a steady state upset by a defined perturbation will ultimately be restored and that the dynamics of steady-state restoration will only depend on the PR and the rate of catabolism. Lp(a) concentration can be perturbed by selective apheresis of apoB-containing lipoproteins, and the rebound of plasma Lp(a) concentrations can be described by a monoexponential function in which the exponent defines the fractional catabolic rate (FCR) and by

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which a steady-state concentration is predicted. However, this approach implies two essential assumptions: (i) that the perturbation itself has no impact on the metabolic parameters characterizing the steady-state and (ii) that Lp(a) metabolism can be described by a one-pool model. From the form of the rebound curve one cannot deduce whether these assumptions are fulfilled. However, if these assumptions are valid, observed (concentration before first apheresis) and predicted steady-state concentrations should be the same. A particular advantage of this approach is that metabolic parameters can be determined repeatedly in the same subjects.

We evaluated Lp(a) metabolism with this methodology in 17 patients with heterozygous familial hypercholesterolemia or severe mixed hyperlipidemia treated with regular apheresis and compared predicted to observed steady-state concentrations. Furthermore, in eight patients we determined Lp(a) metabolism on two different occasions to determine intraindividual variability.

MATERIALS AND METHODS

Seventeen patients with heterozygous familial hypercholesterolemia or severe mixed hyperlipidemia were included. Inclusion criteria were regularly performed apheresis (weekly or biweekly) for more than 3 mon and steady-state Lp(a) concentration > 10 mg · dL^{-1}. The characteristics of the patients are shown in Table 1. All but three patients (patients #2, #5, #8 in Table 2) were on concomitant hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor therapy (lovastatin, simvastatin, atorvastatin). Patients were treated with three different LDL-apheresis techniques [immunoadsorption—Therasorb (Baxter, Unterschleissheim, Germany); heparin-induced precipitation—HELP (B. Braun, Melsungen, Germany); and adsorption to dextran-sulfate—(Kanegafuchi, Osaka, Japan)], as indicated in Table 2. Details concerning the apheresis protocol are outlined elsewhere (11). During the index apheresis preceding the observed rebound of Lp(a) concentration 3683 ± 1324 mL plasma was processed.

Lp(a) concentration was determined prior to the first apheresis (observed steady-state concentration). After regular apheresis was performed for at least 3 mon Lp(a) concentration in plasma was determined before, immediately after, and then daily for 1 wk following a typical apheresis (index apheresis). In some of the patients Lp(a) concentrations were determined at additional time points for up to 4 wk. In these cases the regular rhythm of weekly or biweekly apheresis was interrupted and the first apheresis following the index apheresis was delayed for up to 4 wk. However, this schedule could not be applied in all patients. Thus, different numbers of data points (between 6 and 12) were used to define the rebound of Lp(a) concentration in plasma following the index apheresis. The number of data points used to define the rebound is indicated in Table 3.

In a subgroup of eight patients metabolic parameters of Lp(a) were determined twice following two different aphereses. The index aphereses following which the parameters were determined were at least 6 mon and up to 10 yr apart.

Lp(a) concentrations were determined in duplicate at each time point by radio immunoassay (until 1997) or nephelometry (since 1998, patients #9, #10, #12, #14, and for all repeated analysis performed in eight patients). For radioimmunoassay a commercially available test kit (intra- and interassay variation of 5 and 9%, respectively; Pharmacia Diagnostics, Uppsala, Sweden) was used as described before (12). Nephelometric determinations were performed using a Behring nephelometer and material (intra- and interassay variation of <2.0 and <3.5%, respectively; Behring, Marburg, Germany). Lipid concentrations were determined by commercially available enzymatic tests (Boehringer Mannheim, Mannheim, Germany). Apo(a) phenotypes were determined with sodium dodecylsulfate polyacrylamide gel electrophoresis followed by immunoblotting and categorized as previously described (5,13).

By using the SAAM-II program (version 1.1, SAAM Institute, Seattle, WA) a curve defined by the following monoexponential equation

$$\text{Lp(a)} = A + [B\cdot(1 - e^{-k\cdot t})] \quad [1]$$

was fitted to the observed Lp(a) concentrations, where Lp(a) = concentration at time t after apheresis, A = Lp(a) concentration immediately after apheresis, B = difference between postapheresis concentration and steady-state concentration, thus: $A + B$ = predicted steady-state Lp(a) concentration, k = FCR. PR is estimated by multiplying the FCR with the pool size at steady-state. Pool size of Lp(a) was determined by multiplying the measured Lp(a) concentration by the estimated plasma volume (0.04 · body weight). Predicted steady-state concentrations were compared to observed steady-state concentrations (concentration before first apheresis), and correlations between kinetic parameters and steady-state concentrations as well as Lp(a) phenotypes were tested.

Fitting a monoexponential equation to Lp(a) concentrations following apheresis to determine kinetic parameters implies that Lp(a) plasma metabolism can be described by a one-pool model, which implies that plasma Lp(a) is kinetically homogenous. This methodology furthermore assumes that apheresis does not alter Lp(a) metabolism, thus that apheresis does not induce a change in FCR or PR.

TABLE 1
Characteristics of Patients (n = 17)

Parameter	Value
Age (yr)	54 ± 11
BMI (kg·m^{-2})	26.2 ± 3.5
Cholesterol[a] (mg·dL^{-1})	360 ± 130
LDL-cholesterol[a] (mg·dL^{-1})	285 ± 127
HDL-cholesterol[a] (mg·dL^{-1})	50 ± 9
Triglycerides[a] (mg·dL^{-1})	126 ± 59
Lp(a)[a] (mg·dL^{-1})	110 ± 71
	(median 110; range 11–245)

[a]Determined prior to first apheresis. Values represent means ± SD. SMI, body mass index; LDL, low density lipoprotein; HDL, high density lipoprotein; Lp(a), lipoprotein (a).

TABLE 2
Lp(a) Phenotypes and Concentrations

Patient	Lp(a) concentration before first apheresis ($mg \cdot dL^{-1}$)	Lp(a) concentration before index apheresis ($mg \cdot dL^{-1}$)	Lp(a) concentration after index apheresis ($mg \cdot dL^{-1}$)	Lp(a) phenotype	Apo(a) MW[a] (kD)	Apheresis[b] technique
#1	28	10	5	S2/S3	610	D
#2	64	41	19	S3/S4	670	D
#3	11	7	3	S2	610	H
#4	150	111	41	S1/S2	530	I
#5	169	78	26	S1-2	560	I
#6	78	62	21	S1/S4	530	H
#7	131	78	38	S1/S4	530	H
#8	182	86	29	S2	610	I
#9	110	81	32	S1-2/S3	560	D
#10	245	91	28	S1-2/S3	560	D
#11	176	133	50	S1/S4	530	I
#12	175	101	39	S1/S2-3	530	H
#13	94	54	16	S1-2	560	I
#14	17	13	6	S2/S4	610	I
#15	162	105	31	S1/S3	530	I
#16	40	18	9	S2/S5	610	I
#17	32	26	13	S4-5	730	D
Mean ± SD	110 ± 71	64 ± 39	24 ± 14	NA[c]	NA[c]	
Median; range	110; 11–245	78; 7–133	26; 3–50			

[a]Molecular weight of the smaller isoform.
[b]D, dextran-sulfate; H, HELP (heparin-induced precipitation); I, immunoadsorption; Apo(a), apoprotein (a); for other abbreviation see Table 1.
[c]Not applicable.

In the subgroup of patients in whom parameters were determined twice, parameters from the two analyses were tested for correlation and concordance (intraclass correlation coefficient). The intraclass correlation coefficient was calculated according to the method of Kramer and Feinstein (14). A coefficient value (R_i) of above 0.73 was considered to indicate concordance.

Correlation analysis tests for interdependence or linear relatedness between two parameters, however, it does not test to which extent the two approaches yield the same result. For quantitative comparisons between the results of the two approaches a concordance index such as the intraclass correlation coefficient is preferable (14,15).

Values are expressed as mean ± SD; in addition Lp(a) concentrations are given as median and range. Correlations were calculated using the Minitab program (Minitab, State College, PA).

TABLE 3
Metabolic Parameters of Lp(a)

Patient	Observed Lp(a)[a] ($mg \cdot dL^{-1}$)	Predicted Lp(a) ($mg \cdot dL^{-1}$)	FCR (d^{-1})	PR ($mg \cdot kg^{-1} \cdot d^{-1}$)	Data points[b]
#1	28	14	0.02	0.43	8
#2	64	140	0.03	1.83	8
#3	11	9.3	0.14	0.51	8
#4	150	151	0.16	9.9	7
#5	169	118	0.24	11.1	9
#6	78	85	0.17	5.7	11
#7	131	87	0.48	10.9	9
#8	182	351	0.03	4.7	12
#9	110	83	0.25	8.3	6
#10	245	216	0.05	4.5	7
#11	176	193	0.22	17	9
#12	175	115	0.36	16.5	9
#13	94	89	0.14	5	9
#14	17	30	0.07	0.78	6
#15	162	184	0.07	5.3	8
#16	40	31	0.12	1.5	8
#17	32	31	0.22	2.8	8
Mean ±SD	110 ± 71	113 ± 88	0.16 ± 0.12	6.27 ± 5.26	8.4 ± 1.5
Median; range	110; 11–245	89; 9–351			

[a]Observed Lp(a) concentration refers to Lp(a) concentration determined before first apheresis.
[b]Number of data points used to fit the monoexponential function. FCR, fractional catabolic rate; PR, production rate; for other abbreviation see Table 1.

RESULTS

Lp(a) concentrations determined before the first apheresis (observed steady-state concentration) as well as before and after the index apheresis are shown in Table 2. On average LDL-apheresis decreased Lp(a) plasma concentration by 59.1 ± 8.3%. This decrease was independent of the apheresis method. These patients were treated regularly with apheresis at weekly or biweekly intervals. Since rebound to steady-state is not complete within this period preapheresis concentrations decrease over time and are usually lower than steady-state concentrations.

In Figure 1 Lp(a) concentrations following apheresis are shown in three patients, representing a wide range of FCR. A monoexponential function (solid line) was fitted to observed Lp(a) concentrations (symbols). Predicted steady-state concentration (dotted line) and Lp(a) concentration prior to the first apheresis (observed steady-state concentration—dashed line) are also shown. There is good agreement between observed and predicted steady-state concentration in one patient (Fig. 1A) whereas these values were different in the other two patients (Figs. 1B and 1C). In Figure 2 Lp(a) concentrations are shown for three of the eight patients in whom Lp(a) metabolism was determined twice. In two of the three patients (patterns C–F) the rebound is similar, whereas in the other patient (pattern A and B) rebound curves were different on the two occasions.

Table 3 shows the calculated metabolic parameters. The estimated FCR (0.16 ± 0.12 d^{-1}) and PR (6.27 ± 5.26 $mg \cdot kg^{-1} \cdot d^{-1}$) varied over a wide range. Metabolic parameters

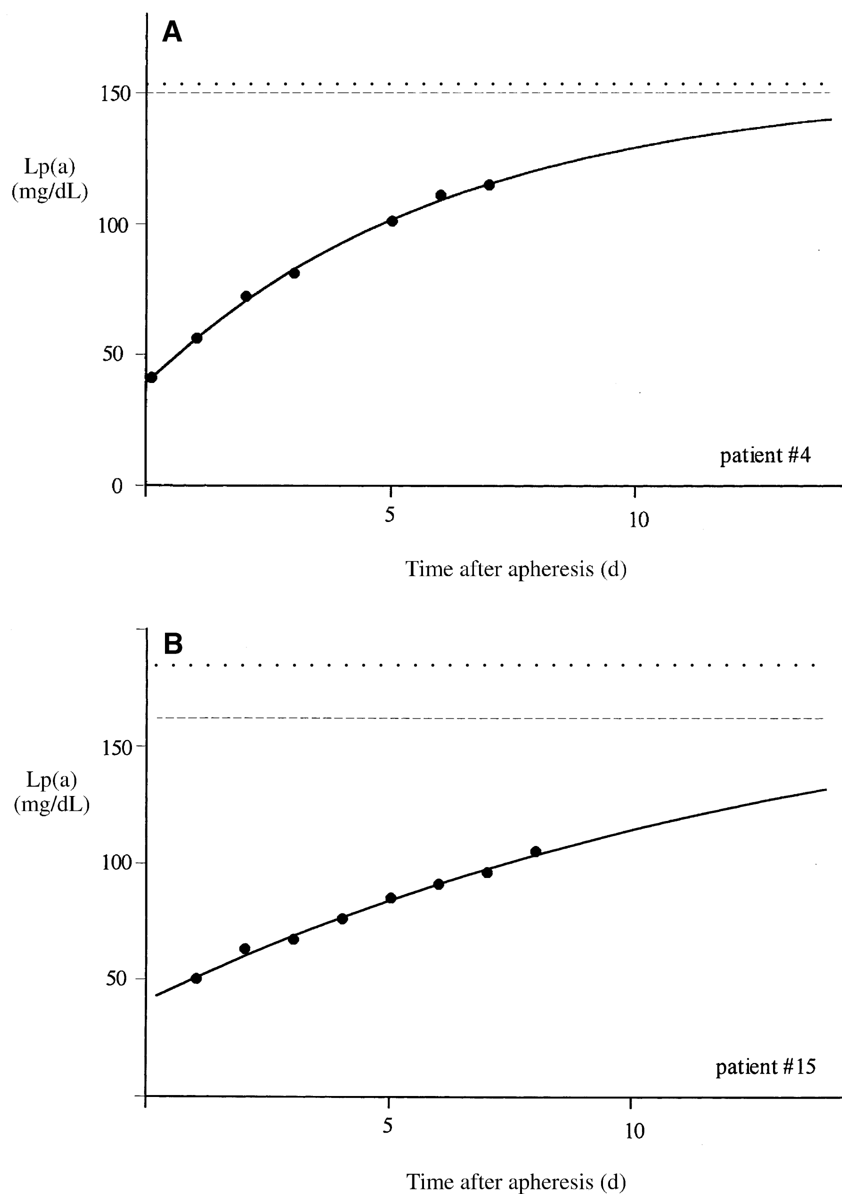


FIG. 1. (continued)

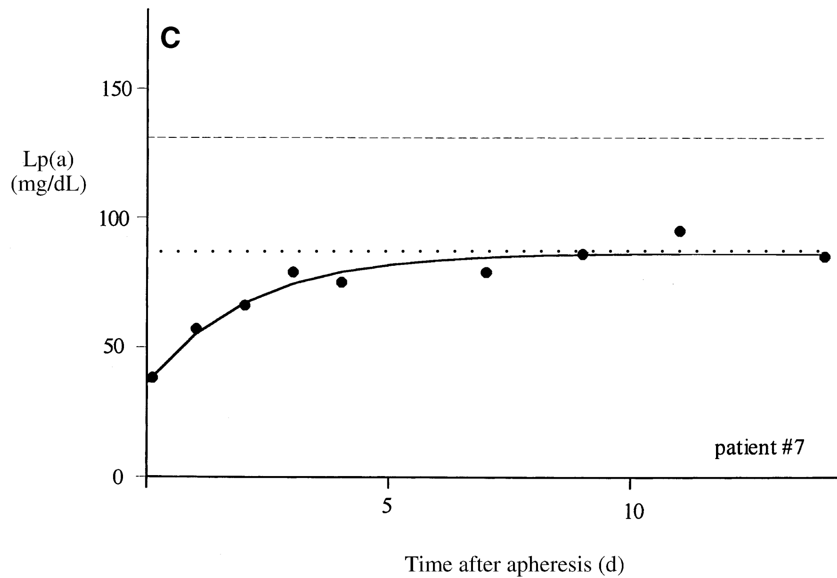


FIG. 1. Time course of lipoprotein (a) [Lp(a)] plasma concentration in three patients representing a wide range of fractional catabolic rate (A, patient #4: 0.16 d^{-1}; B, patient #15: 0.07 d^{-1}, C, patient #7: 0.48 d^{-1}). Symbols represent observed concentrations during rebound, solid line (—) indicates the fit of the monoexponential function and dashed and dotted lines correspond to observed (concentration before first apheresis) and predicted steady-state concentration, respectively.

were not related to the apheresis technique used (data not shown). The analysis predicted steady-state Lp(a) concentrations, which in 9 out of 17 patients were considerably different (more than 20%) from observed steady-state concentrations (concentration before first apheresis). Whether or not predicted and observed steady-state concentrations were similar did not depend on whether one or two apo(a) isoforms were detectable and did not depend on the Lp(a) concentration.

Figure 3 shows observed steady-state Lp(a) concentrations as dependents of Lp(a) PR. PR (r^2 = 0.43, P = 0.004) but not FCR (r^2 = 0.03, P = 0.5) was correlated with plasma Lp(a) concentration. Similarly, PR (r^2 = 0.41, P = 0.005) but not FCR (r^2 = 0.13, P = 0.15) was correlated with the molecular weight of the apo(a), thus the apo(a) isoform (Fig. 4). Furthermore, in the subgroup of five patients with the apo(a) isoform S2, Lp(a) concentration was correlated with Lp(a) production (r^2 = 0.98, P = 0.002) but not with Lp(a) FCR (r^2 =

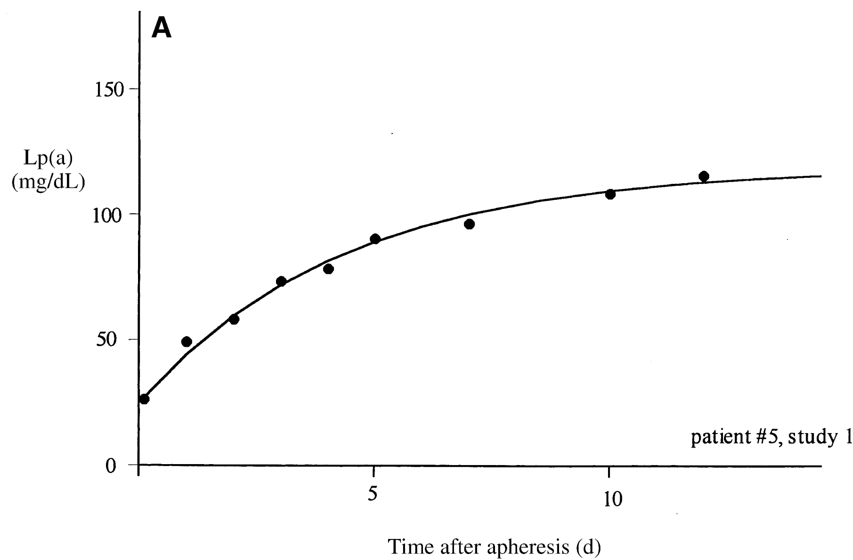
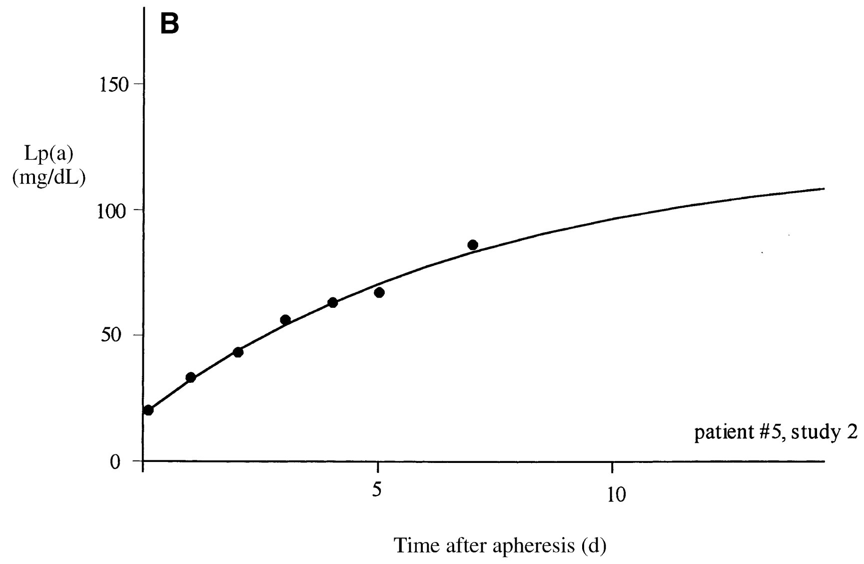
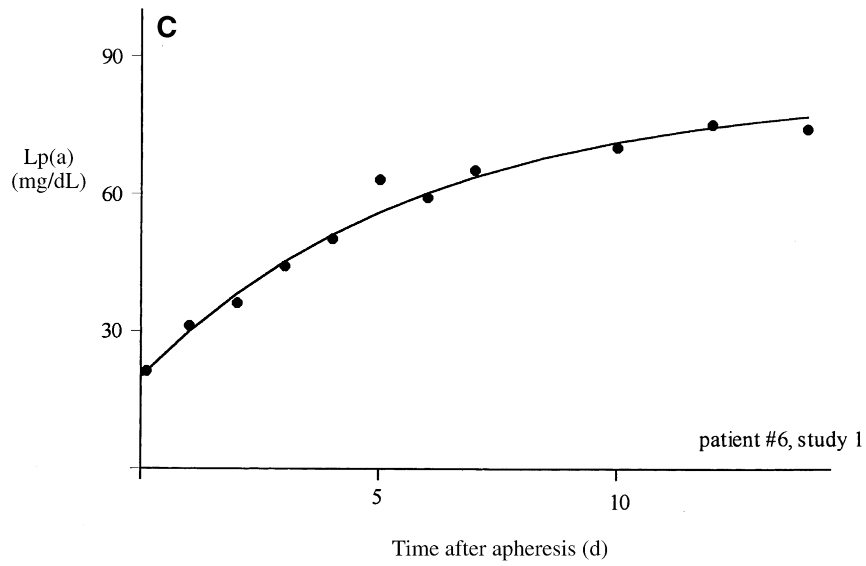


FIG. 2. (continued)





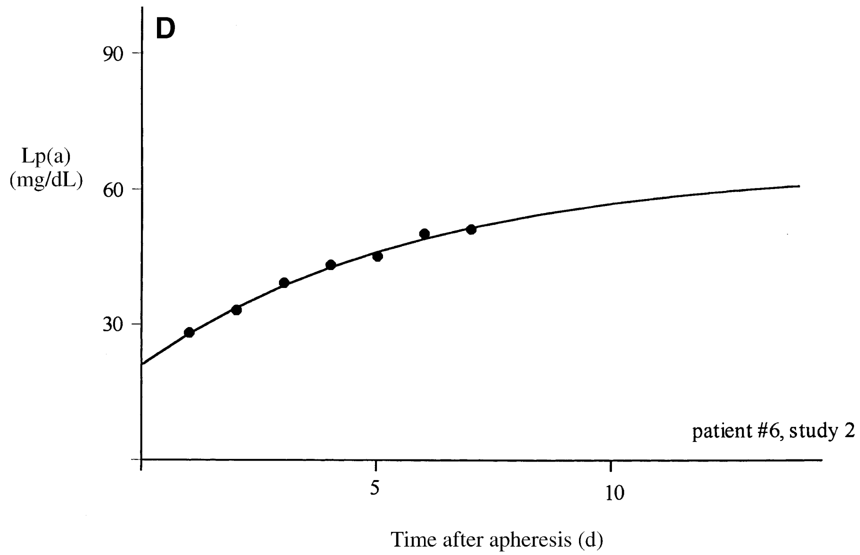


FIG. 2. (continued)

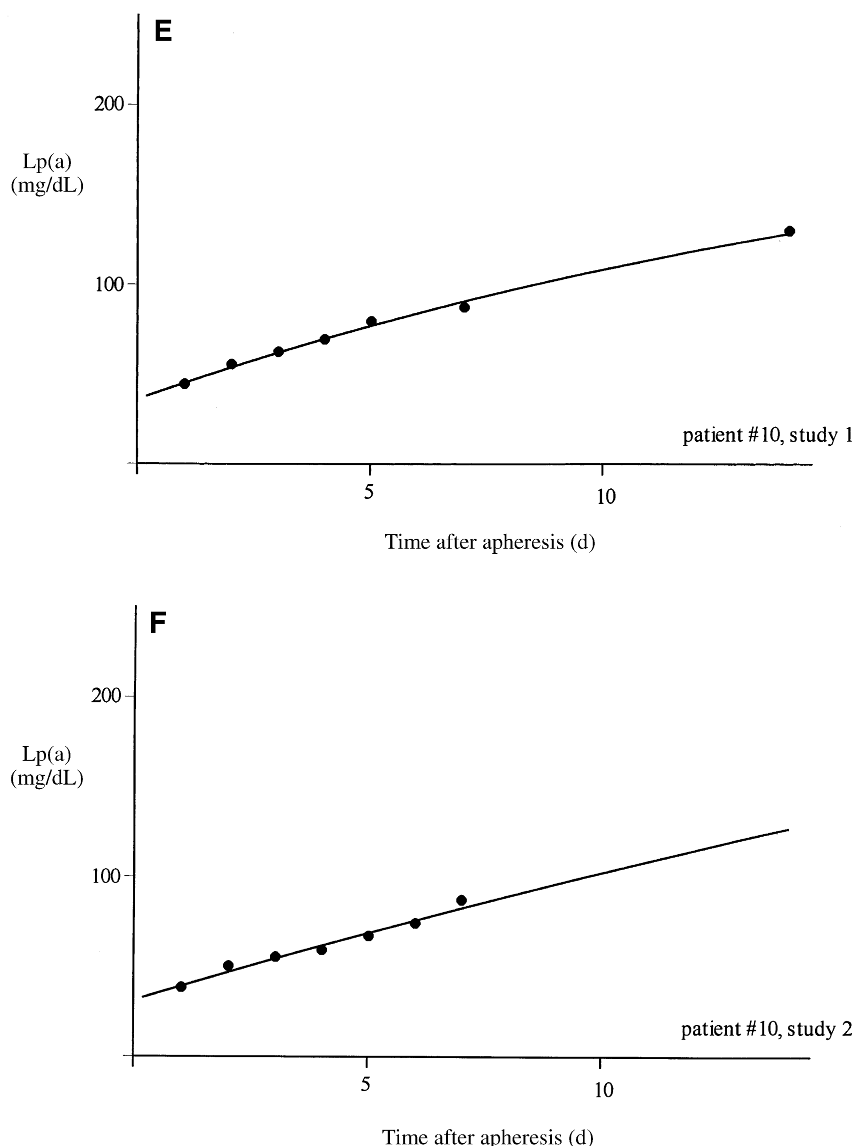


FIG. 2. Time course of Lp(a) plasma concentration in three patients (#5, #6, #10) in whom Lp(a) metabolism was determined twice following two different aphereses. Symbols represent observed concentrations during rebound and the solid line indicates the fit of the monoexponential function. While in two patients (#6, patterns C and D; #10, patterns E and F) the rebound curves are similar, there is some variation between the two analyses in the other patient (#5, patterns A and B). For abbreviation see Figure 1.

0.25, $P = 0.39$). In patients with more than one isoform the smaller isoform (major isoform) was used for these analyses, since the smaller isoform is predominantly associated with the plasma Lp(a) concentration (4,5).

In eight patients Lp(a) metabolism was determined twice (Figs. 2 and 5, Table 4). PR and FCR determined independently following two different aphereses up to 10 yr apart correlated well (PR, $r^2 = 0.883$, $P = 0.0005$; FCR, $r^2 = 0.856$, $P = 0.001$), but only FCR was concordant ($R_i = 0.731$), while PR was not ($R_i = -1.14$). When the parameters were looked at by pairwise comparison (Fig. 5), PR and FCR were each reproducible in six patients, although we observed some random variation in two subjects (FCR: #5 and #11; PR: #5 and #7).

DISCUSSION

Because elevated Lp(a) concentration in plasma is associated with the risk for premature CAD it is important to determine factors that control its concentration. We determined parameters of Lp(a) metabolism in a group of 17 hyperlipoproteinemic patients without the use of a tracer by estimating the FCR from the analysis of the rebound of Lp(a) plasma concentra-

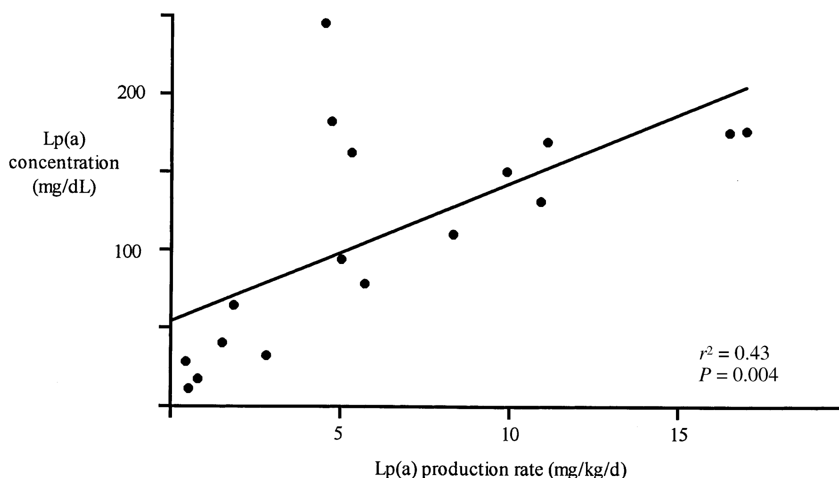


FIG. 3. Correlation (linear regression analysis) of plasma Lp(a) concentration and Lp(a) production rate. For abbreviation see Figure 1.

tion following apheresis. The metabolic parameters obtained by this approach are similar but not identical to those reported from studies using radioactive tracers. As reported in tracer studies we found that (i) Lp(a) production and not catabolism determines plasma concentrations, (ii) the inverse association of Lp(a) concentrations with apo(a) isoforms is due to differences in production and not catabolism, and (iii) differences in Lp(a) concentrations among individuals with the same apo(a) isoform are due to differences in production and not catabolism. Furthermore, in a subgroup of eight patients parameters were determined twice following two different aphereses. Pairwise comparison of FCR and PR determined on both occasions revealed that parameters were similar in six patients but showed some degree of random variation in two patients.

In a series of studies using radiolabeled material Rader *et al.* (6,8) reported FCR of Lp(a) of $0.30 \pm 0.06\ d^{-1}$ (compared to $0.16 \pm 0.12\ d^{-1}$ in our study) and production rates of 2.1 ± 2.7 mg·kg^{-1}·d^{-1} (compared to 6.25 ± 5.26 mg·kg^{-1}·d^{-1} in our study). However, these subjects had considerably lower Lp(a) concentrations (average 18.5 ± 27.5 compared to 98 ± 73 mg·dL^{-1} in this study). A consistent finding in both studies was the observation that PR and not FCR determines Lp(a) concentrations. Despite some discrepancies with regard to absolute values the overall conclusions are very similar: PR and not FCR determines Lp(a) concentrations, the inverse association of Lp(a) concentrations with apo(a) isoforms is due to differences in PR and not catabolism, and finally differences in Lp(a) concentrations among individuals with the same apo(a) isoform seem to be due to differences in production and not catabolism (6,8).

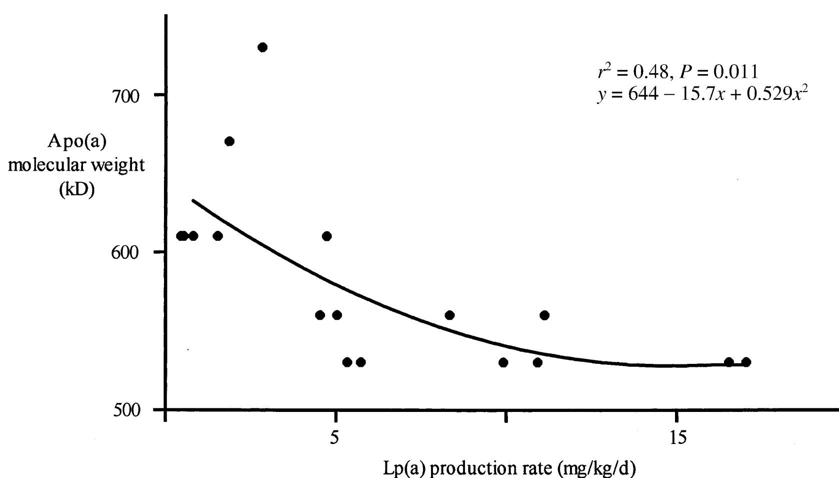


FIG. 4. Correlation (quadratic regression analysis) of the molecular weight of the predominant apoprotein (a) [apo(a)] isoform and Lp(a) production rate. In patients with more than one isoform the molecular weight of the smaller isoform (predominant isoform) was used for analysis. For abbreviation see Figure 1.

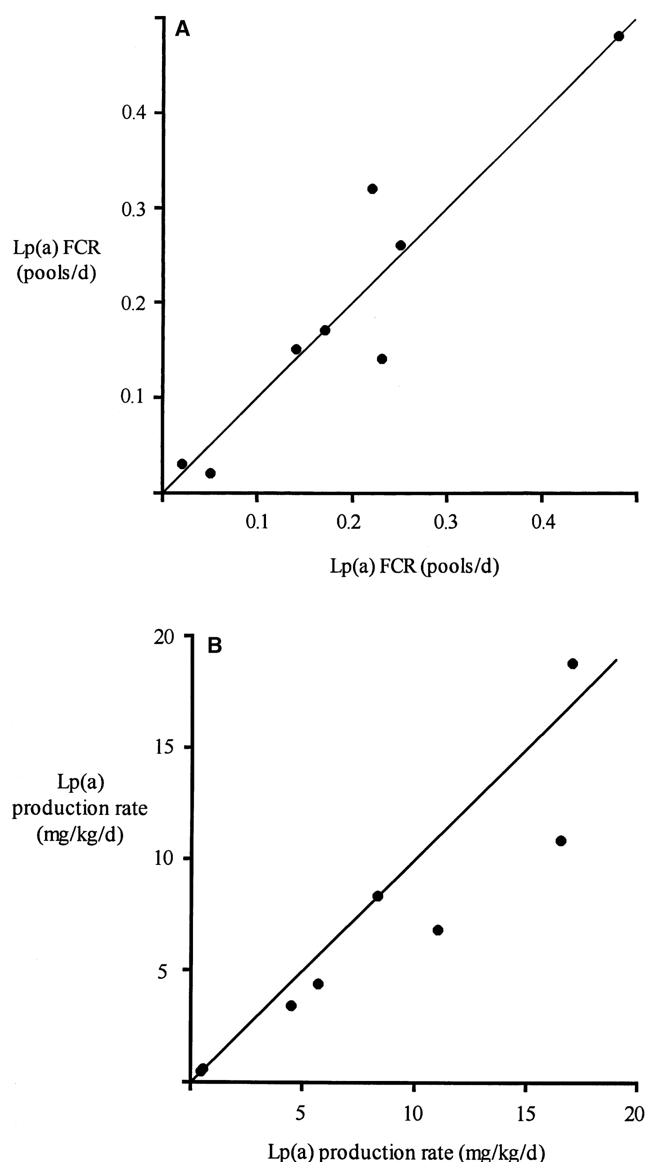


FIG. 5. Fractional catabolic rate (FCR) (A) and production rate (B) estimated on twp different occasions following two different aphereses (between 6 mon and 10 yr apart), with line of equality.

Compared to the parameters published by Rader *et al.* (6,8) the FCR obtained in this study vary over a wider range (0.02–0.48 d^{-1} as compared to 0.22–0.39 d^{-1}). The reason for this variance is unknown, but may be related to several aspects. First, there may be a wide natural variation particularly in such a heterogeneous group of patients as studied here. This heterogeneity is probably further enhanced by the fact that most patients were on concomitant hydroxymethylglutaryl-CoA (HMG-CoA) reductase inhibitor therapy, although such therapy does probably not significantly affect Lp(a) metabolism. Second, in some patients apheresis may affect parameters more than in others. In this respect it is interesting that in the eight patients in whom observed (concentration before first apheresis) and predicted steady-state concentrations were close to each other FCR varied less (FCR, 0.15 ± 0.06 d^{-1}) than in the nine patients in whom predicted and observed steady-state concentrations were different (FCR, 0.18 ± 0.16 d^{-1}). Finally, heparin, which is given as anticoagulant in apheresis, can be considered a further modifying factor perturbing lipoprotein metabolism to some extent.

Our Lp(a) FCR are in better accordance with results published by Armstrong *et al.* (10) who used a similar experimental approach. Values were in the range of 0.05 to 0.18 d^{-1} in a group of five patients with heterozygous familial hypercholesterolemia using a similar approach. Similar to our patients, Lp(a) concentrations were relatively high, but it is unclear whether the analysis predicted steady-state concentrations similar to the observed values.

Our study confirms that metabolic parameters of Lp(a) can be estimated without the use of a tracer. The methodology is based on the concept that metabolic parameters can be estimated from the dynamics by which a steady-state is restored after it has been perturbed by apheresis. However, it is important to understand the limitations of such an approach, which may explain why metabolic parameters obtained with tracer studies and those obtained with this approach differ and why observed (concentration before first apheresis) and predicted steady-state concentrations do not coincide in 9 of 17 patients. The use of the methodology presented here implies several assumptions.

First, this approach implies that apheresis does not alter Lp(a) metabolism, i.e., that apheresis does not affect the production and/or catabolism of this particle. While it has been shown that LDL-apheresis does not alter apoB metabolism in the long term (16), it has been demonstrated *in vivo* (17) and *in vitro* (18) that LDL-receptor activity is increased immediately after apheresis. However, no studies are available examining the effect of apheresis on Lp(a) metabolism. Although Lp(a) is an LDL particle with an additional apoprotein [apo(a)], its metabolism is very different from regular LDL and it is known that the LDL-receptor is not necessary for normal Lp(a) catabolism (19). Whether apheresis changes Lp(a) metabolism is unknown at present. If Lp(a) FCR increases following an acute reduction of pool size (plasma concentration), this would considerably limit the validity of this approach. This could not be overcome by replacing the monoexponential function by a multiexponential one. Such an increase in FCR after apheresis could be observed, if the catabolic pathways operate at saturation before apheresis. For LDL-apoB metabolism it has been pointed out that the rebound analysis does not provide direct information whether LDL-apoB FCR is affected by pool size (20).

Second, the use of a monoexponential equation to describe the rebound of Lp(a) concentration in plasma implies that Lp(a) metabolism can be described by a one-compartment model, with all Lp(a) in plasma being kinetically homogenous. In the case of patients with more than one apo(a) isoform this assumption necessarily represents a simplification, since each Lp(a) contains only one molecule of apo(a) and it is unlikely that Lp(a) particles with different apo(a) moieties behave iden-

TABLE 4
Metabolic Parameters of Lp(a) Determined from Two Independent Rebound Analyses[a]

Patient (months between studies)	Lp(a) before apheresis ($mg \cdot dL^{-1}$)		Lp(a) after apheresis ($mg \cdot dL^{-1}$)		PR ($mg \cdot kg^{-1} \cdot dL^{-1}$)		FCR (d^{-1})	
	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2	Study 1	Study 2
#1 (39)	10	11	5	5	0.43	0.45	0.02	0.03
#3 (33)	7	8	3	3	0.51	0.57	0.14	0.15
#5 (52)	78	71	26	20	11.01	6.82	0.23	0.14
#6 (70)	62	49	21	21	5.66	4.38	0.17	0.17
#7 (69)	78	60	38	22	16.50	10.85	0.48	0.48
#9 (10)	81	72	32	31	8.30	8.33	0.25	0.26
#10 (8)	91	97	28	26	4.46	3.40	0.05	0.02
#11 (125)	133	121	50	50	17.01	18.80	0.22	0.32
Mean ± SD	68 ± 42	61 ± 39	25 ± 16	22 ± 15	8.0 ± 6.5	6.7 ± 6.1	0.19 ± 0.14	0.20 ± 0.15
Median; range	78; 7–133	66; 8–121	27; 3–50	22; 3–50				

[a]For abbreviations see Tables 1 and 3.

tically in kinetic terms. If Lp(a) is not kinetically homogenous a sum of exponentials would be necessary to describe the rebound of Lp(a) following apheresis. However, the concentration data defining the rebound are not sufficient to define a second compartment (second exponent) with sufficient accuracy.

Whether these assumptions are correct cannot be deduced from this study. The observations that the rebound analysis does not predict steady-state concentrations correctly in half of our patients (Table 3) and that there is a discrepancy between the parameters found here compared to those defined by tracer studies indicate that some of these assumptions are not entirely fulfilled in some patients.

Estimating kinetic parameters of Lp(a) metabolism from the rebound following apheresis offers the particular advantage that analyses can repeatedly be performed in the same subject. Up till now, Lp(a) metabolism has not been determined more than once in a given subject, and therefore data on intra-individual variation are not available. We determined Lp(a) metabolism on a second occasion following a different apheresis in eight patients and found, as expected, parameters to be highly correlated. However, the intraclass correlation coefficient may be a more appropriate statistical method to test for concordance and thus reproducibility (14,15). Although both parameters correlated well, only FCR but not PR estimated at the two occasions were concordant. Pairwise comparison revealed satisfactory reproducibility in six out of eight patients (Fig. 5). In two patients (#5 and #11) FCR and in two patients (#5 and #7) PR differed between the two studies. Several explanations are possible: first, some variance in Lp(a) metabolism may exist. The period between the two analyses was longest in those patients in whom parameters differed. Second, the observed difference may be related to the method used for determination of Lp(a) concentration. The estimation of the parameters is obviously dependent on the quality of the concentration measurement and is very sensitive to the data obtained early after the index apheresis. It is well known that the determination of Lp(a) concentration is associated with a considerable error when performed with a radioimmunoassay. The variation coefficients determined in our laboratory (intra-/interassay variation of 5/9% and <2.0/<3.5% for the radio immunoassay and the nephelometric determination, respectively), however, indicate that the differences may not be solely due to the different methods to measure Lp(a) concentration.

Although tracer studies performed in steady state represent the gold standard to determine metabolic parameters, the injection of radiolabeled material is not without problems. Results may be confounded by nonuniform labeling (9), by changes of particle properties by iodination, and by problems associated with the Lp(a) used for labeling (6). Furthermore, a recently published study (21) examining renovascular arteriovenous differences in Lp(a) plasma concentrations indicated much higher production rates of Lp(a) than suggested by the exogenous tracer studies or by the present study. However, studies (22,23) using endogenous labeling with amino acids labeled with stable isotopes, which are now considered gold standard for the evaluation of lipoprotein metabolism (24,25), indicate that Lp(a) production is not as high as suggested from the renal arteriovenous concentration difference.

Given the limitations of the different methods applied, the conclusions from tracer studies and from our own study are remarkably similar.

We conclude that metabolic parameters of Lp(a) can be estimated from the rebound of Lp(a) concentrations following apheresis without the use of a tracer. This approach also allows repeated measurements of Lp(a) metabolic parameters, which in eight patients showed similar albeit not identical results on two occasions. Furthermore, in this group of hyperlipoproteinemic patients differences in Lp(a) plasma concentration are due to differences in production and not in catabolism. Our study also indicates the limitations of such a nonsteady-state approach.

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Fatty Acid Profile of Buccal Cheek Cell Phospholipids as an Index for Dietary Intake of Docosahexaenoic Acid in Preterm Infants

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ABSTRACT: Cheek cells (buccal epithelia) were utilized as a noninvasive index of fatty acid status in a study of the effects of n-3 long chain polyunsaturated fatty acid supplementation on visual function in preterm infants. The fatty acid profile of cheek cell phospholipids was directly correlated with the dietary docosahexenoic acid (DHA) intake of infants receiving: (i) primarily human milk; (ii) n-3 fatty acid-deficient, corn oil-based, commercial formula (CO); (iii) α-linolenic acid-enriched, soy oil-based, commercial formula; or (iv) experimental formula enriched with soy and marine oils providing a DHA level equivalent to that in human milk. In a subset of infants with complete cheek cell fatty acid profiles and visual function assessments, preterm infants at both 36 wk ($n = 63$) and 57 wk ($n = 45$) postconceptional age had significantly ($P < 0.0005$) reduced cheek cell phospholipid DHA levels in the n-3-deficient, CO-fed group compared to the other diet groups. The DHA content in cheek cell phospholipids was highly correlated ($P < 0.0005$) with that of both red blood cell lipids and plasma phospholipids at the 36- and 57-wk time points. The DHA content in cheek cell lipids of infants at 36 wk was significantly correlated with electroretinographic responses ($r = -0.29$; $P < 0.03$) and visual acuity ($r = -0.31$; $P < 0.02$) as measured by visual-evoked potentials (VEP). Cheek cell DHA was highly correlated ($r = -0.57$; $P < 0.0005$) with VEP acuity at the 57-wk time point. These results suggest that the fatty acid profile of cheek cells is a valid index of essential fatty acid status, can be monitored frequently, and is associated with functional parameters in infants.

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Recently, a number of randomized and nonrandomized clinical trials have been conducted to assess the impact of enrichment of formulas with long chain polyunsaturates (LCP) on the biochemical and functional development of both term and preterm infants. Most investigations have utilized either plasma or red blood cell (RBC) lipid profiles to document compliance and to provide indices of tissue fatty acid status. Typically, infant blood samples are obtained from either the antecubital vein or by heel stick; both methods are painful and can be traumatic for the infant and attending parent. As an alternative, human cheek cells (buccal epithelia) can be isolated by noninvasive procedures and represent an accessible tissue for investigating the influence of diet on the infant's fatty acid nutritional status (1,2). Subsequent extraction of lipids and separation of cheek cell phospholipids provide a lipid fraction representative of fatty acids in cellular membranes.

Buccal cheek cells were collected during a clinical trial designed to evaluate the role of the dietary essential n-3 fatty acid, α-linolenic acid (LNA; 18:3n-3), and its long chain metabolite, docosahexaenoic acid (DHA; 22:6n-3), in early visual development in preterm infants. Details of the infant cohort, diets, and methods have been presented previously (3–8). We report here on the utility of cheek cell phospholipids to accurately reflect the impact of fatty acid modifications to formula on cell membrane composition. A second goal was to determine whether, as for RBC and plasma lipids (3–8), these fatty acid modifications are correlated with visual development in preterm infants.

MATERIALS AND METHODS

Subjects. Preterm infants born at 27–33 wk postconception participated in this prospective, randomized clinical trial conducted between 1987 and 1991. Inclusion and exclusion criteria and additional clinical details for the study have been previously reported (3,8).

The current report includes a subset of infants, tested at 36 wk postconception ($n = 63$) and at 57 wk ($n = 45$), that had fatty acid measurements of cheek cell phospholipids, RBC total lipids, and plasma phospholipids as well as complete visual function data.

Diets. Infants were randomized to one of three formula groups or belonged to a reference group whose mothers chose to provide primarily human milk (HM). One group received a standard commercial preterm infant formula based on corn oil (CO) with a relatively low content of n-3 fatty acids [i.e., 24.2% linoleic acid (LA; 18:2n-6); 0.5% LNA; no DHA; and

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Abbreviations: CO, corn oil-based commercial formula; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; ERG, electroretinography; HM, human milk-fed, LA, linoleic acid; LCP, long chain polyunsaturated fatty acids with >18 carbon atom chain; LNA, linolenic acid; MO, soy/marine oil-enriched formula; P/S, polyunsaturate-to-saturate; RBC, red blood cell; SO, soy oil-enriched formula; VEP, visual-evoked potentials.

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an n-6/n-3 fatty acid ratio of 48.2]. The formula enriched with soy oil (SO) supplied 20.8% LA, 2.7% LNA, no DHA, and had an n-6/n-3 ratio of 7.7. The formula enriched with soy and marine oils (MO) provided 20.4% LA, 1.4% LNA, 0.35% DHA, and had an n-6/n-3 ratio of 8.5. The latter formula was designed to provide a fatty acid profile closer to HM (i.e., 12.7% LA, 0.8% LNA, 0.29% DHA, with an n-6/n-3 ratio of 11.4). The "reference standard" HM-fed group received their own mother's milk following Parkland Hospital (Dallas, TX) nursery procedures (including hepatitis B and HIV screening of mothers); an Egnell pump (Lact-e; Hollister, Inc., Libertyville, IL) was used for milk collection with aseptic handling and storing. If milk was intended for use within 24 h, it was refrigerated; otherwise it was frozen and used within 3 wk. This subgroup of six HM-fed infants received more than 75% of their energy intake from HM by 36 wk and 63% through 57 wk. During periods of limited milk supply, formula MO was provided because its fatty acid profile most closely matched that of HM. Details of the diet composition have been published (3,6).

Biological sampling and analysis. Blood samples were obtained by venipuncture from a small arm vein. Cheek cells (buccal mucosal epithelia) were obtained by scraping the inside of the cheek (about 10 strokes) with a sterile wooden spatula (6 × 125 mm) after gently cleaning the mucosa with a gauze soaked in saline. Cells were suspended in 8 mL sterile saline and the process repeated on the second cheek. Cheek cells were precipitated from the suspension by centrifugation at 3,000 × g for 10 min. Lipids from plasma, RBC, and the cheek cell pellet were extracted as previously described (6) within 2–4 hours of harvest, stored in solvent containing 0.02% butylated hydroxytoluene, and kept under N_2 at −20°C for a maximum of 1 wk. Collection of blood and cheek cell samples coincided with visual function measurements at both 36- and 57-wk time points. Both plasma and cheek cell phospholipids were isolated by thin-layer chromatography using a hexane/diethyl ether/acetic acid (80:20:1, by vol) solvent system. Analysis of the phospholipid fraction of buccal cheek cells eliminated contamination of the sample by fatty acids derived from formula present in the mouth during sampling. Fatty acid methyl esters prepared from plasma, RBC, and cheek cell lipids were analyzed by capillary gas chromatography as previously described (6). Data for plasma phospholipids and RBC fatty acids have been published (6,7).

Visual function tests. Electroretinography (ERG) measures the summed electrical response of the retina to a light stimulus and reflects the overall health of the neural retinal layer. Pattern-reversal visual-evoked potential (VEP) visual acuity is a measure of the neural integrity of the pathway from the retina to the primary visual cortex. These procedures have been described in detail elsewhere [ERG (3,4); VEP (5)] and will be summarized briefly here.

Full-field ERG responses to short- and long-wavelength stimuli over an extensive range of retinal illuminances were evaluated at 36- and 57-wk postconceptional age. The ERG response to short wavelength stimuli was used to help isolate rod photoreceptor function. Naka–Rushton plots were computed from rod responses to graded illuminances to derive rod thresholds, defined as the light intensity required to produce a 2 μV response. Thus, lower threshold values reflect healthy, more mature retinal photoreceptors.

VEP were assessed using black-and-white checkerboard stimuli presented on a video monitor with pattern reversals at a rate of 3.8 per second. Responses from three electrodes pasted on the scalp were recorded on tape and subsequently filtered digitally and analyzed. The VEP acuity was derived from peak-to-peak amplitude responses to variations in spatial frequencies of the grating pattern. Visual acuities are reported as Snellen equivalents; the average for normally sighted adults is 20/20 and the average for normally sighted full-term infants at 57 wk postconception is 20/70 (5).

Data analysis. Analysis of variance was used to analyze the effects of the four diets on fatty acid levels of infant groups at each time period. Nonparametric multiple comparison analysis of diet group results was conducted using the Newman–Keuls procedure at an α of 0.05. Visual function data were log transformed prior to Pearson correlation analysis.

RESULTS

Cheek cell fatty acids as a dietary index. Fatty acid profiles in cheek cell phospholipids obtained from infant study groups are presented for the 36- and 57-wk time points in Tables 1 and 2, respectively. Significant differences among infant groups were found for numerous individual fatty acids, fatty acid ratios, and summation data. Of particular importance, LA levels in cheek cells at the 36-wk time point were significantly lower in the HM-fed infants than formula-fed infants, reflecting the 40 to 50% lower level of LA in HM compared to the formulas. Elevated levels of arachidonic acid (20:4n-6), the n-6 end-product docosapentaenoic acid (DPA; 22:5n-6), and the sum of n-6 LCP in the essentially n-3 fatty acid-deficient CO group compared to the infant groups receiving n-3 fatty acid-enriched formula are consistent with competition for incorporation into phospholipids between the n-6 and n-3 series of fatty acids. Furthermore, reduced levels of the n-3 fatty acids and the n-3-to-n-6 LCP ratio in the CO diet group at 36 wk were consistent with the n-3 fatty acid composition of the formulas. The n-6 and n-3 LCP profiles in the experimental MO formula group were nearly identical to those of the HM-fed group with the only exception being the eicosapentaenoic acid level (20:5n-3); this n-3 fatty acid is typically present in menhaden fish oil at twice the concentration of DHA. Second-generation formulas used in current clinical trials to evaluate efficacy of LCP supplementation use DHA sources such as marine algae, egg yolk phospholipids, or select fish oils, all with little or no eicosapentaenoic acid and preferable to menhaden fish oil.

At 57 wk postconception (Table 2), differences in monounsaturates and LA among groups were no longer evident. However, significant differences in major n-3 LCP persisted such that the cheek cell level of DHA and the DHA/DPA ratio

Table 1
Fatty Acid Profiles in Cheek Cell Phospholipids of Study Infants at 36 Wk Postconception[a]

Fatty acid	HM[b] (n = 6)	CO[b] (n = 17)	SO[b] (n = 19)	MO[b] (n = 21)	ANOVA, P
Total saturates[c]	37.9 ± 4.2	40.4 ± 4.3	42.6 ± 4.9	40.1 ± 5.1	0.151
Total monounsaturates[d]	39.2 ± 4.9[e]	33.8 ± 5.5[f]	32.1 ± 3.2[f]	32.8 ± 2.8[f]	0.003
LA (18:2n-6)	12.4 ± 2.1[e]	17.5 ± 3.5[f]	15.5 ± 2.9[e,f]	16.7 ± 4.4[f]	0.002
20:3n-6	1.82 ± 0.60	1.81 ± 0.49	1.49 ± 0.38	1.58 ± 0.55	0.191
20:4n-6	2.54 ± 0.38[e]	3.82 ± 1.53[f]	2.99 ± 0.71[e]	2.67 ± 0.94[e]	0.007
22:4n-6	0.62 ± 0.47	0.69 ± 0.40	0.45 ± 0.28	0.61 ± 0.46	0.330
DPA (22:5n-6)	0.31 ± 0.10[e]	0.57 ± 0.16[f]	0.33 ± 0.19[e]	0.32 ± 0.24[e]	0.001
Sum n-6 LCP	6.12 ± 1.50[e,f]	7.42 ± 2.07[e]	5.67 ± 1.11[f]	5.69 ± 1.45[f]	0.003
LNA (18:3n-3)	0.52 ± 0.17[e,f]	0.50 ± 0.21[e]	0.97 ± 0.27[g]	0.83 ± 0.51[f,g]	0.001
20:5n-3	0.46 ± 0.37[e]	0.17 ± 0.14[f]	0.24 ± 0.17[e,f]	0.70 ± 0.32[g]	<0.0005
DHA (22:6n-3)	0.93 ± 0.42[e]	0.35 ± 0.24[f]	0.65 ± 0.28[e]	0.91 ± 0.43[e]	<0.0005
Sum n-3 LCP	3.19 ± 1.89[e]	1.01 ± 0.98[f]	1.26 ± 0.56[f]	2.72 ± 1.19[e]	<0.0005
DHA/DPA ratio	2.64 ± 1.19[e,f]	0.87 ± 0.57[g]	2.41 ± 1.37[f]	3.75 ± 2.29[e]	<0.0005
n-3/n-6 LCP ratio	0.52 ± 0.20[e]	0.13 ± 0.05[f]	0.22 ± 0.04[f]	0.47 ± 0.22[e]	<0.0005
P/S ratio	0.42 ± 0.07[e]	0.65 ± 0.12[f]	0.57 ± 0.11[e,f]	0.58 ± 0.22[f]	0.028

[a]Values are mean ± 1 standard deviation given as percentage of total fatty acids.
[b]HM, human milk-fed group; CO, corn oil-based commercial formula-fed group; SO, soy oil-based formula-fed group; MO, soy/marine oil-enriched formula-fed group; LCP, long chain polyunsaturated fatty acids of >18 carbon chain length; P/S, polyunsaturate-to-saturate; ANOVA, analysis of variance; LA, linoleic acid; DPA, docosapentaenoic acid; LNA, linolenic acid; DHA, docosahexaenoic acid.
[c]Includes 14:0, 16:0, 17:0, 18:0, 20:0, 22:0, and 24:0.
[d]Includes 16:1, 18:1, 20:1, 22:1, and 24:1.
[e,f,g]Different superscripts indicate significant difference ($P < 0.05$) by Newman–Keuls multiple comparison analysis.

Table 2
Fatty Acid Profiles in Cheek Cell Phospholipids of Study Infants at 57 Wk Postconception[a]

Fatty acid	HM[b] (n = 6)	CO[b] (n = 11)	SO[b] (n = 16)	MO[b] (n = 12)	ANOVA, P
Total saturates[c]	40.5 ± 3.9	39.5 ± 4.0	41.9 ± 3.2	42.9 ± 4.9	0.106
Total monounsaturates[d]	34.1 ± 4.1	30.9 ± 4.9	29.5 ± 4.2	29.2 ± 3.7	0.111
LA (18:2n-6)	16.8 ± 3.8	19.0 ± 4.9	18.0 ± 2.8	18.7 ± 4.6	0.713
20:3n-6	1.60 ± 0.60	1.39 ± 0.53	1.66 ± 0.82	1.32 ± 0.67	0.564
20:4n-6	1.79 ± 0.52[e]	1.74 ± 0.39[e]	2.61 ± 1.14[f]	1.59 ± 0.51[e]	0.006
22:4n-6	0.63 ± 0.17	0.45 ± 0.17	0.57 ± 0.24	0.54 ± 0.45	0.625
DPA (22:5n-6)	0.21 ± 0.10[e]	0.31 ± 0.09[e,f]	0.42 ± 0.18[f]	0.28 ± 0.20[e,f]	0.032
Sum n-6 LCP	4.80 ± 1.22[e,f]	4.52 ± 0.90[f]	5.53 ± 0.94[e]	4.22 ± 0.89[f]	0.005
LNA (18:3n-3)	0.50 ± 0.19[e,f]	0.38 ± 0.13[f]	0.66 ± 0.18[e]	0.71 ± 0.20[e]	<0.0005
20:5n-3	0.65 ± 0.38[e]	0.18 ± 0.14[f]	0.39 ± 0.45[e,f]	0.53 ± 0.19[e]	0.024
DHA (22:6n-3)	1.02 ± 0.14[e]	0.18 ± 0.13[f]	0.45 ± 0.22[g]	1.24 ± 0.45[e]	<0.0005
Sum n-3 LCP	2.26 ± 0.57[e]	0.66 ± 0.32[f]	1.29 ± 0.84[g]	2.85 ± 0.99[e]	<0.0005
DHA/DPA ratio	5.22 ± 1.39[e]	0.70 ± 0.71[f]	1.24 ± 0.48[g]	8.23 ± 7.67[e]	<0.0005
n-3/n-6 LCP ratio	0.47 ± 0.12[e]	0.14 ± 0.02[f]	0.23 ± 0.07[f]	0.68 ± 0.19[g]	<0.0005
P/S ratio	0.62 ± 0.12	0.67 ± 0.17	0.65 ± 0.12	0.63 ± 0.18	0.90

See Table 1 for footnote information.

(an index of n-3 fatty acid sufficiency) in the CO group differed from that in the MO and HM groups by 6- to 12-fold.

Correlations between blood and cheek cell fatty acid profiles. Numerous fatty acids and major indexing values were significantly correlated between cheek cell phospholipids and both total RBC lipids and plasma phospholipids at the 36- and 57-wk time points [see Table 3; RBC and plasma lipid data given previously (6,7)]. The DHA content, as well as the n-3 LCP-to-n-6 LCP ratio (Table 3), in cheek cells was significantly correlated with that in both RBC lipids and plasma phospholipids at 36 and 57 wk postconception (Fig. 1A,B). At this later time point, cheek cell/RBC and cheek cell/plasma phospholipid correlation coefficients (r = 0.72 and 0.83, respectively) were higher than that found for RBC vs. plasma phospholipid DHA (r = 0.58; P < 0.0005). At 36 wk, the arachidonic acid content in cheek cells did not correlate with that in RBC and plasma phospholipids. However at 57 wk, the r value between cheek cells and both blood components was significant (r = 0.40; P < 0.01) but not to the extent of the association between arachidonic acid in RBC lipids and plasma phospholipids [r = 0.51; P < 0.0005; RBC and plasma fatty acid data previously reported (6, 7)].

DHA and visual function. Results of the trial have been reported in detail regarding the impact of n-3 fatty acid nutri-

Table 3
Major Fatty Acid Correlations[a]

Cheek cell phospholipids correlated with:	Total RBC Lipids[b]		Plasma Phospholipids	
	36 wk	57 wk	36 wk	57 wk
Saturates	$r = 0.29$ $P < 0.05$	$r = 0.32$ $P < 0.03$	$r = 0.32$ $P < 0.05$	$r = 0.31$ $P < 0.05$
Monounsaturates	n.s.	$r = 0.33$ $P < 0.03$	$r = 0.30$ $P < 0.05$	$r = 0.31$ $P < 0.05$
Sum n-6 LCP	n.s.	$r = 0.33$ $P < 0.03$	$r = 0.31$ $P < 0.05$	$r = 0.49$ $P < 0.001$
Sum n-3 LCP	$r = 0.39$ $P < 0.003$	$r = 0.48$ $P < 0.001$	$r = 0.41$ $P < 0.001$	$r = 0.39$ $P < 0.01$
n-3/n-6 LCP	$r = 0.48$ $P < 0.0005$	$r = 0.55$ $P < 0.0005$	$r = 0.50$ $P < 0.0005$	$r = 0.54$ $P < 0.0005$
AA (20:4n-6)	n.s.	$r = 0.39$ $P < 0.01$	n.s.	$r = 0.40$ $P < 0.01$
DHA (22:6n-3)	$r = 0.46$ $P < 0.005$	$r = 0.72$ $P < 0.0005$	$r = 0.53$ $P < 0.0005$	$r = 0.83$ $P < 0.0005$

[a]LCP, long chain polyunsaturates >18 carbons; n.s., not significant at $P < 0.05$.
[b]RBC and plasma phospholipid fatty acid data reported previously (6,7).

tional intervention on ERG (3,4), visual acuity (5), total RBC and plasma lipid fatty acid distribution (6), and growth (8) of the entire preterm infant cohort. Data presented here are for the 36- and 57-wk subsets of infants with complete visual function, cheek cell, RBC, and plasma lipid data (Fig. 2). Retinal function was less mature in the n-3-deficient CO formula group as evident from elevated rod ERG thresholds at 36 wk postconception (Fig. 2B). By 57 wk, significant differences in rod ERG thresholds are no longer apparent (Fig. 2E) and may be attributable to the inability to record functional differences during a plateau in development of retinal tissues (3,4). Maturation of visual function was delayed in both the 36-wk and 57-wk CO-infant groups as determined electrophysiologically using VEP responses (Fig. 2C,F). Visual acuities in the MO and HM infants were nearly equivalent whereas the SO group (receiving ample LNA) had intermediate values.

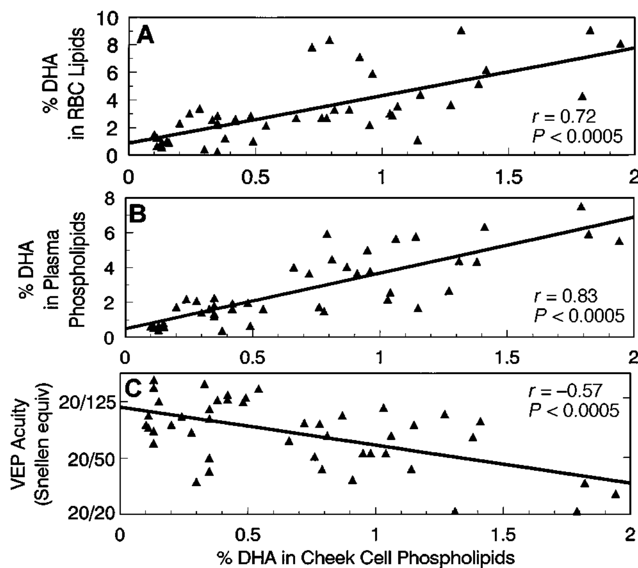

FIG. 1. Correlations between docosahexenoic acid (DHA) in cheek cell phospholipids and DHA in total red blood cell (RBC) lipids (A), DHA in plasma phospholipids (B), and with visual-evoked potential (VEP) acuity (C) at 57 wk postconception. VEP visual acuity given as Snellen equivalents where 20/20 is the average value for normally sighted adults. Equations defining the regression lines in A, B, and C are $y = 0.21 + 0.72x$ ($r^2 = 0.52$), $y = 0.11 + 0.83x$ ($r^2 = 0.68$), and $y = 0.76 - 0.57x$ ($r^2 = 0.33$), respectively. Fatty acid results for RBC and plasma fatty acids reported previously (6,7).

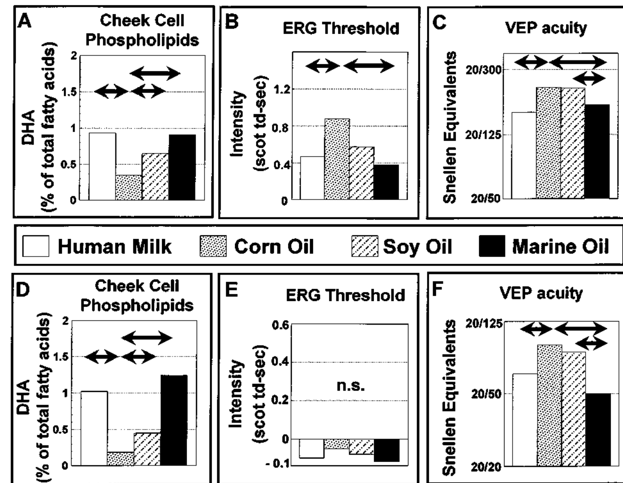

FIG. 2. Cheek cell DHA levels and visual function results of preterm infant subgroups (see Materials and Methods section) at 36 wk (A, B, C) and 57 wk (D, E, F). Rod electroretinography (ERG) thresholds given as light intensity (log scotopic troland-seconds) necessary to obtain a 2 μV response. Comparison of Snellen equivalents for VEP visual acuity on the *y*-axes of (C) vs. (F) demonstrate maturation of visual processes between 36-wk and 57-wk infants; higher Snellen equivalents (e.g., 20/20) are associated with better visual function. Arrows indicate significant differences ($P < 0.05$) between diet groups using Newman–Keuls multiple comparison analysis. n.s., not significant. See Figure 1 for other abbreviations.

At 36 wk postconception, DHA in cheek cell phospholipids was correlated with both rod ERG threshold and VEP acuity (ERG: $r = -0.29$, $P = 0.03$; VEP: $r = -0.31$, $P = 0.02$). Similarly, DHA in plasma phospholipids was correlated with these electrophysiologic functions (ERG: $r = -0.45$, $P < 0.0005$; VEP: $r = -0.38$, $P = 0.002$); however, the association between total RBC lipid DHA values and either ERG thresholds or VEP acuities at 36 wk was not significant (4,5) ($P > 0.09$). At 57 wk, rod ERG thresholds were no longer associated with cellular fatty acid levels; however, VEP acuity was correlated ($P < 0.0005$) with DHA in total RBC lipids ($r = -0.50$) (5), plasma phospholipids ($r = -0.61$) and cheek cell phospholipids ($r = -0.57$; see Fig. 1C). VEP acuity was also correlated with the n-3 sufficiency index (DHA/DPA ratio) in cheek cell phospholipids at both 36 wk ($r = -0.26$, $P < 0.05$) and 57 wk ($r = -0.50$, $P < 0.001$). Thus, elevations in both blood and cheek cell DHA levels were associated with maturation of visual function, based on electrophysiological measurements.

DISCUSSION

In order to better understand the cellular biochemistry associated with early development or pathology of the human visual system, it would be preferable to analyze tissue from the eye, retina, or visual cortex. However, since this is impractical, an alternative source is necessary to provide a fatty acid index for studies of development, disease, or dietary intake. In addition, biochemical sampling permits direct monitoring of compliance in clinical trials instead of relying on self-reporting measures. Blood lipids are commonly used for this purpose and, as reported previously (3–5), provide excellent correlations between fatty acid levels and developing visual function in infant trials. Nevertheless, in some clinical trials, blood samples may not be available or scheduling of repeated sampling may be excessively invasive. The present results suggest that phospholipids from cheek cells (buccal mucosal epithelia) may provide a suitable alternative or supplemental index to the individual's fatty acid status.

Increasing attention has recently been given to buccal cheek cells as a source for identifying various biological characteristics in individuals. Cheek cell preparations are now commonly used in DNA screening to detect gene mutations for diseases such as cystic fibrosis (9) and more recently, Alzheimer's disease (10). Other clinical investigations have utilized cheek cells to evaluate the vitamin E nutritional status in newborns, children, and adults (11–13). For example, Yokota *et al.* (11) found that α-tocopherol levels in buccal cheek cells were closely correlated with both RBC and plasma α-tocopherol levels in young adults when expressed on a per g cellular lipid basis.

Two previous clinical studies have employed cheek cell phospholipids as an index for fatty acid status during nutritional intervention. McMurchie *et al*. (1) reported a significant elevation in the proportion of LA in cheek cell phospholipids in subjects consuming a diet enriched with LA [polyunsaturate-to-saturate (P/S) ratio of 1.0] compared to those receiving a low P/S diet (P/S = 0.4). These authors also noted that vegetarians with low saturated fatty acid intakes compared to omnivores had significantly lower levels of saturates in cheek cell lipids. As part of a larger nutritional study, Sampugna *et al*. (2) evaluated cheek cell lipids as indicators of fatty acid status in women on high (1.0) or low (0.3) P/S diets for 4 mon. The authors reported no significant differences in individual cheek cell fatty acids from subjects on the two diets. The results of our studies are consistent with the studies of McMurchie *et al.* (1) but differences in subject ages, degree of maturation (preterm infants vs. adults), and strict regulation of the nutritional intervention (use of liquid diets in the current study) may have led to a more sensitive response in our study. Variations in sample preparation may also contribute to different results among studies; for example, centrifugation of cheek cells at 12,000 × *g* for 30 min (2), 6,000 × *g* for 10 min (1), or 3,000 × *g* for 10 min (current study) may result in analysis of different cell populations. Furthermore, cell storage may influence the outcome measure; McMurchie *et al.* (1) stored cells frozen at −20°C for 2 wk prior to lipid isolation, Sampugna *et al.* (2) stored cells briefly overnight at −80°C, and we extracted the lipids immediately and stored the extracts for up to 1 wk under N_2 at −20°C before analysis on the chromatograph. These technical variations among laboratories would need to be standardized before cheek cells become a routine measure of fatty acid status in the body.

The fatty acid profiles of cheek cell phospholipids in the present study were found to reflect intake of dietary fatty acids. At 36 and 57 wk postconception, diet-associated differences in several major fatty acids and indices were found in cheek cell phospholipids including numerous individual n-3 and n-6 LCP, total n-3 and n-6 LCP, DHA/DPA ratios, and n-3-to-n-6 LCP ratios.

An elevation in arachidonic acid and other n-6 LCP in cheek cell lipids of the SO formula group at 57 wk also was observed in total plasma lipids (6) and plasma phospholipids (data not shown) but not in RBC lipids (6). Thus a greater association between plasma and cheek cell lipid metabolism is suggested by these data together with: (i) a high correlation between cheek cell-DHA and plasma phospholipid-DHA, and (ii) similar correlations of VEP acuity with DHA in cheek cells ($r = -0.57$) and plasma phospholipids ($r = -0.61$) at 57 wk compared to that in RBC lipids. A common factor to both may be the relatively rapid turnover rates of plasma lipids (7–8 d) (14) and cheek cells (5 d) (15) in comparison to RBC (108–135 d) (15). Considering the different origins, environments, and characteristics of RBC, plasma phospholipids, and cheek epithelial cells, distinct fatty acid profiles may be expected for each entity. None of these indices should be expected to totally reflect the fatty acid profile of brain or retinal tissue, both of which vary regionally. Cheek cells may, however, provide a supportive role along with RBC and/or plasma phospholipids as indices to neural fatty acid status based on significant correlations between visual function and cheek cell, RBC, and plasma phospholipid DHA content. These results support the validity of cheek cells as an index for essential fatty acid status and may be of importance for investigators who need a noninvasive procedure to support blood lipid analysis data in clinical trials.

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Acyl-CoA Synthetase Activity in Liver Microsomes from Calcium-Deficient Rats

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ABSTRACT: A study on the kinetic properties of the nonspecific acyl-coenzyme A (CoA) synthetase activity in liver microsomal vesicles from both normal and calcium-deficient Wistar rats was carried out. After a 65-d treatment, the calcium-deficient diet reflected a 75% increase in the synthetase activity with respect to control animals. The apparent Vm was significantly enhanced, while the Km remained unchanged. We also provided experimental evidence about various fatty acids of different carbon length and unsaturation which depressed the biosynthesis of palmitoyl-CoA following different behaviors in control or calcium-deprived liver microsomes. In addition, we studied in detail the inhibition reflected by stearic, α-linolenic, or arachidonic acids, in the biosynthesis of palmitoyl-CoA in microsomal suspensions either from control or hypocalcemic rats. In control microsomes, stearic acid produced a pure competitive effect, while the other fatty acids followed a mixed-type inhibition. The competitive effect of stearic acid was not observed in calcium-deprived microsomes. At the same time, a mixed-type inhibition produced by either α-linolenic or arachidonic acid was diminished in deprived microsomes due to an increase in the noncompetitive component (αKi). These changes observed in apparent kinetic constants (Km, Vm, Ki, and αKi), as determined by Lineweaver-Burks and Dixon plots, were attributed to the important alterations in the physicochemical properties of the endoplasmic reticulum membranes induced by the calcium-deficient diet. The solubilization of the enzyme activity from both types of microsomes demonstrated that the kinetic behavior of the enzyme depends on the microenvironment in the membrane, and that the calcium ion plays a crucial role in determining the alterations observed.

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The importance of long-chain acyl-coenzyme A (CoA) derivatives as intermediates and regulators of lipid metabolism has prompted a number of investigators to study the long-chain acyl-CoA-synthetase (ACS) AMP-forming (EC 6.2.1.3), which was first demonstrated by Kornberg and Pricer (1) in particulate preparations from guinea pig liver. Subsequent works extended research to different sources such as rat tissues (2–9), yeasts (10,11), infected erythrocytes (12), murine T lymphocytes (13), and various human cells and tissues (14–16). In rat livers, ACS is located in microsomes (1,17–19), outer mitochondrial membranes (20,21), and peroxisomal membranes (22,23). Purified ACS from these three different organelles are identical regarding all molecular and catalytic properties (2,24). The identity of the enzymes was also confirmed immunologically (25). Wilson *et al.* (14) and Laposata *et al.* (16) demonstrated the occurrence of an arachidonic acid-specific enzyme activity, and they suggested its potential role in regulating free arachidonic acid within the cell. Another enzyme activity requiring GTP instead of ATP was reported to occur in the mitochondrial fraction (26), though the significance of this enzyme reaction is controversial (27,28). More recently, Sugiura *et al.* (29) have suggested that certain types of fatty acyl chains of membrane lipids are continuously being converted transiently into acyl-CoA esters by an ATP-independent acyl-CoA synthetase. In any case, it is clear that the long chain acyl-CoA synthetase activity existing in liver microsomes is attributed to a well-characterized protein different from other enzyme activities (12,13,16,30). This enzyme, called nonspecific ACS, is very important because it catalyzes the initial reaction for the overall fatty acid metabolism, and it further regulates the rate of fatty acid biosynthesis (31,32). It has been recently demonstrated that acyl-CoA is necessary for the thyroid hormone receptor function (33), the budding of transport vesicles from Golgi cisternae (34), and the modulation of several enzyme activities such as Na^+,K^+-ATPase (35), Ca^{2+}-ATPase (36), and protein kinase C (37). In addition, three recent reports have indicated that acyl-CoA esters are involved in the intracellular handling of Ca^{2+} (38–40). We should also bear in mind that acyl-CoA have substantial, complex, and differing effects on Ca^{2+} movements in rat liver microsomes. These facts led us to study the relationship between calcium availability and the ACS activity in liver microsomal vesicles from both normal and calcium-deficient Wistar rats. In subsequent experiments, the catalytic properties of this enzyme were also examined under a calcium-deprived diet and the results obtained were discussed in terms of their kinetic significance.

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Abbreviations: ACS, acyl-CoA synthetase; CD, calcium-deficient; S, standard (control); SEM, standard error of the mean.

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MATERIALS AND METHODS

Fatty acids and other chemicals. All the unlabeled fatty acids were obtained from Nu-Chek-Prep. (Elysian, MN). ATP (disodium salt), dithiothreitol, coenzyme A (lithium salt), and Triton X-100 were purchased from Sigma Chemical Co. (St. Louis, MO). [1-^{14}C]Palmitic (58.0 mCi/mmol, 99% radiochemically pure) and [1-^{14}C]stearic acids (56.5 mCi/mmol, 98% radiochemically pure) were supplied by Amersham International (Buckinghamshire, United Kingdom). Other chemicals used were of analytical or chromatographic grade, and they were provided by commercial sources. The concentration and purity degrees of fatty acids were routinely checked by liquid-scintillation counting and gas–liquid chromatography of fatty acid methyl esters prepared in the presence of internal standards. Fatty acids were dissolved in ethanol and stored in the dark at −20°C under an atmosphere of N_2 until they were used.

Animal treatment. Female Wistar rats from Comisión Nacional de Energía Atómica (Buenos Aires, Argentina) weighing 170 ± 10 g were bred and maintained on a control diet (Cargill type "C", Rosario, Argentina) throughout gestation and lactation. The dams were housed in plastic cages (one animal per cage) in a vivarium kept at 22 ± 1°C with a 12-h light/dark cycle and a relative humidity of 60 ± 10%. After weaning, 24 female pups (weighing 47 ± 4 g/animal) were randomly divided into two groups of 12 animals each, and fed *ad libitum* either on a calcium-deficient diet (group CD), or on a balanced diet (group S). The composition of the calcium-deficient diet prepared in our laboratory is shown in Table 1. The Ca^{2+} content (0.5 g/Kg) was determined by a Shimadzu Atomic Absorption Spectrophotometer AA-630-12 (Kyoto, Japan) following the mineralization procedure described elsewhere (41). Control animals were fed on a standard balanced diet supplemented with 5.0 g/Kg calcium in order to supply the mineral at a level equivalent to that recommended by The American Institute of Nutrition (AIN)-93 Purified Diets for Laboratory Rodents (42). The content of Ca^{2+} in drinking water (given *ad libitum*) was determined either by atomic absorption or by calcium-selective electrode Orion model 93-20, Orion Research Inc. (Cambridge, MA), and it was generally below 5 ppm. During the feeding period, body weights, water consumption, and food intakes were determined every day. Samples of blood were collected in order to determine plasma calcium levels. The food intake and water consumption relative to body weights were not significantly different between both groups of animals at the time the rats were killed, after 60 d of feeding. Calcium-deficient animals grew at a rate similar to the control group during the initial 20 d of feeding, then they grew at a reduced rate until day 59 when differences between groups became significant. In order to avoid individual differences among animals that might have resulted from *ad-libitum* feeding, on day 59 all the rats were fasted for 24 h, refed with the corresponding diet for 2 h, and then killed by decapitation without prior anesthesia 12 h after the refeeding period.

TABLE 1
Composition of Calcium-Deficient Diet

Ingredients	g/kg
Casein, high protein (free of calcium)	200.0
Sucrose	505.9
Corn starch	150.0
Cellulose	50.0
Corn oil[a]	50.0
Mineral mixture (calcium-deficient)[b]	13.4
Sodium phosphate, monobasic	8.9
Potassium phosphate, monobasic	8.8
Vitamin mixture[c]	10.0
DL-Methionine	3.0

[a]Contained 11.5% 16:0, 2.1% 18:0, 26.6% 18:1, 57.3% 18:2n-6, 1.4% 18:3n-3, 0.2% 20:3n-6, 0.2% 20:4n-6, 0.2% 20:1, and other fatty acids as trace amounts (<0.2%).
[b]Contained (g/kg mix): NaCl, 183.7; potassium citrate, 576.0; K_2SO_4, 136.1; MgO, 62.8; $MnCO_3$, 9.2; ferric citrate, 16.7; $ZnCO_3$, 4.2; $CuCO_3$, 0.80; KIO_4, 0.03; $Na_2SeO_3 \cdot 5H_2O$, 0.03; $CrK(SO_4) \cdot 12H_2O$, 1.40.
[c]Contained (g/kg mix): choline dihydrogen citrate, 349.7; ascorbic acid, 101.7; vitamin E acetate, 24.2; p-aminobenzoic acid, 11.0; inositol, 11.0; niacin, 9.9; panthothenate, 6.6; menadione, 5.0; vitamin A palmitate, 4.0; vitamin B_{12}, 3.0; pyridoxine HCL, 2.2; riboflavin, 2.2, thiamine, 2.2; vitamin D_3, 0.44; folic acid, 0.2; biotin, 0.04; and corn starch, 466.7.

All the diet components used were purchased from Carlo Erba (Milan, Italy) or Mallinckrodt Chem. Works (NY). The casein was deprived of calcium by EGTA treatment and then defatted with boiling acetone. The calcium content in the extracted casein was negligible. Animal maintenance and handling were in accordance with the National Institutes of Health guide for the care and use of laboratory animals (43).

Preparation of liver microsomal suspensions. Livers were rapidly excised and immediately placed in an ice-cold homogenizing medium (44). The homogenate from each liver was processed individually at temperatures varying from 0° through 2°C, and the microsomal fractions were separated by differential centrifugation at 110,000 × *g*, as described previously (44). Microsomal pellets were resuspended in a cold homogenizing solution up to a final protein concentration of 30–40 mg/mL.

Assay of long-chain fatty acyl-CoA synthetase activity. The enzyme activity was radiochemically determined basically following the method of Tanaka *et al.* (2), consisting of the enzymatic conversion of heptane-extractable radioactively labeled fatty acid into water-soluble acyl-CoA esters. The standard reaction mixture contained 0.1 M Tris-HCl buffer (pH 8.0), 5 mM dithiothreitol, 0.15 M KCl, 15 mM $MgCl_2$, 10 mM ATP(Na_2), 1 mM CoA (lithium salt), and the substrate (1 mM palmitic acid, in a final volume of 0.2 mL.). To prepare the palmitic acid solution, labeled and unlabeled acids were dissolved in ethanol and mixed to obtain 200,000 dpm/incubation tube. The solvent was evaporated to dryness under N_2 and the fatty acids resuspended by sonication in 375 mM Tris-HCl buffer, pH 8.0, containing 1% polyethyleneglycol. The reaction was started by adding 0.04 mg liver microsomal protein in a solution of 10 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, and 1 mM EDTA. After 3 min of incubation, the reaction was stopped by adding 2.25 mL of a mixture of isopropanol/heptane/2 M-sulfuric acid (40:10:1, vol/vol). Then 1.5 mL heptane and 1 mL distilled water were added and the

upper layer was discarded. The lower layer was washed 3 times with 2 mL heptane containing 1 mg/mL of carrier palmitic acid to remove the unreacted radioactive fatty acid. The radioactivity of the aqueous phase was counted in a Wallac 1214 Rackbeta Liquid Scintillation Counter (Turku, Finland). In some experiments, different amounts of unlabeled fatty acids (tested as enzyme inhibitors) were added to the incubation mixture at the concentration indicated in the figures, while in other experiments palmitic acid was replaced by stearic acid under the incubation conditions described above. Blanks were routinely run in all incubations. These blanks consisted of tubes in which boiled microsomes (10 min at 100°C) were added. In preliminary experiments using different experimental conditions, the recovery of radioactivity from the aqueous phase (labeled acyl-CoA) of the blanks did not exceed 1 to 2% of the added radioactivity. The aqueous extract from the reaction mixture was reacted with neutral hydroxylamine, and the hydroxamate derivative was chromatographed on silica gel G with chloroform/methanol/water (95:5:0.5, vol/vol), following the procedure described elsewhere (10). The reaction products were identified by means of authentic standards. Ninety percent of the radioactivity in the reaction product was found in palmitoyl-CoA and *ca.* 3% in free palmitic acid; the remaining 7%, which migrated below the acyl-CoA ester, may be in oxidation products (5).

Solubilization of enzyme activity. The ACS activity was solubilized from the microsomal membrane of control or calcium-deficient rat livers as described by Nagamatsu *et al.* (30) with minor modifications. Briefly, microsomes were diluted in Tris-HCl buffer (50 mM, pH 8.0) containing 5 mM dithiothreitol (instead of 2-mercaptoethanol as indicated in the original method) and 1 mM EGTA, so that the protein concentration was 4.0 mg/mL. A solution of Triton X-100 was added to make the final detergent concentration 5 mM (various detergent concentrations were previously tested). The mixture was allowed to stand at 0–2°C for 60 min and centrifuged at $105{,}000 \times g$ for 1 h. The enzyme activity was partially purified by ammonium sulfate precipitation as described elsewhere (2). The excess of Triton X-100 was removed by washing the pellet three times with 10 vol of the ammonium sulfate solution. The washed pellet was dialyzed overnight at 2°C against 0.02 M sodium phosphate buffer (pH 7.0), 130 mM NaCl, and 1 mM EGTA. This enzyme preparation had a specific activity of 177 ± 6 nmol of palmitoyl-CoA synthesized per milligram of protein per minute, which was similar to that reported by other authors (45) in the presence of residual amounts of Triton X-100. Solubilization of the ACS was also performed by means of the modified method of Juarez *et al.* (46). The extraction was carried out using a high ionic strength solution of KCl. The liver microsomal pellet was resuspended in a buffer containing 0.05 M K_2HPO_4 (pH 7.00), 1.50 M KCl, 2 mM EDTA, and 0.5 mM dithiothreitol. The suspension (4.0 mg protein/mL) was incubated with stirring at 2°C for 30 min in the presence of 10% glycerol. At the end of this period, the solubilized enzyme was separated from the pellet by centrifugation in the cold at $110{,}000 \times g$ for 60 min.

Diphenylhexatriene labeling of microsomes and steady-state fluorescence anisotropy determinations. The $110{,}000 \times g$ microsomal pellet from either control or calcium-deficient diets was washed according to the method of Glaumann *et al.* (47) with minor modifications (48). Fluorescence anisotropy was determined in these washed microsomal suspensions, as previously described (49). All measurements were performed at 37°C in an Aminco-Bowman spectrofluorometer equipped with two glan prism polarizers. Both methodology and calculations were described in a previous paper (48).

Other analytical determinations and statistical treatment of the data. The fatty acid composition of lipids from microsomal suspensions was determined as described previously (48). Total lipids were extracted from microsomes according to the procedure of Folch *et al.* (50), and determined gravimetrically after evaporating aliquots to constant weight (48). The cholesterol content was measured by the method of Allain *et al.* (51). Phospholipid and neutral lipid fractions were isolated from the total lipid extract by silicic acid microchromatography (Bio-Rad Lab., Richmond, CA) according to the method of Hanakan *et al.* (52). Phosphorus analysis was performed following the method of Chen *et al.* (53). Protein content was determined by the micromethod of Lowry *et al.* (54) with crystalline bovine albumin as standard. Samples for gas–liquid chromatography analysis were transesterified as previously described (48), and they were analyzed in a Hewlett-Packard 5840-A gas–liquid chromatography apparatus (Avondale, PA) equipped with a 5840-A terminal computer integrator system and with a 6-ft glass column packed with 10% SP-2330 on a 100–200 mesh Chromosorb WAW-DMCS (Supelco Inc., Bellefonte, PA). Eicosa-11-monoenoic acid (1 μg/tube) was used as an internal standard.

All values represented the mean of 3 to 6 individual determinations (assayed in triplicate) ± 1 standard error of the mean (SEM). In order to test the statistical significance of numerical differences in results, data were analyzed by either the Student's *t*-test or by ANOVA (analysis of variance), with the aid of the GB-STAT Professional Statistics Program (version 4.0) from Dynamic Microsystems, Inc. (Silver Springs, MD). Data were plotted using Sigma Plot Scientific Graphing Software (version 2.0) from Sigma Chem. Co., (St. Louis, MO).

RESULTS

In the present experiments, the ACS activity was studied in liver microsomal suspensions from both normal and calcium-deprived rats. At weaning, CD animals were fed on a diet whose calcium content was 10-fold lower than that recommended for animal breeding (42). During the feeding experimental period, calcium levels in the serum of the CD-group progressively decreased up to 1.8 times lower than those of the S-group (1.44 ± 0.10 vs. 2.60 ± 0.10 mmol/L, respectively). Using this kind of microsomal suspension, we optimized the assay design with respect to protein concentration and incubation time. Previous studies with ACS from rat liver microsomes had shown that the interpretation of experimen-

TABLE 2
ACS Activity for Palmitic Acid from Control (S) or Calcium-Deficient (CD) Rat Liver Microsomes[a]

Microsomes	Specific activity (nmol/min·mg)
S	150.1 ± 3.1
CD	261.4 ± 4.0*

[a]Results were obtained as described in the Materials and Methods section and they are given as nmoles of product formed per min per mg of microsomal protein. Each value represents the mean ± 1 SEM of six animals assayed in triplicate.
*Significantly different from S-group, $P < 0.001$.

tal results could become complicated by a high ratio of endogenous fatty acid to enzyme protein, or to an excessively lengthy incubation period (55). Thus, following the procedure of Tanaka *et al.* (2), we verified that the enzyme activity with palmitic acid as substrate (1.0 mM concentration) was completely linear as a function of a microsomal protein up to 0.40 mg/tube or 2 mg/mL (constant incubation time 10 min), and to time for up to 15 min (constant protein concentration of 0.50 mg/mL). Under the experimental conditions stated in the Materials and Methods section, the activity of nonspecific ACS was measured at initial velocity conditions using palmitic acid as substrate. Therefore, the resulting rate of formation of the product was directly proportional to the enzyme activity. As indicated in Table 2, the activity of nonspecific ACS increased *ca.* 75% over control values in animals fed on the calcium-deficient diet.

Then we examined the effect of the calcium-deficient diet on the apparent kinetic parameters by determining the values of Vm and Km for palmitic acid through double reciprocal plots according to the method of Lineweaver and Burk (56). Figure 1 shows that the apparent Vm for the calcium-deficient control or calcium-deficient microsomes, evoked by the presence of varying amounts of different fatty acids, labeled palmitate was used as substrate—at a saturated concentration of 1 mM—while another fatty acid was simultaneously added as an alternative substrate within the range 0.05 to 0.40 mM. The results obtained are illustrated in Figure 2A for control microsomes and Figure 2B for calcium-deficient ones. In control microsomes, the sole saturated fatty acid tested as alternative substrate (stearic acid) evidenced a weak inhibitory effect only at concentrations greater than 0.3 mM. Unsaturated fatty acids derived from linoleate or α-linolenate showed a greater inhibition capacity of nonspecific ACS activity than that observed for fatty acids of the n-9 family. In calcium-deficient microsomes the pattern of the inhibitory effects displayed by the tested fatty acids was less noticeable than that observed in control microsomes. Moreover, in the case of the

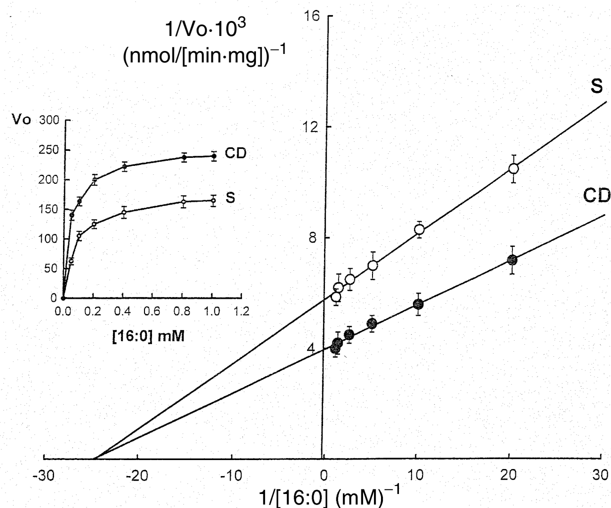

FIG. 1. Linear regressions for the Lineweaver-Burk plots of acyl-coenzyme A-synthetase ACS activity for palmitic acid from control or calcium-deficient rat liver microsomes. Enzyme activity was assayed as described in the Materials and Methods section. Typical Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 standard error of the mean (SEM) of three different incubations assayed in triplicate.

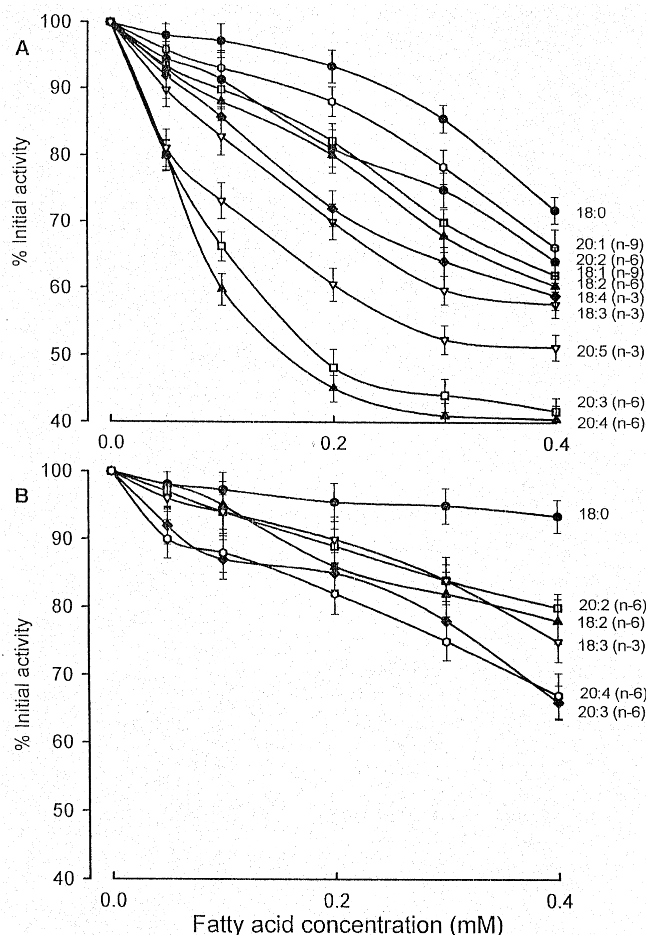

FIG. 2. Inhibition of nonspecific ACS activity for palmitic acid from control (A) or calcium-deficient (B) microsomes in the presence of various fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid as the main substrate and varying amounts of unlabeled inhibitor fatty acids at the concentrations indicated. Basal activity was 143.5 ± 4.0 and 271.1 ± 4.4 nmol·min^{-1}(mg protein)$^{-1}$ in control and calcium-deficient microsomes, respectively. Each point represents the mean ± 1 SEM of three different incubations using microsomes from three animals. For abbreviations, see Figure 1.

saturated fatty acid (stearic), there was no inhibitory effect within the range of concentrations assayed. In spite of the quantitative differences observed, the relative capacity shown by the alternative substrates to reduce palmitate activation was the same in both types of microsomal preparations.

In order to further investigate the type of inhibition provoked by the alternative substrates and the calcium-induced modifications in the lipid composition of the endoplasmic reticulum membranes, we carried out a series of experiments using palmitic acid as substrate with stearic, α-linolenic, and arachidonic acids, as weak, medium, or strong inhibitors, respectively, of the nonspecific ACS activity. In these incubations, for each concentration of palmitic acid studied (0.05 to 1.00 mM), various concentrations of inhibitors were tested (stearic acid: 0 to 0.36 mM or 0 to 0.40 mM in either control or calcium-deficient microsomes; α-linolenic acid: 0 to 0.12 mM or 0 to 0.20 mM; and arachidonic acid: 0 to 0.08 mM or 0 to 0.20 mM). In all instances initial velocities were measured and plotted according to the method of Lineweaver and Burk (56) (Figs. 3 and 4 for control or calcium-deficient microsomes, respectively). The analysis of the double reciprocal plots shown in Figure 3 indicates that the stearic acid displayed a competitive behavior only at relatively high fatty acid concentrations, whereas α-linolenic and arachidonic acids evidenced a mixed-type inhibition consisting of uncompetitive and competitive components (57). In calcium-deficient microsomes (Fig. 4) the stearic acid was not effective as a competitive inhibitor even at higher concentrations tested. Although α-linolenic and arachidonic acids showed a mixed-type inhibition pattern, the extent of their effects was significantly lower than that observed in control preparations. To estimate quantitatively the magnitude of these inhibitory effects on nonspecific ACS activity, data from Figures 3 and 4 were re-plotted as the inverse of the initial velocities vs. inhibitor concentrations (Dixon plots). Dixon plots are frequently used to identify the type of inhibition and to determine Ki values. The velocity equation for competitive inhibition may be converted into a linear form in which the varied ligand is [i]. The straight lines obtained at different substrate concentrations have positive slopes. Drawing a horizontal line at a height of 1/Vm, these lines can be intercepted at the −[i] value representing the kinetic parameter "αKi". This parameter considers the noncompetitive component of a linear-mixed-type inhibition (57). Figures 5 and 6 show the results obtained for control or calcium-deficient microsomes, respectively. The slopes of these plots are given by Km/−[S].Vm.Ki. Since Km [S] and Vm are constants, the slope is inversely proportional to Ki. Comparing the plots obtained for both types of microsomal preparations, relative Ki and αKi values were obtained. These data were consistent with the apparent kinetic parameters obtained from the Lineweaver-Burk plots shown in Figures 3 and 4. All these kinetic data are summarized in Table 3. These results clearly indicate that the calcium-deficient diet produced a significant decrease in the inhibitory effects presented by all the tested fatty acids. In the case of the stearic acid, Vm was increased *ca.* 80% over control values, while

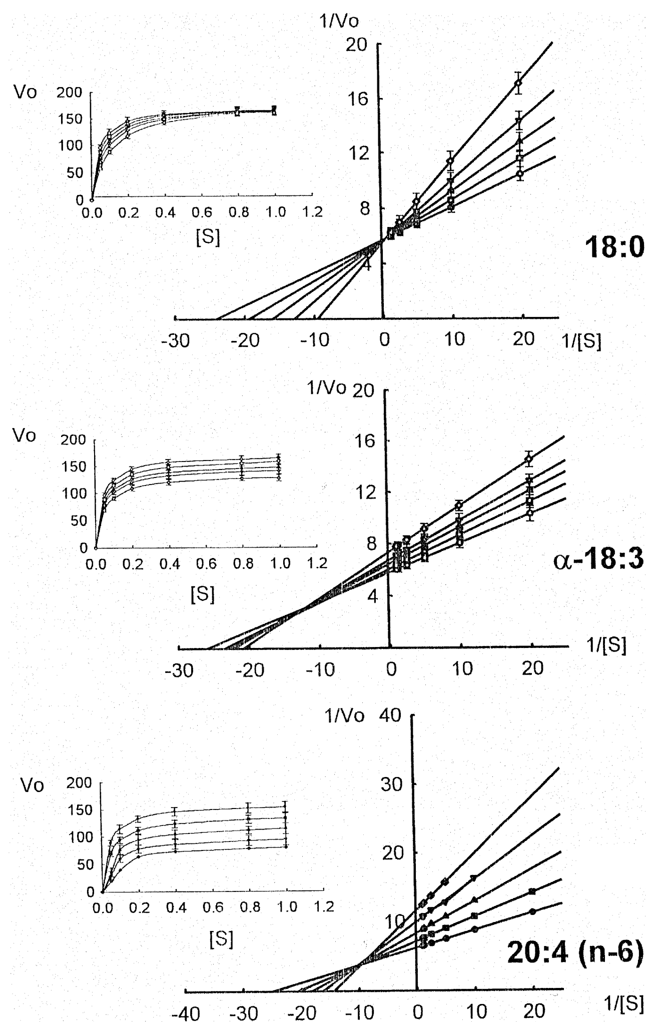

FIG. 3. Linear regressions for Lineweaver-Burk plots of ACS activity for palmitic acid from control microsomes in the presence of various concentrations of inhibitory fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid and varying amounts of the unlabeled inhibitory fatty acid as indicated. Typical Michaelis-Menten plots are given in the inserts. 1/Vo was expressed as $[\text{nmol}/(\text{min}\cdot\text{mg})]^{-1}\cdot 10^3$ and 1/[S] was given as $(\text{mM})^{-1}$. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

its competitive effect disappeared at the concentrations assayed. The increase in Vm values observed with hepatic microsomes of the CD-group, even in the presence of the α-linolenic or arachidonic acids as inhibitors, was produced through a decrease in the competitive component. Although this finding was markedly evident in the case of arachidonic acid, Ca deficiency significantly raised both Ki and αKi in all the studied fatty acids.

We also studied the linear regressions for Lineweaver-Burk plots of nonspecific ACS activity using either palmitic or stearic acid as substrate (Fig. 7). It was observed that the Km value for stearic acid (a pure competitive inhibitor) increased in calcium-deficient microsomes, compared to controls, while Km for palmitic acid remained unchanged in both

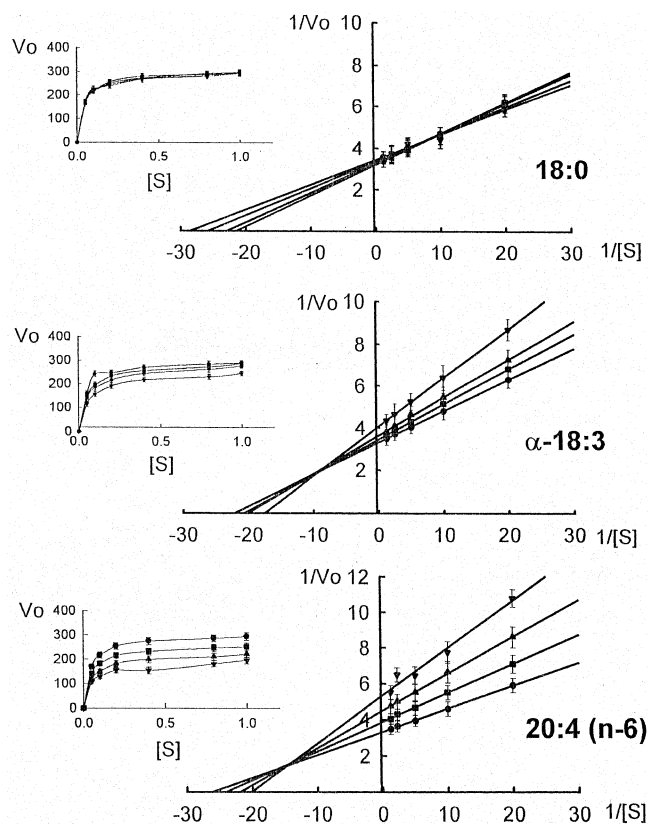

FIG. 4. Linear regressions for Lineweaver-Burk plots of nonspecific ACS activity for palmitic acid from calcium-deficient microsomes in the presence of various concentrations of inhibitory fatty acids. The enzyme was assayed as described in the Materials and Methods section. The reaction mixture contained saturated amounts of labeled palmitic acid and varying amounts of the unlabeled inhibitory fatty acid as indicated. Typical Michaelis-Menten plots are given in the inserts. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and 1/[S] was given as $(mM)^{-1}$. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

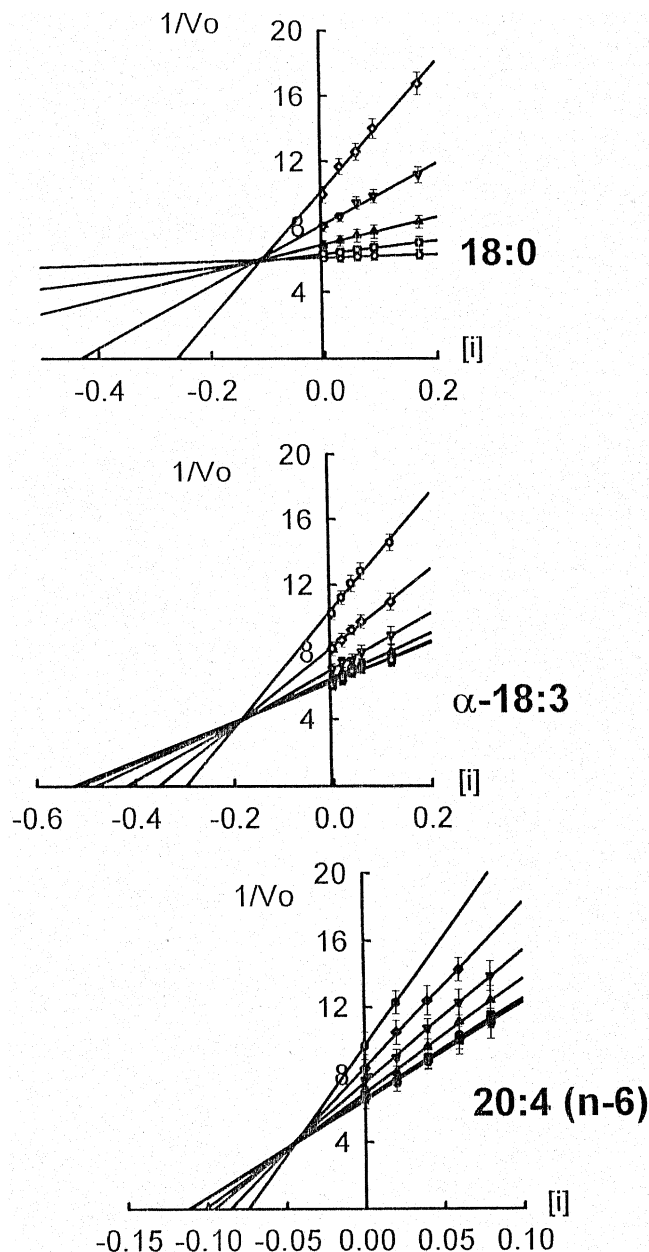

FIG. 5. Linear regressions of Dixon plots from control microsomes. Data were taken from the reciprocal plots shown in Figure 3. 1/Vo was expressed as $[nmol/(min \cdot mg)]^{-1} \cdot 10^3$ and [i] was given as mM. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

kinds of microsomes.

Table 4 shows the interlipid relationships and fluorescence anisotropy of liver microsomal membranes from either control or calcium-deficient rats. The results clearly indicate that in fact the lipid bilayer of the endoplasmic reticulum membrane was significantly modified by calcium deficiency. The relative content of lipids was decreased at the time that the neutral lipid was significantly raised. The proportion of cholesterol and phospholipids was diminished by calcium deficiency. On the other hand, Table 5 shows that the fatty acid composition of the different lipid fractions was significantly modified by calcium deprivation. The amounts of saturated fatty acids significantly increase in both triglyceride and phospholipid fractions, while a concomitant decrease was observed in the content of phospholipid polyunsaturated fatty acids. The decrease produced in the unsaturation index suggested that the quality and/or quantity of the fatty acid chains acylated to the lipid structures were substantially modified in the microsomes of the CD-group. These modifications in the physicochemical state of the lipid bilayer, induced by calcium deficiency, could be closely associated with the difference found in the nonspecific ACS activity, and with the altered response of this enzyme to the fatty acid tested as inhibitors. Taking into account these considerations, we investigated the ACS activity deprived of its microenvironment in the biomembrane. The linear regressions for Lineweaver-Burk plots of the solubilized enzyme from control or calcium-deficient microsomes are shown in Figure 8. It is evident that the kinetic behavior exhibited by nonspecific ACS between control and calcium-deficient microsomal preparations was the same after solubilization procedures either with Triton X-100 or

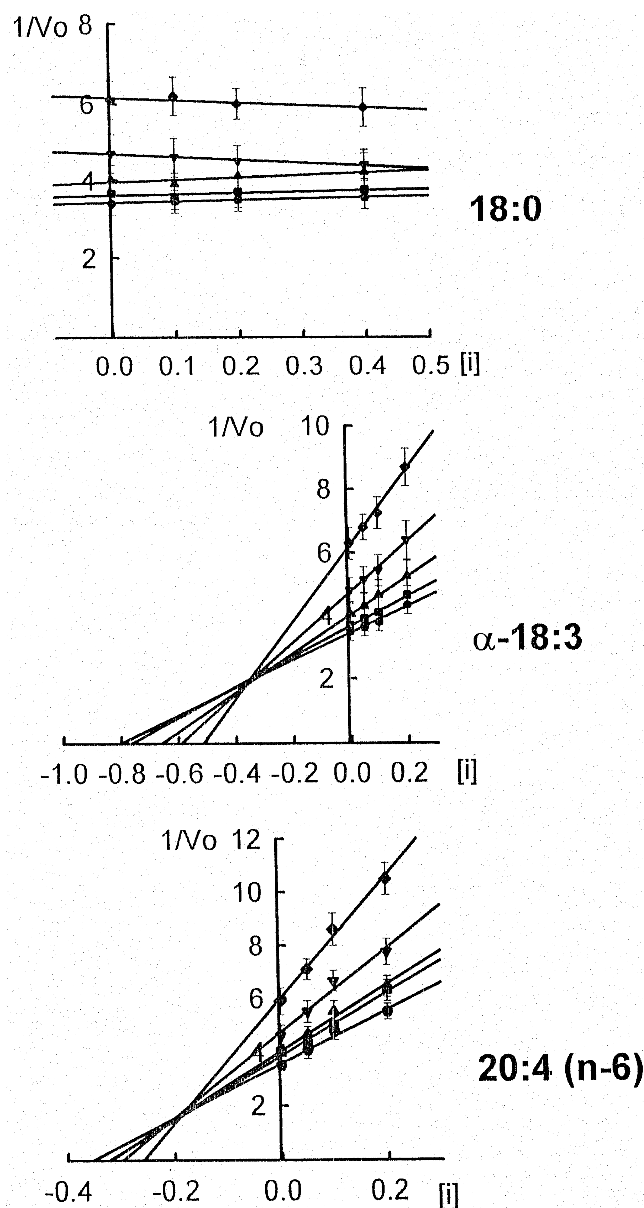

FIG. 6. Linear regressions of Dixon plots from calcium-deficient microsomes. Data were taken from the reciprocal plots shown in Figure 4. 1/Vo was expressed as $[\text{nmol}/(\text{min}\cdot\text{mg})]^{-1}\cdot 10^3$ and [i] was given as mM. Each point represents the mean ± 1 SEM of six different incubations assayed in duplicate. For abbreviations, see Figure 1.

KCl. These apparent kinetic parameters calculated from Figure 8 are summarized in Table 6.

DISCUSSION

An examination of the nonspecific ACS activity in liver microsomal suspensions from both normal and calcium-deprived rats using palmitic acid as substrate revealed that calcium deprivation promotes a significant increase in this enzymatic activity measured at initial velocity conditions. To our knowledge, this is the first experimental evidence of the regulatory effect caused by calcium deprivation on the palmitoyl-CoA synthesis in liver microsomes. Previous studies by other authors clearly indicate that the formation of palmitoyl-CoA causes a significant increase in the release of Ca^{2+} from microsomal vesicles (38–40). Based on these findings, it is reasonable to speculate that in calcium-deficient rats the stimulation observed in the biosynthesis of palmitoyl-CoA would preserve calcium levels within the cells.

From the results shown in Figure 1, we can assume that, under calcium-deficient conditions, the increased Vm resulted from an increment in the specific activity of the enzyme, whereas the substrate affinity remained unchanged. Previous studies from other laboratories showed that ACS seems to be largely dependent on the extent of substrate unsaturation and the carbon chain length (2–5,8,12). On the other hand, studies on the fatty acid composition carried out in platelets (4), lymphocytes (13), rat brain microsomes (5), and plasmodium-infected erythrocytes (12) demonstrated that the activation of palmitic acid is markedly decreased in the presence of unlabeled unsaturated fatty acids.

The interpretation of the competition studies shown in Figures 2–4 is rather difficult because of unknown factors, such as fatty acid solubility, critical micellar concentration of the different fatty acids, and the form in which fatty acids are present in the incubation medium. However, it was shown that in calcium-deficient microsomes the pattern of the inhibitory effects displayed by the tested fatty acids was similar but less noticeable than the one observed in control microsomes (Fig. 2A,B). The highest inhibitory capacity of unsaturated fatty acids provoked in the activation of palmitic acid (especially the polyunsaturated fatty acids of the n-6 or n-3 series), compared with the other fatty acids studied, may be due to their relatively easy accessibility and higher affinity for the membrane-bound enzyme. This explanation was first suggested by Reddy *et al.* (5) to justify the fatty acid specificity of long chain ACS from rat brain microsomes and mitochondria. Thus the minor response obtained for each fatty acid in calcium-deficient microsomes would be the consequence of some alterations in the physical and/or chemical state of these membranes.

The different kinetic behavior induced by calcium deficiency seems to be the consequence of a change in the concentration range at which the inhibitory effects are produced. In this regard, the kinetic pattern in both types of microsomes (Figs. 3 and 4) was similar, having a minor response in the CD-microsomal suspensions despite the highest concentrations of the fatty acids used as inhibitors. Taking into account that the three fatty acids studied exhibited special variations in their inhibition mechanisms, we may presume that there exist at least two binding sites in the nonspecific liver ACS. α-Linolenate, which behaves like arachidonate, would be activated at the same site and would have a better affinity for polyunsaturated species, whereas palmitate and stearate would be activated at the other site. The results summarized in Table 3 would support this hypothesis because of the differential effect on the palmitoyl-CoA synthesis caused by the unsaturated inhibitors, compared to stearic acid. On the other

TABLE 3
Apparent Kinetic Parameters of ACS for Palmitic Acid from Control or Calcium-Deficient Microsomes[a]

	Control			Calcium-deficient			Change (%)		
	18:0	α-18:3	20:4	18:0	α-18:3	20:4	18:0	α-18:3	20:4
Km	41 ±3	39 ±4	43 ±2	39 ±4	43 ±5	37 ±5	−5	10	−14
Vm	176 ±11	170 ±8	163 ±9	306* ±18	308* ±16	312* ±21	74	81	92
Ki	117 ±9	178 ±13	47 ±3	∞*	292* ±17	189* ±11	∞	64	302
αKi	—	537 ±22	113 ±7	—	789* ±53	347* ±29	—	48	207

[a]Apparent kinetic parameters are expressed as mean ± 1 SEM of six different incubations assayed in duplicate using at least five different substrate concentrations and four inhibitor concentrations (see Figures 3 and 4 for control or calcium-deficient diet, respectively). Km, Ki , and αKi are given in μM concentrations. Vm was expressed as nmoles/min.mg.protein. The inhibition constants (Ki for the competitive and αKi for the noncompetitive component) of the linear-mixed-type inhibition were calculated as described in the text. Percent changes were calculated with respect to the corresponding value of the control group. —, no inhibition observed; ∞, too high to be calculated; *significantly different with respect to control ($P < 0.01$).

hand, if saturated fatty acids had been activated at the same molecular site, the Km value of stearic acid as substrate would have had a value similar to its Ki in inhibiting the activation of palmitate. As shown in Figure 7, the Km for stearic acid, a pure competitive inhibitor, was 52.0 ± 4.1 and 101.3 ± 6.5 μM for control and CD-microsomes, respectively, whereas the Km for palmitic acid in both kinds of microsomes remained unchanged (41.6 ± 3.3 vs. 40.9 ± 4.1 μM). This finding indicates that, under calcium-deficient conditions, the affinity for stearic acid significantly declines; consequently, this acid works as a bad competitor for other substrates. It is worth noticing that the abnormal changes introduced by calcium deficiency only imply a significant increase in the Vm for palmitic acid, with no substantial modifications in Km values. On the contrary, in the case of stearic acid, Vm modification was less important than that observed in Km val-

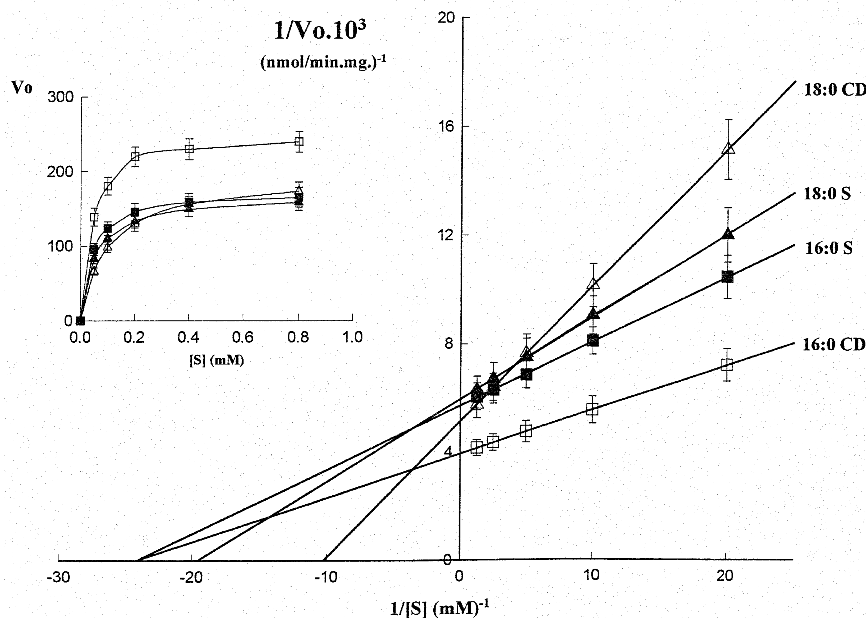

FIG. 7. Linear regressions for Lineweaver-Burk plots of ACS activity using palmitic acid (squares) or stearic acid (triangles) as substrates, from control (S) (solid symbols) or calcium-deficient (CD) (open symbols) microsomes. Enzyme activities were assayed as described in the Materials and Methods section. Typical Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 SEM of three different incubations assayed in triplicate. For abbreviations, see Figure 1.

TABLE 4
Interlipid Relationships and Fluorescence Anisotropy of Liver Microsomal Membranes from Control or Calcium-Deficient Rats[a]

	Control	Calcium-deficient	*n*	*P* <
Cholesterol/phospholipid (μmol/μmol)	0.31 ± 0.02	0.23 ± 0.01	3	0.02
Total lipid/phospholipid (mg/μmol)	0.79 ± 0.03	0.98 ± 0.04	3	0.02
Total lipid/cholesterol (mg/μmol)	2.48 ± 0.02	3.00 ± 0.03	4	0.001
Phospholipid/protein (μmol/mg)	0.45 ± 0.03	0.30 ± 0.01	3	0.01
Neutral lipid/protein (μg/μg)	0.06 ± 0.003	0.11 ± 0.01	3	0.01
Neutral lipid/polar lipid (μg/μg)	0.13 ± 0.01	0.36 ± 0.02	5	0.01
Fluorescence anisotropy (r_s)	0.1405 ± 0.0010	0.1112 ± 0.0008	6	0.01
Unsaturation index	3.80 ± 0.05	2.90 ± 0.1	4	0.001

[a]Results were expressed as the mean ± 1 SEM of the experiments indicated as "*n*". For details see the Materials and Methods section. Unsaturation index was calculated as the total double bonds in total unsaturated fatty acids/mol saturated fatty acids. *P* <, indicates the value at which calcium-deficient results are significantly different from the respective control value.

ues (Fig. 7). Whether changes in affinity and/or in the number of active enzyme molecules are the consequence or the cause of calcium-induced alterations in the lipid bilayer awaits further clarification.

Previous studies have demonstrated that the lipid domain fluidity is determined by the cholesterol/phospholipid molar ratio, lipid/protein ratio, and degree of unsaturation of lipid acyl chains (58,59). Such changes were reflected in our experimental system through a modification in the diphenylhexatriene steady-state fluorescence anisotropy (r_s) (Table 4), as detected by an increase in the rotational mobility of the probe within the membrane lipid phase when compared to those values observed in the microsomal membranes from control animals. As previously reported, this parameter depends on the overall membrane lipid mobility (60) and earlier studies with diphenylhexatriene have demonstrated that this probe is a useful tool to estimate membrane fluidity (61). Storch and Schachter (62) reported that an increase in calcium ion evokes a decrease in lipid fluidity of isolated rat hepatocyte plasma membranes by the activation of phospholipase A_2, demonstrating that the cation decreased the arachidonic acid content and the overall double bond index of membrane lipids. The effect of calcium deficiency on the physicochemical state of the microsomal membrane has not been shown until now, and further investigation on the precise role of this ion seems to be needed.

Previous studies carried out with long-chain ACS from rat liver microsomes showed that Triton X-100 enhanced fourfold the Vm value without affecting Km (5). Taking this fact into account, it seemed vital to investigate the ACS activity deprived of its microenvironment in the biomembrane.

Our results demonstrated that the enzyme preparation obtained after a solubilization with Triton X-100 exhibited a similar specific activity as compared with the one shown by a fraction solubilized with KCl. In accordance with data previously reported by other authors (45,63), the results presented

TABLE 5
Fatty Acid Composition (μg/mg microsomal protein) of Triglycerides and Phospholipids in Liver Microsomes from Control (S) or Calcium-Deficient (CD) Rats[a]

	Triglycerides		Phospholipids	
Fatty acids	S	CD	S	CD
14:0	0.6 ± 0.1	0.6 ± 0.1	0.1 ± 0.01	2.1 ± 0.1*
16:0	38.9 ± 1.2	44.9 ± 1.5*	16.0 ± 0.6	25.6 ± 1.0*
16:1n-7	3.3 ± 0.1	4.2 ± 0.2	0.9 ± 0.1	1.0 ± 0.1
18:0	7.8 ± 0.2	11.6 ± 0.9*	19.4 ± 0.2	27.1 ± 0.4*
18:1n-9	36.2 ± 2.0	34.5 ± 1.8	5.8 ± 0.1	5.6 ± 0.2
18:1n-7	3.0 ± 0.1	4.0 ± 0.2	4.1 ± 0.2	5.1 ± 0.2
18:2n-6	6.9 ± 0.3	13.6 ± 0.7*	11.3 ± 0.2	15.6 ± 0.4*
18:3n-3	0.1 ± 0.0	traces	0.2 ± 0.0	0.3 ± 0.02
20:3n-6	0.3 ± 0.1	1.1 ± 0.1*	2.3 ± 0.1	2.6 ± 0.1
20:4n-6	0.4 ± 0.1	traces	25.6 ± 0.7	15.7 ± 0.5*
20:5n-3	0.1 ± 0.01	traces	0.4 ± 0.1	0.6 ± 0.1
22:5n-3	traces	traces	1.0 ± 0.2	0.3 ± 0.1*
22:6n-3	traces	traces	9.6 ± 0.1	4.4 ± 0.2*

[a]Results are expressed as the mean ± 1 SEM from six animals assayed in duplicate. Asterisks indicate significantly different (*P* < 0.01) with respect to the corresponding control value. GLC analyses were performed as described in the Material and Methods section. Only relevant fatty acids were included.

TABLE 6
Apparent Kinetic Parameters of Solubilized ACS Activity for Palmitic Acid, from Control (S) or Calcium-Deficient (CD) Microsomes[a]

	Treatment			
	KCl		Triton X-100	
	S	CD	S	CD
Km (μM)	42.6 ± 4.1	39.2 ± 5.2	56.8 ± 4.3	60.4 ± 5.5
Vm (nmol/min·mg)	177.1 ± 8.7	176.9 ± 6.6	167.3 ± 9.8	158.6 ± 5.5

[a]Results were obtained from the linear regressions shown in Figure 8. Data are given as the mean ± 1 SEM of four different incubations assayed in triplicate.

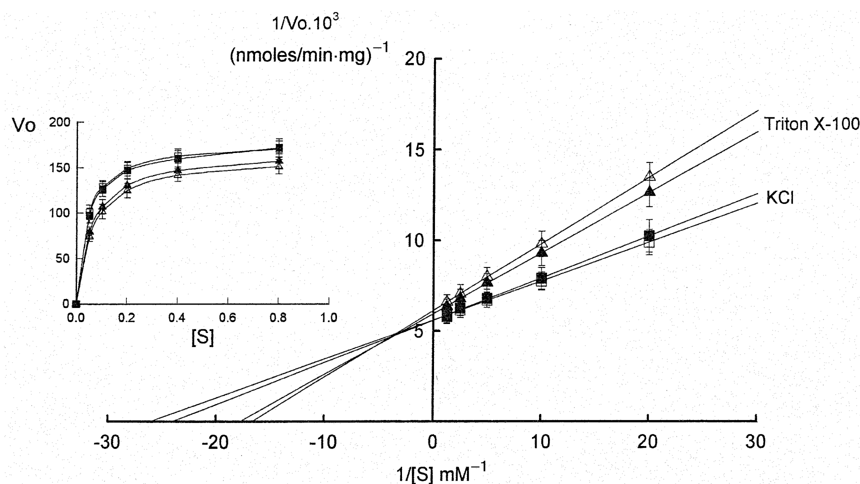

FIG. 8. Linear regressions for Lineweaver-Burk plots of solubilized ACS activity for palmitic acid, from control (open symbols) or calcium-deficient (solid symbols) microsomes. Both types of microsomes were treated with Triton X-100 (triangles) or a buffer with KCl (squares). After partial purification, the activity was assayed as described in the Materials and Methods section. Michaelis-Menten plots are shown in the insert. Each point represents the mean ± 1 SEM of four different incubations assayed in triplicate. For abbreviations, see Figure 1.

in this paper also show that residual amounts of Triton X-100 have no significant effect on the palmitoyl-CoA synthetase activity, but they promote an increase in Km values without affecting Vm (Table 5).

Considering that membrane enzymes such as ACS interact with surrounding lipids and proteins in the membrane, it is conceivable that the configuration of the molecule would be altered if its microenvironment changed. It is important to remark that the different behavior exhibited by ACS between S- and CD-microsomal preparations was eliminated after solubilization procedures. From the results shown in Figure 8 and Table 5, we may assume that both modifications in the specific activity of the enzyme and the differential response observed in competition experiments were the consequence of the alterations in the reticulum membranes induced by the calcium-deficient diet.

In conclusion, this work shows experimental evidence of the role of calcium ions in relation to fatty acid activation. These studies would indicate an alteration in the physicochemical state of the lipid bilayer, evoked by calcium deficiency, that indirectly modifies the kinetic properties of the enzyme. The results presented here would be of interest when examining the intracellular regulation of acyl-CoA synthesis, from the lipid metabolism as well as cell biology and physiology points of view.

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The Inhibitory Guanine Nucleotide-Binding Protein $G_{i2}\alpha$ Induces and Potentiates Adipocyte Differentiation

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ABSTRACT: The present study further elucidates the involvement of the α-subunit of the GTP-binding protein G_{i2} in the differentiation of murine 3T3-L1 cells. Control and vector-transfected cells attained a fully differentiated adipocyte phenotype showing ample lipid droplets. Cells expressing wild type (WT)-$G_{i2}\alpha$ or the constitutively active R179E-$G_{i2}\alpha$, however, became enlarged, less confluent, and produced large amounts of lipids. Differentiation consistently increased the triglyceride (TAG) content in control cells. In both WT-$G_{i2}\alpha$ and R179E-$G_{i2}\alpha$ clones, a marked increase in TAG could be detected even prior to insulin/dexamethasone/isobutyl methylxanthine exposure. The activity of palmitoyl-CoA synthetase (PCS) and glycerophosphate acyltransferase (GPAT) also increased upon differentiation. WT-$G_{i2}\alpha$ and R179E-$G_{i2}\alpha$ overexpression also enhanced PCS and GPAT activities even before differentiation medium was added. The total amount of phospholipids (PL) generally increased upon differentiation; however, pre- and postdifferentiation values were insignificantly different in cells expressing WT-$G_{i2}\alpha$ and R179E-$G_{i2}\alpha$. Differentiation altered the PL profile with a relative shift from phosphatidylcholine and phosphatidylethanolamine to phosphatidylinositol (PI) in differentiated cells. Finally, differentiation yielded a general increase in the activity of basal PI-phospholipase-C activity. Again, cells expressing WT-$G_{i2}\alpha$ and R179E-$G_{i2}\alpha$ demonstrated elevated enzyme activity and enhanced second messenger accumulation subsequent to differentiation. In summary, cells with the R179E-mutants of $G_{i2}\alpha$ exhibited stimulated lipid turnover and accumulation in both undifferentiated and differentiated cells.

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The G-proteins (guanine nucleotide-binding proteins) were discovered in 1971 (1). Since then, more than 20 α-subunits have been described (2,3), which constitute four distinct classes or families (i.e., G_s, G_i, G_q, and G_{12}). The G-proteins, which are more or less ubiquitous (3), are able to communicate with each other (4,5), and there is also extensive cross-talk between G-protein-mediated and several other signaling systems (5,6). Malfunctions of G-proteins may result in altered communication within cells, leading to aberrations in hormonal signalling (6,7). In turn, endocrine diseases like acromegaly may result (8). Therefore, naturally occurring mutations in G-proteins have been sought and discovered in different endocrine tumors and osteosarcoma cells (9,10). This has led to speculations about G-proteins as possible proto-oncogenes, implicating them as discriminant factors in both cellular growth and differentiation (11,12).

Murine 3T3-L1 cells are preadipocytes that differentiate to mature adipocytes (13,14) after 5 to 7 d of treatment with insulin, dexamethasone, and the phosphodiesterase inhibitor isobutyl methylxanthine. G_{i2} of the G_i-subspecies has previously been implicated in the modulation of DNA-synthesis and transcription rates (15,16), and has also been shown to couple to the insulin-like growth factor (IGF) type-II receptor (17). We recently transfected 3T3-L1 cells with the stimulatory R179E- and Q205L-$G_{i2}\alpha$ [constitutively activated (GTPase deficient) $G_{i2}\alpha$ mutant] constructs, which reduced the rate of DNA-synthesis prior to cell confluence (16). These missense mutations strongly affected the differentiation process measured by increased hormone-sensitive lipase mRNA, increased isoprenalin- and corticotropin-elicited adenylate cyclase activity, and elevated 2-deoxy glucose uptake (16).

The goal of the present work is to elucidate to what extent $G_{i2}\alpha$ influences deposition of triglycerides (TAG), fat-metabolizing enzymes, and phospholipid (PL) content and turnover as markers of the differentiation process.

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Abbreviations: DAG, diacylglycerol; G204A-$G_{i2}\alpha$, constitutively inactivated (receptor dissociated) $G_{i2}\alpha$ mutant; GPAT, glycerophosphate acyltransferase; GPDH, glycerophosphate dehydrogenase; IGF, insulin-like growth factor; IP_3, 1,4,5-inositol trisphosphate; MAPK, mitogen-activating protein kinase; PBS, phosphate-buffered saline; PCS, palmitoyl-CoA synthetase; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PI-PLC, phosphatidylinositol phospholipase-C; PKC, protein kinase C; PL, phospholipid; pZipNeo, pZipNeo(SX)V (retroviral vector); R179E- and Q205L-$G_{i2}\alpha$, constitutively activated (GTPase deficient) $G_{i2}\alpha$ mutant; TAG, triglyceride; WT, wild type.

MATERIALS AND METHODS

Site-directed $G_{i2}\alpha$ mutations and construction of expression vectors. The insert of plasmid DJG18, encoding a rat α_{i2} cDNA (provided by Reed, R., Johns Hopkins University, Baltimore, MD) was subcloned into M13mp19, and site-directed mutagenesis was performed by a modification of the method of Nakamaye and Eckstein (18). The mutagenic oligonucleotide had the following sequences: R179E-α_{i2}, 5′-GC-

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CTGTGGTCTTCACTTCGGTC-CGCAGCACATC-3′, and G204A-α_{i2}, 5′-CCGCTCAGATCGCTGGGCGCCCACAT-CAAACAT-3. Mutations were confirmed by dideoxy sequencing. Wild type (WT) and mutant α_{i2} cDNA were ligated into the EcoRI site of a shuttle vector pSP72NotI and then into the NotI site of a modified retroviral vector, pZipNeoSV(X) (19). In the latter constructs, the transcription of α_{i2} cDNA and the G418-resistance gene is directed by the Moloney murine leukemia virus long terminal repeat.

Transfection and clone selection. Cell lines were transfected with 1.0 µg of linearized DNA per 10-cm plate by calcium phosphate precipitation. Twenty-four hours after transfection, cells were grown in selective medium containing G418 (0.60 mg/mL) for at least 3 wk. G418-resistant clones were selected by serial dilution. Ten clones were selected for each construct, and the studies described here were performed with one of the three clones demonstrating the highest level of $G_{i2}\alpha$ expression.

Cell culture. 3T3-L1 cells were grown in monolayer cultures in petri dishes 6 cm in diameter, obtained from Costar (Cambridge, United Kingdom). Dulbecco's modified Eagle medium (DMEM) with high glucose, purchased from GibcoBRL (Grand Island, NY), was used as growth medium. To this medium were added 10% fetal calf serum, penicillin-streptomycin (50 µg/mL), fungizone (2.5 µg/mL), and anti-pleuropneumonia-like organisms agent (10 µg/mL) from GibcoBRL. The cells were grown in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Medium was changed three times a week. Differentiation was accomplished with insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM) present in the growth medium for 7 d after cell confluence. The hormones were obtained from Sigma Chemical Company (St. Louis, MO). Growth medium was changed every 24 h for the last 3 d before the cells were fully differentiated. Cells were prewashed with 2 × 1.0 mL phosphate-buffered saline (PBS), pH 7.4, and then finally harvested in 2.0 mL PBS.

Measurement of DNA content. The DNA content was measured by the method of Labarca and Paigen (20).

Analysis of protein level. The protein amount was measured by the method of Lowry *et al.* (21) using bovine serum albumin (BSA) as a standard.

Determination of TAG. For the determination of TAG amounts, a commercial kit delivered by Boehringer (Mannheim, Germany), was used. The assay is based on spectrophotometric end point glycerol measurements subsequent to hydrolysis.

Assay of palmitoyl-CoA synthetase (PCS). The enzyme velocity was assayed as described by Krisans *et al.* (22). Washed cells were homogenized using an Ultra-Turrax for 10 s and centrifuged for 30 min at 27,000 × *g*. The supernatant was discarded. The reaction was started by the addition of crude, resuspended membrane protein (20–50 µg) to ice-cold incubation mixture. The mixture had a final volume of 400 µL and consisted of 150 mM Tris-HCl, pH 7.4, 6.2 mM $MgCl_2$, 0.5 mg/mL Triton X-100, 2 mM ATP, 600 mM CoA, 1 mM dithiothreitol, and 100 µM [1-^{14}C]-palmitic acid ($5 \cdot 10^4$ dpm). After 6.5 min incubation at 37°C, the reaction was terminated by addition of 3.0 mL of methanol/chloroform/heptane (1.4:1.25:1.0) and 1.0 mL of 0.1 M sodium acetate, pH 4. After vortexing and centrifugation for 5 min at 270 × *g* at room temperature, 1.0 mL of the aqueous phase was mixed with 10 mL of scintillation fluid, and radioactivity was determined by counting in a Packard counter for 5 min.

Assay of glycerophosphate acyltransferase (GPAT). The enzyme activity was essentially assayed as described by Sooranna and Saggerson (23). Crude membrane protein was prepared as indicated above. Crude membrane protein (0.5 mg) was incubated in a total volume of 1.0 mL for 7.5 min at 30°C. The incubation mixture contained 0.1 M Tris-HCl, pH 7.4, 0.7 mM L-[U-^{14}C]-glycerol 3-phosphate (0.06 mCi/mmol), 2 mM $MgCl_2$, 2 mM KCN, 0.7 mM dithiothreitol, bovine serum albumin (1.2%, wt/vol) and 70 µM palmitoyl-CoA. The reaction was stopped by the addition of 4.0 mL of 6% $HClO_4$. After vortexing and centrifugation at 12,100 × *g* for 10 min, the supernatant was decanted. The walls of the incubation tubes were wiped with tissue paper before the pellet was redissolved in 1.6 mL distilled water and shaken for 1 h. Butanol (1.0 mL) was added, followed by vortexing, the addition of 4.0 mL $HClO_4$, and repeated vortexing. After centrifugation for 5 min at 480 × *g*, 0.5 mL of the supernatant butanol layer was mixed with 10 mL scintillation fluid and radioactivity determined in a Packard counter.

Measurement of PL. The growth medium was removed from the cell culture dishes after 7 d of treatment subsequent to confluency. Growth medium containing 10 µCi/mL of [^{32}P]-labeled phosphate was added to the dishes and the cells were incubated for 2 h. Finally, the medium was removed, and the cells were washed twice with PBS, pH 7.4. Then the cells were harvested in 1.0 mL PBS, the lipids were extracted using the procedure of Folch *et al.* (24), and the organic layer was dried under a stream of N_2. The lipid residues were dissolved in 100 µL of chloroform and applied to thin-layer chromatography plates delivered by Merck (Darmstadt, Germany). The plates were chromatographed for 2 h in a tank containing 65 mL methanol, 35 mL chloroform, 5.0 mL 40% methylamine, and 5.0 mL distilled water (25). The radiolabeled PL were visualized by autoradiography. Autoradiographic film was delivered by Amersham International (Buckinghamshire, United Kingdom). The spots representing the different PL were scraped, 4.5 mL of Insta-Gel II was added, and the vials were counted in a Packard scintillation counter for 5 min.

Assay of diacylglycerol (DAG). The assay was performed using a commercial kit (RPN200) furnished by Amersham, and designed to analyze tissue and cellular levels of DAG. The assay procedure is based on a radioenzymatic assay, employing the enzyme DAG kinase which quantitatively converts DAG to phosphatidic acid in the presence of [^{32}P]-γ-ATP.

Phosphatidylinositol phospholipase C (PI-PLC) assay. This method was adapted from Jackowski *et al.* (26).

Aliquots (30 μL) of diluted crude membrane suspensions (35 μg protein) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, were mixed with 10 μL incubation mixture (100 mM Tris-HCl, pH 6.5, 400 μM GTP, 2.2 mM $CaCl_2$, 1.0 mM $MgCl_2$ in 2.4-mL microfuge tubes on ice. Three microliters (42,000 cpm) of a $[^3H]$phosphatidylinositol 4,5-bisphosphate (PIP_2) (New England Nuclear, Boston, MA) stock solution in 2% sodium cholate was added to each tube, and incubations were carried out at 35°C for 5 min. The reaction was stopped by adding in succession 150 μL $CHCl_3/CH_3OH/HCl$ (1:2:0.02), 50 μL $CHCl_3$, and 50 μL 2 M KCl. After vortexing and phase separation at 5000 × *g* in a microfuge, 100-μL aliquots of the aqueous layers were counted in a Packard liquid scintillation counter. In the assay blanks, some 2% of the radioactivity was retained in the aqueous phase.

RESULTS

The gross appearance of the 3T3-L1 cells changed markedly subsequent to differentiation (Fig. 1). Cells, transfected with the retroviral vector pZipNeoSV(X), attained the characteristics of fully mature adipocytes upon differentiation. However, the WT-$G_{i2}\alpha$ and the activating mutant R179E-$G_{i2}\alpha$ cell clones became larger upon transfection and accumulated much larger lipid droplets subsequent to differentiation. The inactivating mutant G204A-$G_{i2}\alpha$ did not affect the 3T3-L1 preadipocytes significantly (not shown).

Consistent with the profile of lipid droplet deposition seen in the light microscope, differentiation increased the TAG contents in control and vector-transfected cells as well as in cells expressing WT-$G_{i2}\alpha$ and R179E-$G_{i2}\alpha$. However, the differentiation medium did not enhance ($P > 0.05$) TAG contents in G204A-$G_{i2}\alpha$ transfected cells (Fig. 2). Characteristically, a distinct elevation of TAG content could be detected in WT-$G_{i2}\alpha$- and R179E-$G_{i2}\alpha$-expressing clones even before differentiation ($P < 0.05$).

To further elucidate the impact of $G_{i2}\alpha$ on TAG accumulation, the two lipid-synthesizing enzymes PCS (Fig. 3A) and GPAT (Fig. 3B) were studied. The activity of each enzyme exhibited the same pattern as the TAG deposition profile. The

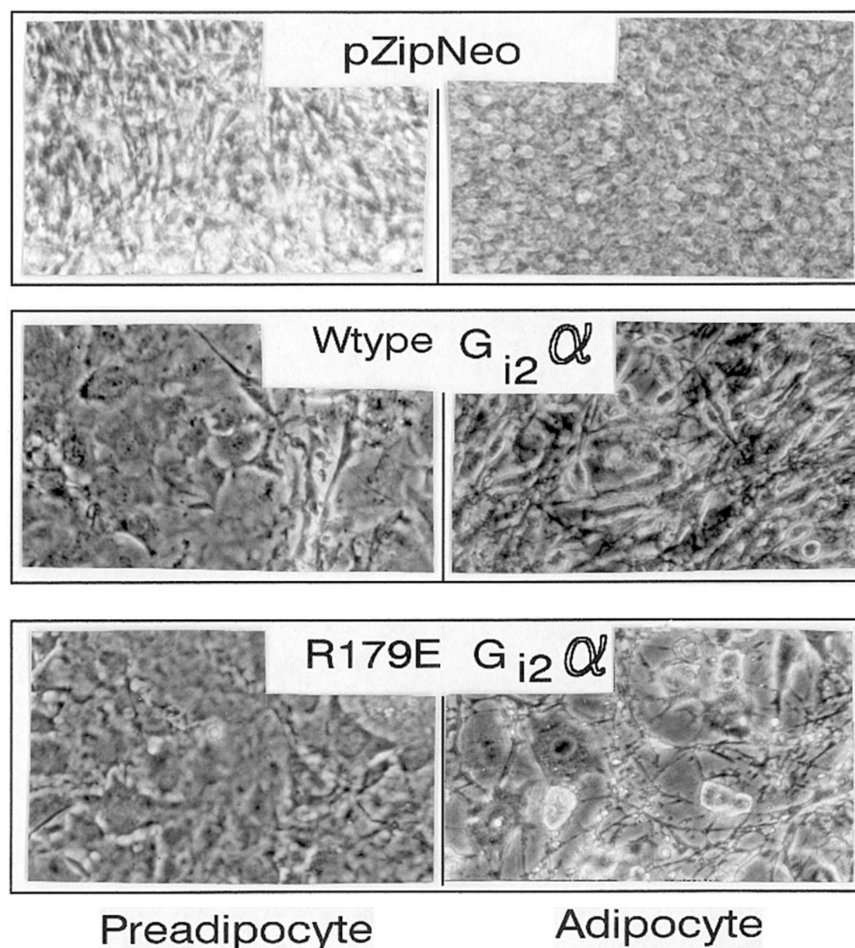

FIG. 1. Stably $G_{i2}\alpha$-transfected 3T3-L1 cells at confluence subsequent to 7 d of treatment with insulin (10 μg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). All clones were grown in Dulbecco's modified Eagle medium containing 10% fetal calf serum and standard penicillin-streptomycin, with antipleuropneumonia-like organisms agent and fungizone supplementation. Abbreviations: pZipNeo = pZipNeo(SV)X (retroviral vector); Wtype $G_{i2}\alpha$ = wild type $G_{i2}\alpha$; R179E-$G_{i2}\alpha$ = constitutively activated (GTPase deficient) $G_{i2}\alpha$ mutant.

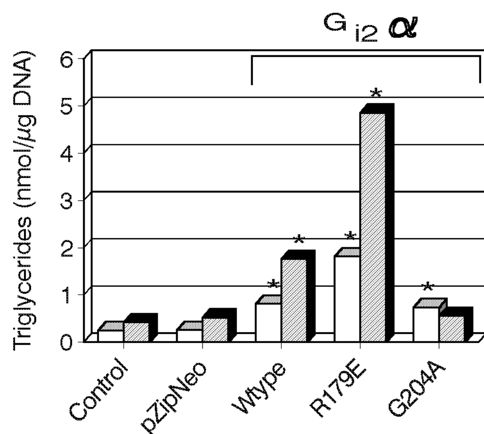

FIG. 2. Triglyceride contents (nmol/µg DNA) in stably $G_{i2}\alpha$-transfected 3T3-L1 preadipocytes (open bars) and adipocyte (cross-hatched bars) measured before and after 7 d of incubation in Dulbecco's modified Eagle medium supplemented with 10% fetal calf serum, insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). Washed cells were harvested in phosphate-buffered saline, pH 7.4, and homogenized for 10 s. Lipids were extracted at room temperature by the procedure of Folch *et al.* (24), and triglyceride content assayed using a commercial kit from BioMerrieux. *Significant differences ($P < 0.05$) from nontransfected control cells. (Student's *t*-test, $n = 6$). Average coefficient of variation (CV) for this study equaled 13.4%. G204A: G204A-$G_{i2}\alpha$, constitutively inactivated (receptor dissociated) $G_{i2}\alpha$ mutant. For other abbreviations see Figure 1.

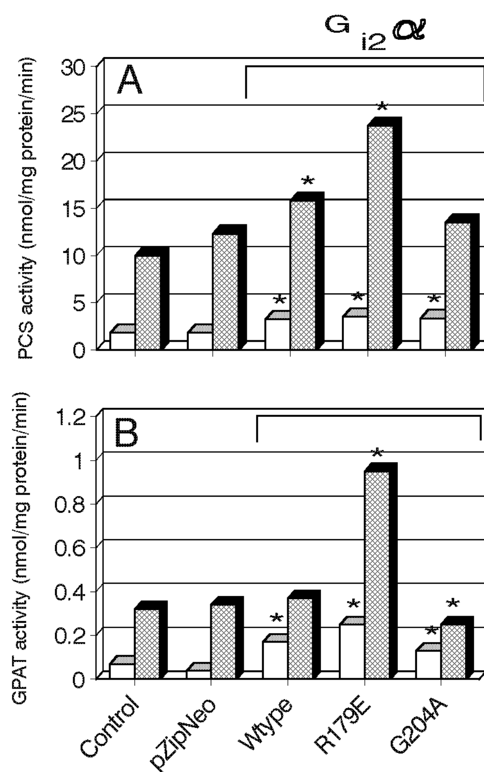

FIG. 3. (A) Palmitoyl-CoA synthetase (PCS) activity measured as nmol · min^{-1} · mg^{-1} in membranes from stably $G_{i2}\alpha$-transfected undifferentiated cells and cells treated with insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). Cells harvested in 1.0 mL of phosphate-buffered saline (PCS), pH 7.4, were homogenized for 10 s and centrifuged for 30 min at 27,000 × *g* to isolate membranes. PCS was determined according to the method of Krisans *et al.* (22). Protein (20–50 µg) was incubated for 6.5 min at 37°C in a mixture of 150 mM Tris-HCl, pH 7.4, 6.2 mM $MgCl_2$, 0.5 mg/mL Triton X-100, 2 mM ATP, 600 mM CoA, 1 mM dithiothreitol, 100 µM [1-^{14}C]-palmitic acid (5 × 10^4 dpm) in a total volume of 400 µL. *Significant differences ($P < 0.05$) from nontransfected control cells (Student's *t*-test, $n = 6$). Average CV for this study equaled 17.2%. (B) Glycerophosphate acyltransferase (GPAT) activity (nmol · min^{-1} · mg^{-1}) measured in undifferentiated 3T3-L1 cells and after treatment with insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). GPAT was determined essentially according to Sooranna and Saggerson (23). Membrane protein fractions were incubated in a total volume of 1.0 mL at 30°C for 7.5 min. The incubation solution contained 0.1 M Tris buffer, pH 7.4, 0.7 mM of L-[U-^{14}C]-glycerol 3-phosphate (0.06 mCi/mmol), 2 mM $MgCl_2$, 2 mM KCN, 0.7 mM dithiothreitol, bovine serum albumin (1.2 %, wt/vol), and 70 µM palmitoyl-CoA ester. *Significant differences from nontransfected control cells (Student's *t*-test, $n = 4$). Average CV for this study equaled 14.7%. Open bars: preadipocytes; cross-hatched bars, adipocyctes. Abbreviation: Wtype-$G_{i2}\alpha$ = wild type $G_{i2}\alpha$. For other abbreviations see Figures 1 and 2.

activating enzyme PCS showed a marked rise after differentiation in both control and transfected cells ($P < 0.05$). Not unexpectedly, the most notable increase was elicited by the R179E-$G_{i2}\alpha$ construct. A similar phenomenon with increased enzyme activity subsequent to differentiation ($P < 0.05$) was also observed for GPAT. Again the highest rate of activity was exhibited by cell cultures expressing R179E-$G_{i2}\alpha$. The somewhat unexpected finding that G204A-$G_{i2}\alpha$ mutant also brought about changes of PCS and GPAT activities in 3T3-L1 cells is discussed later.

The documented two- to fourfold stimulation of the lipid-synthesizing machinery, induced by R179E-$G_{i2}\alpha$, stimulated speculations as to whether the total amount of PL was also augmented to the same extent. As can be interpreted from Figure 4, preadipocyte cell transfection and differentiation led to a marked elevation in total amount of PL ($P < 0.05$). Compared to control and vector-transfected cells, the R179E-$G_{i2}\alpha$-expressing clones exhibited the most striking increase, both before and after differentiation. Despite elevation of predifferentiation contents of PL in G204A-$G_{i2}\alpha$-expressing cells, enhancement of PL accumulation upon incubation in differentiation medium was not observed ($P < 0.05$). Figure 5 portrays the percentage shift in the PL profile subsequent to 7 d of attempted differentiation. All the clones transfected with activating $G_{i2}\alpha$-containing constructs reduced ($P < 0.05$) their relative phosphatidylethanolamine (PE) and phosphatidylcholine (PC) contents, while relative phosphatidylinositol (PI) levels were enhanced ($P < 0.05$).

Increased steady-state amounts of PI moieties (i.e., PI, phosphatidylinositol 4-phosphate PIP_2) may signify chronically enhanced protein kinase (i.e., protein kinase C = PKC; calmodulin-dependent protein kinase = CamPK) activities. Hence, analyses of accumulated DAG and rate of 1,4,5-inositol trisphosphate (IP_3) synthesis were conducted (see Fig. 6A and B, respectively). The cells expressing the R179E-$G_{i2}\alpha$-activating mutant exhibited much higher levels of DAG and

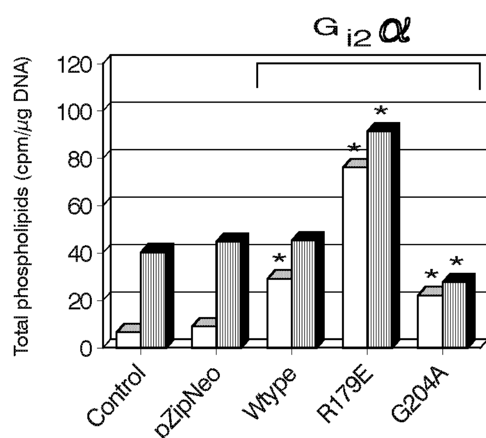

FIG. 4. Total amounts of phospholipids measured as [^{32}P]-cpm/µg DNA in undifferentiated cells and after treatment with insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). The cells were incubated for 2 h in 0.5 mL of Dulbecco's modified Eagle medium supplemented with 10 µCi/mL [^{32}P]-labeled phosphate. The cells were washed twice in 1.0 mL phosphate-buffered saline (PBS), pH 7.4. Then the cells were harvested 1.0 mL PBS, pH 7.4 and the lipids extracted at room temperature using the procedure of Folch *et al.* (24) and dried under a stream of N_2. The lipids were dissolved in 100 µL of $CHCl_3$ and applied to thin-layer chromatographic (TLC) plates. After chromatography and autoradiography were completed, the TLC plates were cut and counted in a Packard scintillation counter for 5 min. *Significant differences ($P < 0.05$) from nontransfected control cells (Student's *t*-test, $n = 4$). Average CV for this study equaled 12.0%. Open bars, preadipocytes; lined bars, adipocytes. For abbreviations see Figures 1–3.

rate of IP_3 production after differentiation ($P < 0.05$). No other construct modulated the differentiation process in terms of DAG amounts and IP_3 production to a significant extent ($P > 0.05$).

DISCUSSION

According to Wang *et al.* (27,28), differentiation of 3T3-L1 preadipocytes can be enhanced by insulin or by dexamethasone and a methyl xanthine; however, insulin is the main anabolic hormone taking part in regulation of metabolism in the liver, muscle, and adipose tissue (29). *In vivo*, insulin also plays a role in cellular proliferation (30). GTP-binding proteins (G-proteins) mediate transmembrane signaling from receptors on the cell surface to different effector units (2–6). The levels of G-proteins often change upon cell differentiation (31–34). However, we (16) and others (27,28,35) have previously shown that $G_s\alpha$ inhibits, whereas $G_{i2}\alpha$ enhances the extent to which 3T3-L1 cells differentiate. In this work, we assess the impact of $G_{i2}\alpha$ on the differentiation process in terms of actual TAG deposition, fat-metabolizing enzymes, PL accumulation, PL profile, and second messengers derived from PI-PLC-elicited turnover of PI trisphosphate (PIP_2).

Generally, this paper demonstrates that overexpression of constitutively active $G_{i2}\alpha$ enhanced TAG-synthesizing enzyme activities as well as TAG steady-state levels. However, inconsistent data with regard to lipid synthesis rate and accumulation in the G204A-$G_{i2}\alpha$ expressing clones were observed. These may be due to an inherent variability of the 3T3-L1 cells to respond to differentiation stimuli (27,35). In a previous report, we showed that the inactive G204A-$G_{i2}\alpha$ construct completely inhibited 3T3-L1 cell differentiation in terms of isoproterenol- and corticotropin-susceptible adenyl-

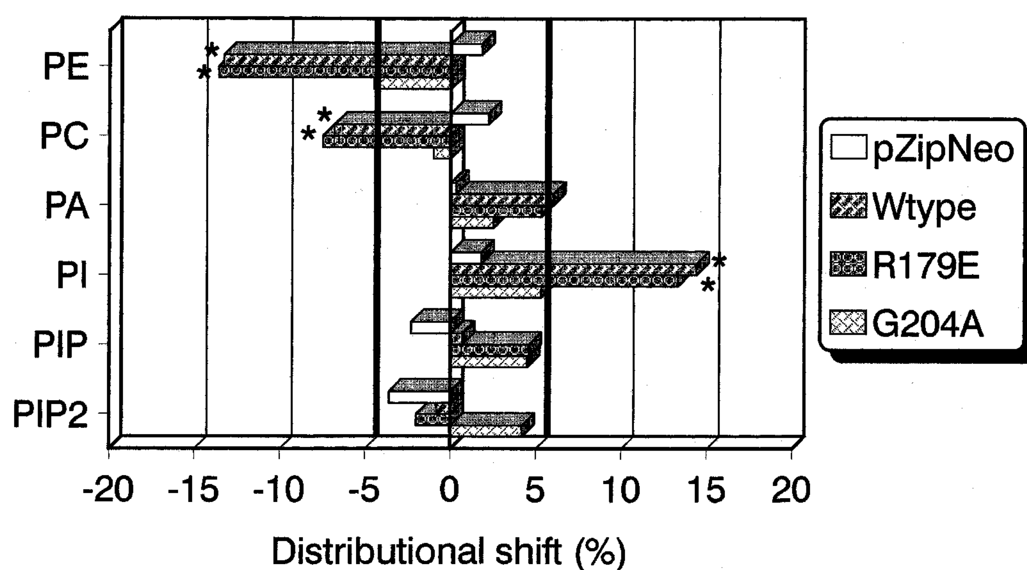

FIG. 5. Changes in phospholipid profile in 3T3-L1 cells after treatment for 7 d with insulin (10 µg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM) as compared to control. The figure shows percentage (%) distribution of different phospholipids. *Significant differences ($P < 0.05$) from nontransfected control cells (Student's *t*-test, $n = 6$). Average CV for this study equaled 12.0%. Abbreviations: PE = phosphatidylethanolamine; PC = phosphatidylcholine; PA = phosphatidic acid; PI = phosphatidylinositol; PIP = phosphatidylinositol 4-monophosphate; PIP_2 = phosphatidylinositol 4,5-bisphosphate. For other abbreviations see Figures 1–3.

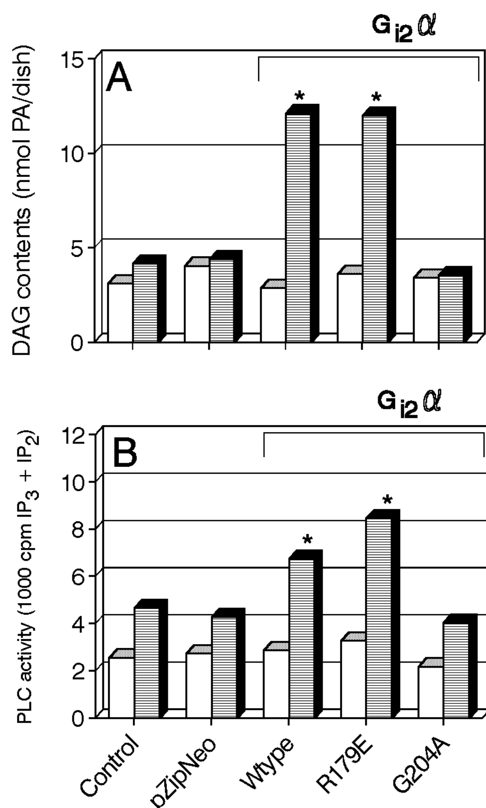

FIG. 6. (A) Diacylglycerol (DAG) contents (nmol/dish) measured as phosphatidic acid (PA) in undifferentiated cells and after treatment with insulin (10 μg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM) for 7 d. Lipids, extracted according to the procedure of Folch *et al.* (24), were incubated with DAG kinase and $[^{32}P]$-γ-ATP as described in a commercial kit obtained from Amersham (Buckinghamshire, United Kingdom). $[^{32}P]$-PA was isolated using thin-layer chromatography and autoradiography followed by counting in a Packard liquid scintillation counter. *Significant differences ($P < 0.05$) from nontransfected control cells (Student's *t*-test, $n = 3$). Average CV for this study equaled 11.4%. (B) Phosphatidylinositol phospholipase-C (PLC) activity measured in undifferentiated and differentiated cells incubated for 7 d with insulin (10 μg/mL), dexamethasone (10 nM), and isobutyl methylxanthine (0.5 mM). Aliquots (20 μL) of crude membrane suspensions (35 μg protein) in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, were mixed with 10 μL incubation mixture (containing GTP, $CaCl_2$, and $MgCl_2$) on ice. $[^3H]$-PIP_2 stock solution (42,000 cpm) was added to each tube and incubated for 5 min at 35°C. After additions of organic solvents and phase separation, 100 μL of the aqueous phase was counted in a liquid scintillation counter. *Significant differences ($P < 0.05$) from nontransfected control cells (Student's *t*-test, $n = 6$). Average CV for this study equaled 6.8%. Open bars, preadipocytes; lined bars, adipocytes. IP_3, 1,4,5-inositol trisphosphate; IP_2, inositol 1,4-bisphosphate; for other abbreviations see Figures 1–3.

ate cyclase activation and hormone-sensitive lipase mRNA levels (16). Finally, overexpression of constitutively active $G_{i2}\alpha$ enhanced the overall contents of phosphatidyl moieties, as well as steady-state levels of the second messengers DAG and IP_3.

Differentiation of the preadipocytes has been linked to insulin or IGF type I (IGF-I) activation of the mitogen-activating protein kinase (MAPK) cascade (2,36). Since the G_{i2} heterotrimer was shown to couple to the IGF-II receptor subtype (17), the impact of $G_{i2}\alpha$ probably also involves MAPK activation through *raf*- and *ras*-proteins (36,37). However, this model of preadipocyte differentiation presents a similar G-protein profile as adipocytes from obese mice (38,39) and from hypothyroid rats (40), where the $G_{i2}\alpha/G_s\alpha$ ratio is enhanced. Contrastingly, no difference in the $G_{i2}\alpha/G_s\alpha$-ratio could be detected in adipocytes from insulinopenic diabetic rats (41), thyroidectomized patients suffering from papillary carcinoma (42), or obese subjects (42). Nonetheless, evidence has been published that $G_i\alpha$ in adipocytes from streptozocin-induced diabetic rats display far less efficient coupling to prostaglandin E_1- and A_1-receptors (43). All in all, the recent literature indicates that enhanced differentiation of adipocytes by $G_{i2}\alpha$ involves enhanced growth factor receptor coupling to the MAPK pathway with a concomitant reduction in basal cAMP production.

Others have demonstrated that preadipocytes enhance their levels of acetyl-CoA carboxylase, fatty acid synthetase, and glycerophosphate dehydrogenase (GPDH) activity (44–46) upon differentiation. The activities of these and other enzymes, including PCS and GPAT, are also augmented in adipocyte differentiation *in vivo* (14). Hence, it may be asserted that $G_{i2}\alpha$ plays a major role in the phenotypic change of fibroblast-like cells to mature adipocytes, both as a solitary signal and as an amplifier of insulin.

Like the TAG, the steady-state level of PL rose as a consequence of the differentiation process induced by $G_{i2}\alpha$. When the cells enter their resting state as adipocytes, their content of PI, PIP, and PIP_2 increases markedly. It has been reported that insulin stimulates the activity of PI 3-kinase, thus leading to an increased amount of PIP_2 (47). As PI 3-kinase can be considered a key enzyme in the polyphosphoinositide cycle (48,49), changes in the content of other PL in this cycle may be a result of PI 3-kinase modulation. Apparently, enhanced $G_{i2}\alpha$ activity may have induced PI 3-kinase by itself and potentiated the stimulatory effect of insulin. Interestingly, wortmannin, which inhibits PI 3-kinase activity, was demonstrated also to reduce 3T3-L1 lipid deposition and GPDH activity (50). Furthermore, pioglitazone, which normally interferes with cAMP-dependent pathways, was shown to obliterate the isoproterenol-induced reduction in PI 3-kinase activity (51). In summary, the relative increase in PI-derived moieties over the levels induced by insulin itself may reside with $G_{i2}\alpha$-elicited potentiation of the insulin effect or also may be due to inhibition of basal adenylate cyclase activity in the preadipocytes.

DAG has been reported to be generated in response to insulin in the 3T3-L1 clone and in many other cell types (52,53). DAG is a signal molecule and is made from the same precursor (PIP_2) as IP_3 through hydrolysis by PI-PLC. Thus, this pathway is bifurcate, as the external message is split into two second messengers. DAG is lipophilic and induces translocation of PKC. After stimulation, DAG kinase removes DAG by converting it to phosphatidic acid. IP_3 is water-soluble and is released into the cytosol where its role is

to mobilize Ca^{2+}. Recently, it was demonstrated that pertussis toxin treatment of 3T3-L1 cells reduced insulin-elicited DAG release, GPDH activity, and 2-deoxy glucose uptake (54). Hence, $G_{i2}\alpha$-induced adipocyte differentiation involves chronically enhanced PIP_2 turnover. It was shown that PKC seeks to attenuate PI-PLC activity in differentiated 3T3-L1 cells (55); however, it seems that basal PI-PLC activity in cells expressing the constitutively activated $G_{i2}\alpha$ stays at a 2.5-fold higher level. Phorbol-12-myristate-13-acetate and the ionophore ionomycin synergistically counteract adipocyte marker gene expression (56). Since PKC is known to inactivate $G_{i2}\alpha$ (6), it seems that a reduction in $G_{i2}\alpha$ during normal adipocyte differentiation is a natural mechanism used by the preadipocyte to counteract excessive phenotype alteration. In fact, treatment of mature 3T3-L1 adipocytes with pertussis toxin reduced the level of GPDH activity by 50% after 7 d of exposure (54). The introduction of a constitutively active $G_{i2}\alpha$ that escapes inactivation by PKC thus accounts for the preservation of the two- to fourfold enforcement of 3T3-L1 adipocyte characteristics reported in this paper.

PLC also degrades other phosphatidylinositides into inositol phosphates, DAG, and 1-alkyl,2-acyl glycerol (57,58). By this action, the enzyme takes part in the regulation of the amount of substrate(s) available for the kinases (e.g., PI 3-kinase) in the polyphosphoinositide cycle and also yields bioactive acyl glycerols. According to recent reports (59,60), PI-PLC activity seems to be regulated by several G-proteins such as $G_{q/11}$ and G_o. Hence, G_{i2} may also be instrumental in modulating the steady-state level of adipocyte differentiation by restraining the impact of PKC activation.

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Isolation and Identification of a Mouse Brain Protein Recognized by Antisera to Heart Fatty Acid-Binding Protein

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ABSTRACT: Although a novel brain-specific fatty acid-binding protein (B-FABP) was recently cloned, the identity of a second fatty acid-binding protein detected with antibodies to the heart (H-FABP) has not been clearly resolved. The present investigation, using matrix-assisted laser desorption mass spectrometry, showed that this protein was a form of H-FABP whose N-terminal amino acid was neither methionine nor was it acetylated. Furthermore, isoelectric focusing revealed two major isoforms, a major band pI 7.4 and a minor band pI 6.4, in a distribution pattern opposite to that observed for H-FABP in the heart. Tryptic peptide mass maps of the in-gel digested SDS polyacrylamide gel electrophoresis protein bands showed that the two isoforms differed only in a single peptide corresponding to residues 97–106 of the heart H-FABP sequence. This peptide had an $[M + H]^+$ ion of either 1205.62 (pI 7.4) or 1206.53 (pI 6.4), consistent with a single amino acid substitution, Asp98 or Asn98. Whereas it is well established that both H-FABP and B-FABP interact with polyunsaturated fatty acids, we showed that they also significantly alter plasma membrane cholesterol dynamics in a manner opposite to that of another brain lipid-binding protein, sterol carrier protein-2. In summary, the data demonstrated for the first time that the H-FABP from brain, while nearly identical to H-FABP from heart, differed significantly in isoform distribution and in amino terminal structure from heart H-FABP. This suggests that the brain and heart H-FABP may not necessarily function identically in these tissues.

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Western and Northern blottings indicate that brain cytosol contains at least four members of the fatty acid-binding protein (FABP) family, with the brain-specific (B)-FABP (1–3) and a protein crossreactive to heart (H)-FABP being the most prominent (4–7). Comparison of the cDNA sequences reveals that B-FABP shares only about 65% sequence homology with H-FABP (1–3). FABP bind a wide variety of hydrophobic ligands involved not only in lipid metabolic and structural pathways but also in cellular signaling and differentiation (4–6,8–10). B-FABP preferentially binds polyunsaturated fatty acids (e.g., arachidonic acid and docosahexaenoic acid, K_d ~ 10 nM) (11), fatty acyl CoA, and lysophosphatidic acid (4). H-FABP from mouse heart also preferentially binds polyunsaturated fatty acid such as arachidonic acid (4). Finally, some intracellular proteins that bind fatty acids also interact with cholesterol and modulate membrane cholesterol dynamics (12–18). Whether the brain FABP acts similarly is not known.

In contrast to B-FABP, almost nothing is known regarding the cDNA or amino acid sequence, isoform pattern, definitive structural identification, or function of the protein crossreactive to anti-H-FABP. Structural identification of this protein is especially important for several reasons: First, H-FABP is present in vascular muscle (19,20), suggesting that H-FABP detected in brain may in part originate from vascular tissue. Although the quantitative contribution of the latter is most likely small given the neural/glial volume relative to vascular, noncapillary volume, the contribution of vascular vs. neural/glial derived H-FABP may influence the isoform pattern of H-FABP in brain. Second, the presence of polymorphism (21) and impurities in native FABP preparations (22–24) has made it difficult to resolve the identity of anti-H-FABP crossreactive proteins. For example, a mammary gland FABP (originally identified as a new protein differing only slightly from H-FABP) was recently shown to be a mixture of H-FABP and another FABP, rather than being a new protein or H-FABP isoform (22–25). Third, native FABP often display isoform patterns not evident from cDNA sequence analysis alone (reviewed in Ref. 26). Fourth, the different isoforms may have distinct functions. For example, the two H-FABP isoforms present in bovine heart have distinct intracellular localization in mitochondria and cytosol, respec-

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Abbreviations: B-FABP, brain-specific fatty acid-binding protein; DEAE, diethylaminoethyl; FABP, fatty acid-binding protein; H-FABP, heart fatty acid-binding protein; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; L-FABP, liver-FABP; MALDI, matrix-assisted laser desorption ionization; PVDF, polyvinylidene difluoride; SCP-2, sterol carrier protein-2; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid.

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tively (reviewed in Ref. 24). Similarly, two rat liver (L)-FABP isoforms differentially modulate microsomal fatty acyl CoA incorporation into phosphatidic acid (26,27). The primary purpose of the present work was to isolate the B-FABP immunoreactive to anti-heart H-FABP, resolve its isoform pattern, mass map the peptide sequence to resolve its identity, and examine the effect of H-FABP as well as B-FABP on plasma membrane cholesterol dynamics.

MATERIALS AND METHODS

Materials. Tris base, SDS, urea, acrylamide, bis-acrylamide, bovine serum albumin, glycine, Tween 20, 20% Nonidet P-40 (octylphenol-ethylene oxide condensate), Ponceau S, dibasic potassium phosphate, monobasic potassium phosphate, EDTA, phenylmethylsulfonyl fluoride, leupeptin, pepstatin, sodium azide (NaN_3), dithiothreitol, ammonium persulfate, *N,N,N′,N′*-tetramethylethylenediamine, ammonium sulfate $(NH_4)_2SO_4$, 2-(*N*-morpholino)ethanesulfonic acid, piperazine-*N,N′*-bis(2-ethanesulfonic acid), and cholesterol were obtained from Sigma Chemical Company (St. Louis, MO). Trifluoroacetic acid (TFA), acetonitrile [high-performance liquid chromatography (HPLC) grade], β-mercaptoethanol, glycerol, and methanol were from Fisher (Pittsburgh, PA). Polyvinylidene difluoride (PVDF) membranes were purchased from Millipore Inc. (Bedford, MA). Horseradish peroxidase-conjugated goat-anti-rabbit-immunoglobulin and Brilliant Blue G were from Bio-Rad (Richmond, CA). Murine recombinant H-FABP and affinity-purified antibody against this protein were prepared as described (25). Murine recombinant B-FABP and affinity-purified antibodies against this protein were prepared as described (28). Amicon stirred ultrafiltration cell (Model 8050) and Amicon Centriprep 10 concentrator tubes were obtained from Amicon (Beverly, MA). Ampholytes pH 3.5–9.5, Sephadex G-25 (coarse), Sephadex G-50 (superfine), diethylaminoethyl (DEAE) CL-4B Sepharose, and carboxymethyl Fast Flow Sepharose were purchased from Pharmacia (Piscataway, NJ). C4 reversed-phase HPLC column was from Vydac (Hesperia, CA).

Animals. Swiss Webster mouse brains, purchased frozen and shipped on dry ice, were obtained from Hazleton Research Products (Denver, PA). In order to remove plasma proteins that bind fatty acids (e.g., albumin) and may interfere with isolation of the H-FABP, blood was removed by rinsing the brains with phosphate-buffered saline prior to freezing. Blood does not normally contain H-FABP as a contaminant since this is an intracellular protein expressed in skeletal or vascular muscle. The experimental protocols for the use of laboratory animals were approved by the appropriate institutional review committee and met guidelines of the American Association for Accreditation of Laboratory Animal Care.

Purification of H-FABP from mouse brain. The H-FABP was isolated at 4°C from mouse brain by a modification of an earlier described protocol (4). In addition to removing extraneous proteins, the purification procedure had to remove B-FABP copurifying in the early steps of the procedure. Purification was monitored by Coomassie Blue stain/silver stain, and Western blotting using affinity-purified specific antibodies against recombinant mouse H-FABP and recombinant mouse B-FABP.

Briefly, the procedure was as follows: Frozen mouse brains (700) brains were thawed, rinsed, and homogenized in 50 mM phosphate buffer, pH 7.4, with 1.0 mM EDTA, 0.02% NaN_3, 0.5 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL leupeptin, and 1.5 μg/mL pepstatin using 10 strokes with a tight-fitting glass Dounce homogenizer. The homogenate was centrifuged at $12{,}000 \times g$ for 30 min, and the supernatant was reserved. Powdered ammonium sulfate was added to this supernatant to a concentration of 25, 50, 75, and 90% saturation. The suspension was centrifuged at $3{,}000 \times g$ for 30 min, the 90% saturation pellet was taken up in 50 mM phosphate buffer and was loaded on a G-25 Coarse Sephadex desalting column (50 mm, 36 cm), equilibrated with 50 mM phosphate buffer, pH 7.4, 0.02% NaN_3, and eluted with the same buffer. Eluant absorbance was monitored at 280 nm. Ammonium sulfate in the fraction was detected by spot tests using barium chloride in HCl. In the initial salt fractionation procedure both H-FABP and B-FABP were mainly precipitated in the 90% ammonium sulfate saturation pellet. Some lower and higher molecular weight contaminants were removed in the other ammonium sulfate pellets and 90% ammonium sulfate saturation supernatant. After removal of ammonium sulfate, the sample was concentrated and loaded onto a G-50 Sephadex size exclusion column (30 mm, 87 cm). Fractions that were immunoreactive with anti-H-FABP and anti-B-FABP were collected, concentrated, buffer-exchanged with 20 mM triethanolamine buffer, pH 7.8, concentrated again, then loaded on a DEAE-Sepharose column (15×3 cm) equilibrated with 20 mM triethanolamine buffer, pH 7.8, eluted with a linear gradient of 0 to 1 M NaCl in 20 mM triethanolamine buffer, pH 7.8. Fractions immunoreactive with only anti-H-FABP and fractions immunoreactive with both anti-H-FABP and anti-B-FABP were collected separately. The latter were concentrated, buffer-exchanged with 20 mM acetate buffer, pH 4.6, concentrated, loaded on a CM-Fast Flow Sepharose (15×3 cm) column, and eluted as for the DEAE-Sepharose to yield additional purified H-FABP. Fractions immunoreactive with only anti-H-FABP from the two columns were maintained separate. Final purity was achieved by C4 reversed-phase (Vydac) HPLC using an aqueous phase (with 0.1% TFA) in a linear gradient with organic phase (0.1% TFA in acetonitrile) from 10 to 80% organic phase over 60 min.

Tricine SDS-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting, Coomassie Blue staining, and silver staining. H-FABP purity was determined by Tricine SDS-PAGE, followed by blotting on PVDF membranes (Millipore Inc.), and detection with affinity-purified anti-H-FABP as described earlier (4,29,30). Protein concentration was determined as described earlier (31).

Isoelectric focusing and pI determination. Isoelectric focusing (IEF) electrophoresis was carried out basically as de-

scribed earlier (32) with the following modifications. IEF gels were cast as 1.5-mm thick polyacrylamide gels [30% T, 5.7% C, where T denotes the total percentage concentration of both monomers (acrylamide and bis-acrylamide), and C denotes the percentage concentration of the crosslinker relative to the total concentration T] containing 9 M urea, 2% (vol/vol) Nonidet P-40, 5% (vol/vol) Pharmacia ampholytes, pH 3.5–9.5, using a Mini Protean II electrophoresis Unit (BioRad). Duplicate gels were run essentially according to published procedures (33). Gels were prefocused at 300 V for 15 min. Samples (20 μL) of H-FABP from the HPLC purification and pH marker were then loaded on the gels. Gels were run at 100 V for 20 min, followed by 300 V for 16 h, in the cold room. One gel was stained with Coomassie stain (0.1% Brilliant Blue R-250), another was transferred onto PVDF membrane to develop a Western blot. Isoelectric points were determined by calibration using Pharmacia broad range (pI 3–10) Isoelectric Point Calibration Kit. IEF gel bands of interest were cut and loaded onto the second dimension 16% SDS-polyacrylamide gel (1-mm thick). The latter was run at 75 V for about 30 min, then at 100 V for about 2 h, and then silver stained as described (34) and submitted to Smith Kline Beecham Pharmaceuticals (King of Prussia, PA) for in-gel digestion and amino acid sequencing by mass spectrometric analysis.

In-gel digestion and mass spectrometric analysis of tryptic peptides. Silver staining of 1-mm thick SDS-PAGE gels in preparation for subsequent mass spectrometric analysis was carried out as described (34). The procedure for the digestion of proteins in polyacrylamide gels was a modification of a published procedure (35). Briefly, the silver-stained band of interest was excised from the gel, equilibrated in 100 mM NH_4HCO_3, and then reduced and alkylated using dithiothreitol and iodoacetamide. Following reduction and alkylation, the gel slice was removed and washed in 50% acetonitrile/100 mM NH_4HCO_3, cut into small pieces, and lyophilized. Modified trypsin in 50 mM NH_4HCO_3 was added to the gel pieces, and the sample was incubated overnight at 37°C. An aliquot of the crude digest supernatant was removed, for direct analysis by matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectroscopy.

Samples were prepared by mixing the crude digest supernatant with an equal volume of matrix solution containing 200 fmol/μL of adrenocorticotropic hormone peptide 18–39 ($[M + H]^+ = 2465.199$) as an internal standard and allowing 0.5 μL of this sample/matrix to dry on the MALDI target. The matrix solution was 10 μg/mL α-cyano-4-hydroxycinnamic acid in 50:50 ethanol/acetonitrile. MALDI mass spectra were recorded on a Micromass (Manchester, United Kingdom) TofSpec SE single-stage, reflectron time-of-flight mass spectrometer. A three-element ion source provided a variable ion extraction region and utilized the time lag focusing principle (36). The instrument had a 3.4-m effective path length. Samples were irradiated by 337 nm photons from a pulsed Laser Science (Cambridge, MA) nitrogen laser at a frequency of 5 Hz. Between 25 and 50 laser shots were averaged per spectrum.

Monoisotopic peptide $[M + H]^+$ values were assigned based on an internal calibration derived from a matrix ion (*m/z* 379.350) and adrenocorticotropic hormone peptide 18–39 (*m/z* 2465.199). Peptide molecular weights were searched against the European Molecular Biology Laboratory nonredundant protein database using the Peptide Search Program (37).

Fluorescence polarization measurement. (i) *Sterol exchange assay*. The exchange of dehydoergosterol for cholesterol was monitored by an assay based on a method (38) modified to determine molecular sterol transfer (18). The plasma membrane lipid composition (17,18) was the same for donors and acceptors except for the nature of the sterol (total sterol in both cases was 35 mol%; 12 mol% of total cholesterol was replaced by dehydroergosterol in the donor membranes). Donor plasma membranes were diluted in piperazine-*N,N′*-bis(2-ethanesulfonic acid) (10 mM, pH 7.4) to 3.5 μg protein/mL. Effector proteins, lyophilized H-FABP or B-FABP were dissolved in sterile distilled water prior to use and were added at this stage. Tenfold excess of acceptor plasma membrane was added to give a final volume of 2 mL and final membrane concentration of 38.5 μg protein/mL. During the exchange assay the sample was continuously stirred at 37°C using a resident micro stir bar (Fisher Scientific Inc., Pittsburgh, PA) in the cuvette. The extent of sterol transfer was monitored by contin-uous measurement of dehydroergosterol polarization for 3–4 h in the T-format with an ISS photon-counting fluorometer (ISS Inc., Champaign, IL) equipped with a 300 watt Xe-arc lamp. Excitation wavelength was 324 nm. Light scattering was reduced through use of KV-389 emission cutoff filters (Schott Glass Technologies Inc., Duryea, PA). The inner-filter effect was made negligible by use of dilute membrane suspensions (absorbance at 324 nm <0.1). Previous studies showed that photobleaching did not occur under these conditions (39). Data were collected automatically every 20 s by a Compaq PC computer through an ISS analog-to-digital interface.

(ii) Dehydroergosterol standard curve for plasma membrane sterol exchange assays. The dependence of polarization, p, on dehydroergosterol concentration can be described in standard curves for plasma membranes with the following polynomial:

$$p = mx_d^2 + n \qquad [1]$$

where x_d is the dehydroergosterol concentration in donor membranes. Parameters $m = 0.1207$ and $n = 0.3187$ for plasma membranes were obtained using computer nonlinear regression.

(iii) Data analysis. Light scatter contributions from the acceptor membranes were subtracted from the entire curve prior to computer analysis. Initial rates were determined from the first 10 min of exchange for both spontaneous and protein-mediated exchange. Earlier results from this laboratory using model membrane systems suggested that sterol exchange occurs *via* kinetics that can be described by the following biexponential equation:

$$x_d = A_2 \exp(-k_{E1}t) + B_2 \exp(-k_{E2}t) + C_2 \qquad [2]$$

When Equation 2 is inserted into Equation 1, we obtain:

$$p = m\,[A_2^2 \exp(-2k_{E1}t) + 2\,A_2B_2 \exp(-k_{E1}t - k_{E2}t) + B_2^2 \exp(-2k_{E2}t) + 2A_2C_2 \exp(-k_{E1}t) + 2B_2C_2 \exp(-k_{E2}t) + C_2^2] + n \quad [3]$$

where A_2, B_2, and C_2 represent the two exchangeable and nonexchangeable fractions, respectively, k_{E1} and k_{E2} represent the corresponding rate constants of exchangeable fractions, and the constants m and n are from Equation 1. Equation 3 was used to fit experimental curves with four fitted parameters, k_{E1}, k_{E2}, A_2, and B_2. C_2 must satisfy the condition that $A_2 + B_2 + C_2 = 1$.

RESULTS

Purification of H-FABP from mouse brain. After separation of the combined FABP from brain cytosol as described in the Materials and Methods section, the key step allowing resolution of H-FABP from B-FABP was ion exchange chromatography. DEAE anion exchange column elution (Fig. 1A) showed fractions immunoreactive to affinity-purified anti-H-FABP eluting with low salt elution (demonstrated by a – sign), whereas fractions immunoreactive to affinity-purified anti-B-FABP were eluted with increasing NaCl (demonstrated by + sign). The remaining fractions immunoreactive to both anti-H-FABP and anti-B-FABP were further separated by CM Sepharose fast flow cation exchange column chromatography (Fig. 1B). In contrast to DEAE anion exchange chromatography, fractions immunoreactive to anti-B-FABP were eluted with no salt and/or low salt (+ symbol, Fig. 1), whereas fractions immunoreactive to anti-H-FABP were eluted with increasing NaCl (– symbol, Fig. 1).

Final purity of H-FABP was achieved by reversed-phase HPLC. The anti-H-FABP immunoreactive fractions obtained from the DEAE anion exchange column and the CM Sepharose cation exchange column were injected separately on a C4 reversed-phase HPLC column and eluted with a stepwise acetonitrile (0.1% TFA) gradient from 10 to 70% in aqueous solution (H_2O, 0.1% TFA) within 35 min. Under the HPLC elution conditions utilized, the H-FABP was eluted at 60% acetonitrile with relative retention time of 30 min (Fig. 2A). The final purity of H-FABP was shown by Western blot (Fig. 2B) and silver staining (Fig. 2C). In contrast, under identical HPLC conditions, B-FABP eluted with relative retention time of 26 min (data not shown).

Two-dimensional electrophoresis. The first dimension of IEF electrophoresis (Fig. 3A) showed that H-FABP from brain (lane 1) migrated primarily as two bands. The major band had an apparent pI 7.4, and the minor band had an apparent pI 6.4. Western blot analysis showed that only the bands pI 7.4 and 6.4 were immunocrossreactive to anti-H-FABP (Fig. 3B). The silver-stained second-dimension SDS-PAGE gel showed primarily a single band for both the pI 7.4 (lane 1) and pI 6.4 (lane 2) isoforms (Fig. 3C). A faint band corresponding to the mass of the dimer was also detectable.

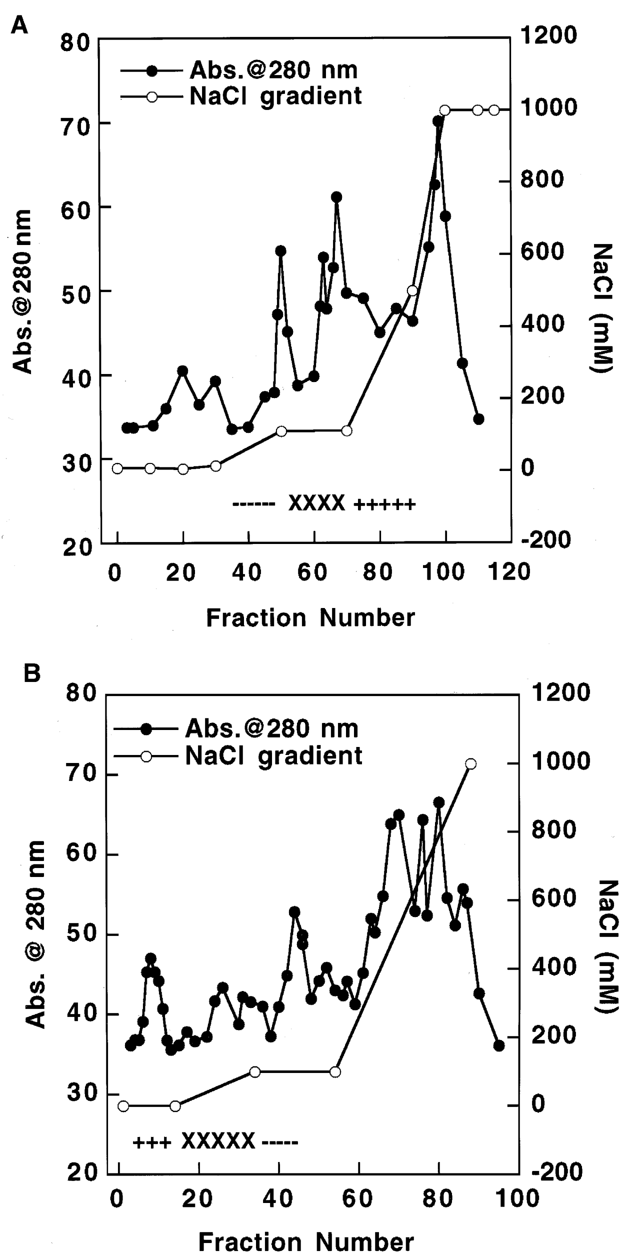

FIG. 1. Ion exchange chromatographic profiles for the separation of heart fatty acid-binding protein (H-FABP) from brain-specific (B)-FABP. Solid circles represent the absorbance at 280 nm, and open circles represent the step gradient of NaCl used to elute the proteins. The minus (–) signs represent the fractions only immunocrossreactive with anti-H-FABP. The × signs represent the fractions immunocrossreactive with both anti-H-FABP and anti-B-FABP. The plus (+) signs represent the fractions only immunocrossreactive with anti-B-FABP. (A) Diethylaminoethyl anion exchange chromatographic profiles. (B) Carboxymethyl cation exchange chromatographic profiles.

Identification of H-FABP from mouse brain by mass spectrometry. The identity of the H-FABP from brain was determined by in-gel digestion of the second-dimension SDS-PAGE bands with trypsin and subsequent MALDI mass spectrometry of resultant peptides (Fig. 4). The pattern of tryptic digest peptides revealed almost complete identity to that ob-

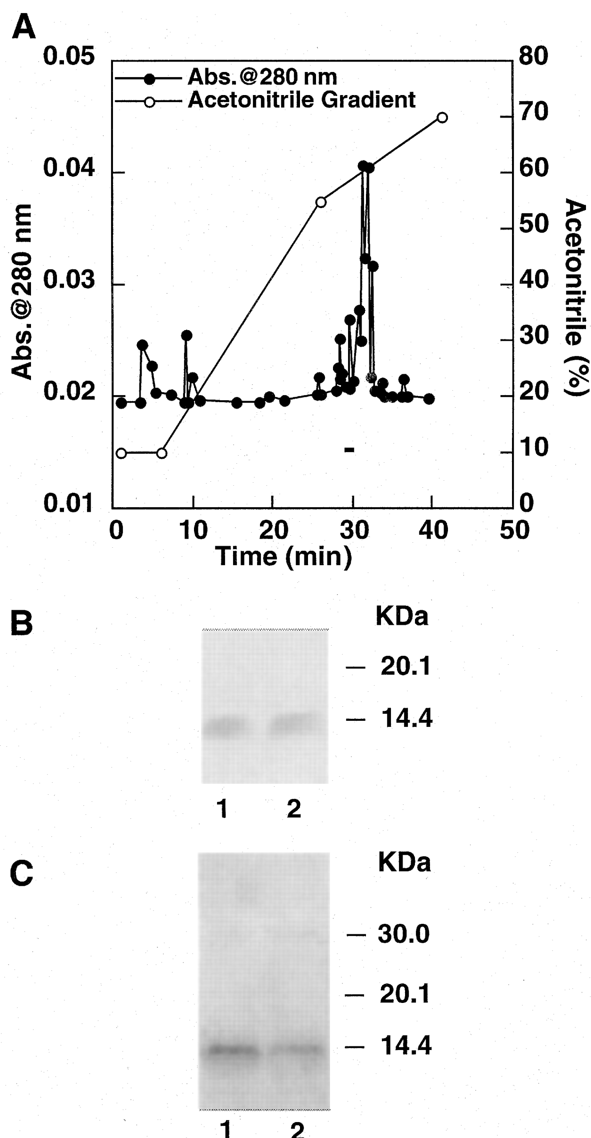

FIG. 2. (A) High-performance liquid chromatographic (HPLC) profile of final purification of H-FABP from mouse brain. A C4 reversed-phase HPLC column was equilibrated with 90% H_2O (0.1% trifluoroacetic acid)/10% acetonitrile (0.1% trifluoroacetic acid). A stepwise gradient to 30% H_2O (0.1% trifluoroacetic acid)/70% acetonitrile (0.1% trifluoroacetic acid) (open circles) was run within 35 min to elute H-FABP. The closed circles represent the absorbance at 280 nm. The final purities of H-FABP from mouse brain are shown in (B), Western blot; (C) silver staining of SDS-polyacrylamide gel electrophoresis (SDS-PAGE) gel. Lanes 1 and 2 represent samples from two different vials collected in series. For other abbreviation see Figure 1.

tained from the H-FABP sequence (Table 1) and recombinant H-FABP (not shown). The only difference was in the N-terminus of the H-FABP of brain vs. heart origin. In contrast to the masses of N-terminal peptides from heart H-FABP, the brain H-FABP did not contain either an acetyl group or a methionine at the N-terminus as revealed by mass of the N-terminal peptide T1 (Table 1).

Identification of native heart H-FABP isoforms from mouse brain by mass spectrometry. There is considerable debate whether true FABP isoforms actually exist or whether they are simply due to posttranslational modifications, degree of ligand binding, or conformation (reviewed in Ref. 26). Therefore, it was important to determine if the H-FABP from brain separated by IEF in the preceding section are true isoforms. As described above and in Figure 3, IEF of the H-FABP from brain revealed two bands. In-gel digestion of the second-dimension SDS-PAGE bands with trypsin and subsequent MALDI mass spectrometry of resultant peptides showed that the pattern of tryptic digest peptides for H-FABP band 1 (Fig. 4A) and band 2 (Fig. 4B) from IEF were almost completely identical between calculated and observed masses (Table 1). The only difference between these two isoforms was noted in peptide T13 (Table 1). The masses of the T13 peptide from band 1 and band 2 of native H-FABP from mouse brain were 1205.62 and 1206.53, respectively (Table 1). A 1-Da difference in the mass of peptide T13 is consistent with a single amino acid substitution, either Asn98–Asp98 or Gln100–Glu100. The partial published amino acid sequence predicted from the cDNA of heart H-FABP was aligned to the partial amino acid sequence of native brain H-FABP (40,41) in Table 2. The Asp/Asn substitution (labeled in bold) is that found in the heart H-FABP (reviewed in Ref. 26). In summary, these data are consistent with the brain H-FABP isoforms resolved by IEF as being true isoforms and not simply altered proteins due to bound ligand or posttranslational modifications as has been observed for several other putative FABP isoforms (reviewed in Ref. 26).

Fluorescence polarization measurement. As described in the introduction, although the fatty acid specificity of both the B-FABP and H-FABP are known, there is evidence that some FABP can also modulate intermembrane sterol transfer. Therefore, this possibility was tested with H-FABP and B-FABP, using a fluorescence polarization assay to examine sterol transfer between isolated plasma membrane vesicles. Sterol carrier protein-2 (SCP-2), a protein that binds cholesterol and increases sterol transport, was used as a positive control in the assay. Both recombinant H-FABP and recombinant B-FABP functionally affected sterol transfer between plasma membranes but in a manner very different from SCP-2. In contrast to SCP-2, which stimulated intermembrane sterol transfer (Fig. 5), H-FABP (Fig. 6A) and B-FABP (Fig. 6B) inhibited intermembrane sterol transfer. Furthermore, analysis of the exchange data as described in the Materials and Methods section showed that the effect of the B-FABP on individual exchange parameters was not simply the reverse of that observed with SCP-2 (Table 3). Although all three proteins increased the size of the exchangeable sterol domain, the effect of both H-FABP and B-FABP was greater than that of SCP-2 (Table 3). In contrast to SCP-2, which decreased the half-time of exchange, both H-FABP and B-FABP increased the half-time of sterol transfer (Table 3). Finally, whereas SCP-2 doubled the initial rate of sterol transfer, H-FABP and B-FABP had no effect on the initial rate of sterol exchange. In summary, both H-FABP and B-FABP may modulate intermembrane sterol transfer.

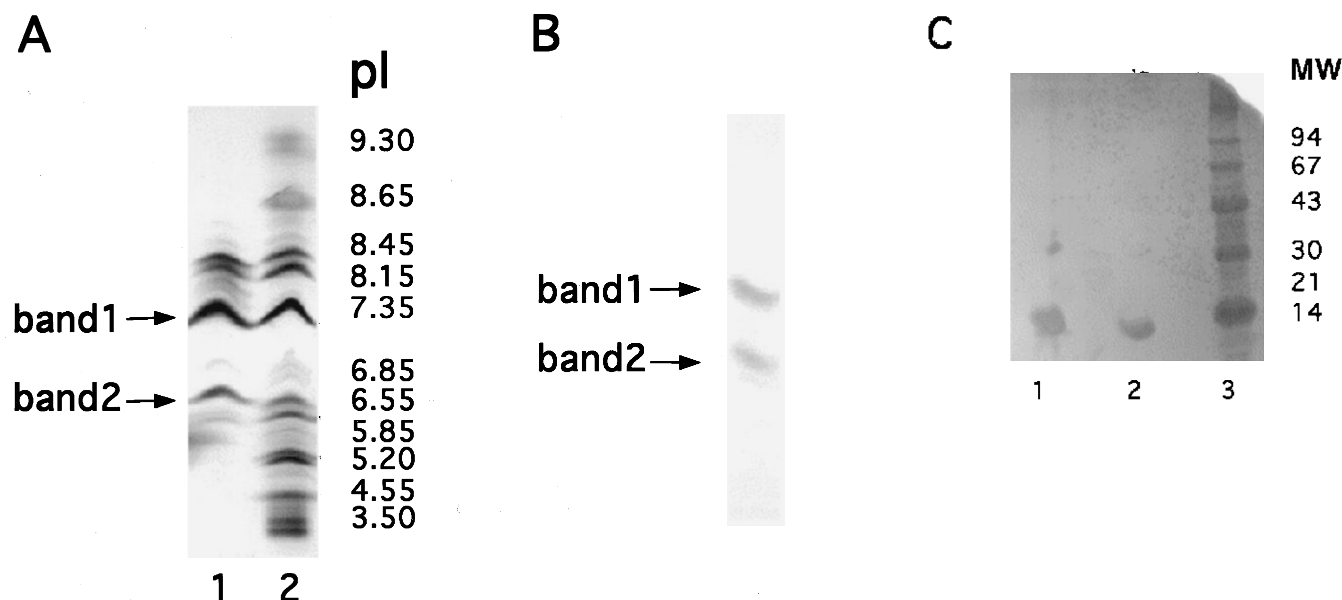

FIG. 3. (A) Isoelectric focusing (first dimension) gel. Isoelectric focusing and Coomassie staining were used to determine the isoelectric points of the H-FABP from mouse brain. Lane 1 is 15 ng of H-FABP from brain. Lane 2 is Pharmacia Broad Range pI Calibration Kit markers, the pI values of which are listed along the right edge of the gel. The isoform pI 7.4 was labeled as band 1, and the isoform pI 6.4 was labeled as band 2. (B) Western blot shows both band 1 and band 2 of H-FABP from brain were immunocrossreactive with anti-H-FABP. (C) Silver staining of second-dimension (SDS-PAGE) gel. Band 1 (pI 7.4) and band 2 (pI 6.4) of H-FABP from brain were cut from isoelectric focusing gel and loaded in lanes 1 and 2 of an SDS-PAGE gel. Lane 3 is Gibco (Grand Island, NY) prestained molecular weight markers. For abbreviations see Figures 1 and 2.

DISCUSSION

Increasing evidence for roles of fatty acids in differentiation and function of brain tissues has stimulated interest in proteins that may be involved in the uptake and/or intracellular trafficking/targeting of fatty acids in nervous system cells. Western and Northern blotting has suggested that brain contains at least four cytosolic FABP (4–7,24) and one integral plasma membrane FABP (42). Prior to the present investigation, only one of these had been structurally identified (B-FABP primary amino acid sequence was predicted from the cDNA cloned from a brain library). In brain, the identity of the second major FABP, which is crossreactive to anti-H-FABP from heart, has not been established. This is a significant problem in view of previous difficulties in identifying FABP based only on cross reactivity with antisera (see introduction and Ref. 4). Results of the present investigation made several important contributions to our understanding of FABP from brain.

First, isolation of the native H-FABP from mouse brain and subsequent mass spectroscopic analysis revealed that it was essentially identical to H-FABP from mouse heart. The major difference was that tryptic peptide data of the native H-

TABLE 1
Mass Spectrometric Data of Tryptic Peptides of H-FABP from Mouse Brain[a]

		Molecular mass			
Tryptic peptide	Residue in sequence	Band 1 (pI 7.4)	Band 2 (pI 6.4)	Calculated	Sequence
T1	1–9	994.51	994.41	994.50	()ADAFVGTWK
T3	15–21	948.38	948.30	948.38	(K)NFDDYM*K
T4	22–30	907.58	907.50	907.50	(K)SLGVGFATR
T6	45–52	861.50	861.47	861.47	(K)NGDTITIK
T7	53–58	711.36	711.43	711.37	(K)TQSTFK
T8	59–78	2326.08	2326.08	2326.08	(K)NTEINFQLGIEFDEVTADDR
T8+9	59–79	2454.13	2454.22	2454.19	(K)NTEINFQLGIEFDEVTADDRK
T11	82–90	889.57	889.51	889.50	(K)SLVTLDGGK
T12	91–96	737.47	737.47	737.46	(K)LIHVQK
T13	97–106	**1205.62**		1205.59	(K)W**N**GQETTLTR
T13	97–106		**1206.53**	1206.56	(K)W**D**GQETTLTR
T15+16	113–126	1496.94	1496.94	1496.88	(K)LILTLTHGSVVSTR

[a]Boldface type indicates difference in the N-terminus of the heart fatty acid-binding protein (H-FABP) of brains vs. heart origin.

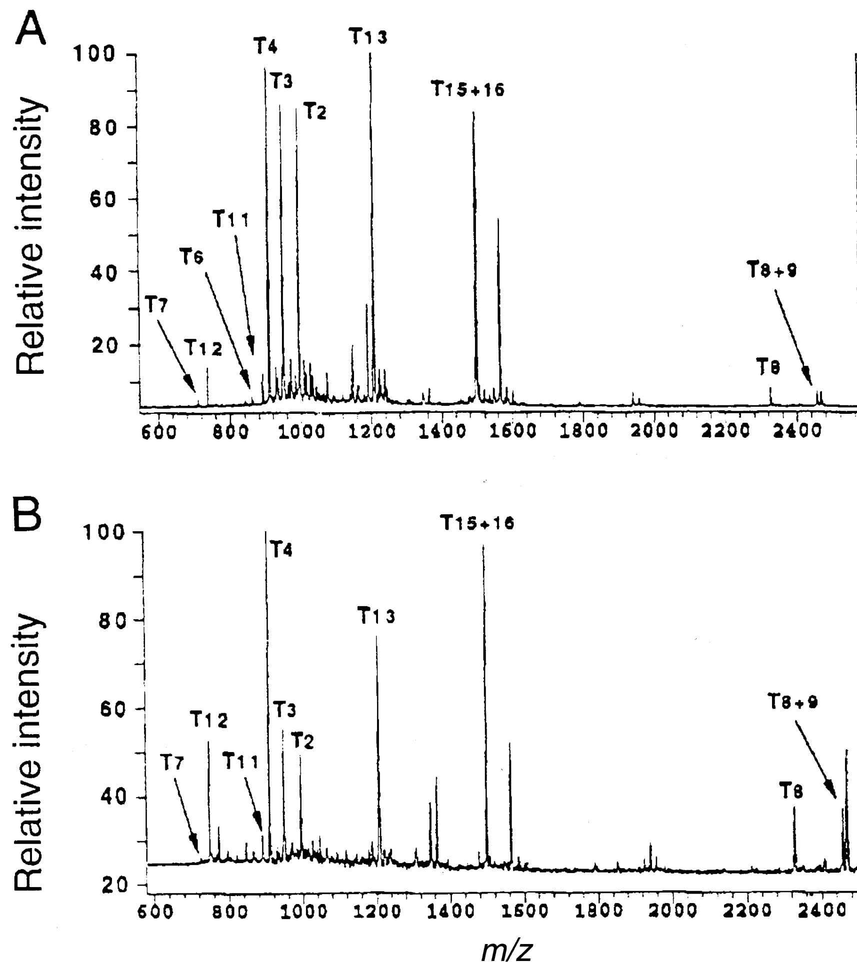

FIG. 4. Delayed-extraction MALDI-reflectron time-of-flight mass spectra of tryptic digest peptides from band 1 (pI 7.4) (A), and band 2 (pI 6.4) (B) of H-FABP from brain after two-dimensional electrophoresis purification. MALDI, matrix-assisted laser desorption ionization; for other abbreviation see Figure 1.

FABP from mouse brain had an N-terminal Ala, unlike native heart H-FABP (43) or the cDNA-derived amino acid sequence (44). This difference in amino terminus is primarily responsible for the major difference in IEF behavior between the native H-FABP and native H-FABP. The isoelectric points, pI 7.4 and 6.4, of native H-FABP from mouse brain were more basic than those of human H-FABP, pI 5.3 and bovine H-FABP, pI 4.9 and pI 5.1 (45). Both pI of native mouse H-FABP were also more basic than the pI 5.82 calculated from the cDNA encoding mouse heart H-FABP (44) and the pI 6.0 of rat mammary epithelial cell heart H-FABP (46). Since the native H-FABP from mouse brain was not N-terminal acetylated, its pI was more basic. In contrast, the isoforms of native H-FABP from human heart, bovine heart, and rat heart have an acetyl group at the N-terminal, which give a more acidic pI. The functional significance of H-FABP with or without N-terminal acetylation is thus far not clear. However, it was recently observed that another cytosolic lipid-binding protein, acyl-CoA-binding protein, isolated from murine cells was N-terminal acetylated and exhibited a 2.1-fold greater stimulation of microsomal phosphatidic acid synthesis than did recombinant rat ACBP which was not N-terminal acetylated (47).

TABLE 2
Aligned Partial Sequences of H-FABP from Mouse Brain and Heart

Protein sources	Tissues	Isoforms	Partial amino acid sequences[a]	References
Native	Brain	pI 7.4	Lys-Trp-**Asn**-Gly-Gln	This work
Native	Brain	pI 6.4	Lys-Trp-**Asp**-Gly-Gln	This work
cDNA-derived	Heart	pI 5.8	Lys-Trp-**Asp**-Gly-Gln	45

[a]Amino acids corresponding to position 98 are in bold. See Table 1 for abbreviation.

Second, the mass spectroscopic data clarified another problem, i.e., whether the two forms of native anti-heart H-FABP immunoreactive proteins from mouse brain represented true isoforms. As shown herein, mass spectroscopic analysis revealed that these bands were true isoforms due to an amino acid substitution. Peptide mass mapping of the two native H-FABP proteins from mouse brain revealed that they differed by only 1 Da in a single tryptic peptide, 97–106. Based on the cDNA-derived amino acid sequence of this peptide, the observed mass difference between the two isoforms

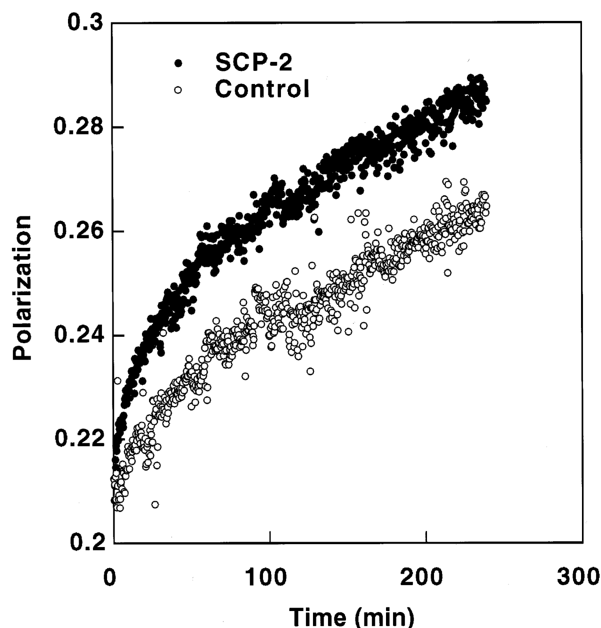

FIG. 5. Effect of sterol carrier protein-2 (SCP-2) on plasma membrane sterol exchange. Plasma membrane sterol exchange was determined as described in the Materials and Methods section. The lower curve represents spontaneous exchange whereas the upper curve was obtained with 1.5 μM SCP-2 present in the assay medium.

could be accounted for by either an Asn exchange with Asp at residue 98 or by a Gln exchange with Glu at residue 100. Asp/Asn exchange seems to be a common event in several members of the FABP family (26,48–50). Two isoforms of native H-FABP from bovine heart have been isolated and shown to result from an Asp/Asn exchange at residue 98 (43). Because the Asn at residue 98 in both bovine heart and mouse brain H-FABP is present in a sequence (Asn–Gly) which is subject to facile nonenzymatic deamination, it is possible that the two forms of H-FABP are an artifact. However, it was shown that in bovine heart, the Asp98 and Asn98 isoforms are coded by distinct mRNA, and that the protein product of the Asn98 gene did not convert to Asp98 over time (48).

Third, the pattern of brain H-FABP isoforms suggests that these altered protein forms may be functionally significant. The most important observation in this regard was that the proportions of the two native H-FABP isoforms from brain differed significantly from those found in heart. For example, in bovine heart the more acidic isoform of H-FABP represents a larger portion of the total (43,48,51). Based on the observation that one of the H-FABP isoforms was exclusively found associated with mitochondria, the latter investigators suggested an important role for this isoform in mitochondrial fatty acid oxidation, the most important energy source for the heart. In contrast, brain acquires most of its energy from glycolysis. Consistent with this, the pattern of the brain H-FABP isoforms was shifted away from that predicted to be associated with mitochondria. While this correlative explanation is appealing, future work is needed to reveal the physiological significance of brain H-FABP isoforms.

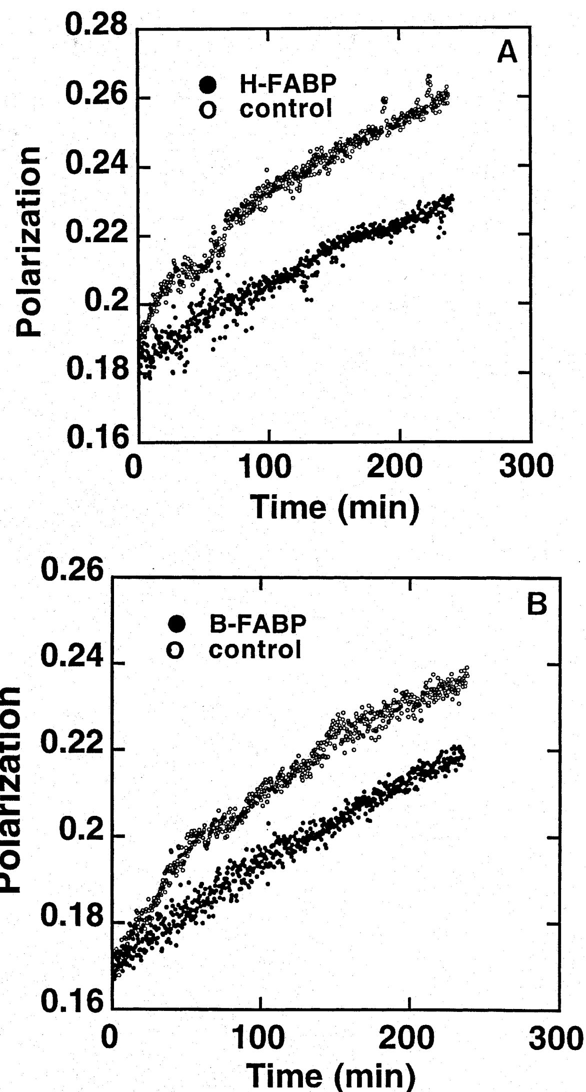

FIG. 6. Effect of H-FABP and B-FABP on plasma membrane sterol exchange. (A) The top curve (○) represents spontaneous exchange, and the bottom curve (●) was obtained with 1.5 μM H-FABP present in the assay medium. (B) The top curve (○) represents spontaneous exchange and the bottom curve (●) was obtained with 1.5 μM B-FABP present in the assay medium. All exchanges were performed at 37°C in 10 mM piperazine-*N,N′*-bis(2-ethane sulfonic acid) (pH 7.4). For abbreviations see Figure 1.

Fourth, native brain H-FABP as well as B-FABP may participate in intracellular sterol trafficking and modulation of membrane sterol domains/membrane function. Another FABP, the liver L-FABP, was shown to alter intracellular cholesterol trafficking, plasma membrane cholesterol domains, and plasma membrane Na^+,K^+-ATPase activity (12,13,18,52). Both B-FABP and H-FABP increased exchangeable sterol domain size, concomitantly increased the half-time of sterol transfer, but did not alter the initial rate of sterol transfer from isolated plasma membranes. The net effect was that H-FABP and B-FABP inhibited intermembrane sterol transfer. This inhibition may be correlated with recent observations that increasing age alters transbilayer fluidity and cholesterol asym-

TABLE 3
Kinetic Parameters of Spontaneous and Protein-Mediated Sterol Transfer Between Plasma Membranes[a]

Samples	Fractional domains f_1 (%)	Fractional domains f_2 (%)	Halftime $t_{1/2}$ (min)	Initial rate (ΔP, 10^{-4} min^{-1})
Control	0.47 ± 0.03	0.53 ± 0.03	137 ± 5	5.13 ± 0.89
SCP-2	0.56 ± 0.11	0.44 ± 0.11	99 ± 1*	11.10 ± 1.10**
H-FABP	0.76 ± 0.07*	0.24 ± 0.07*	267 ± 56**	5.12 ± 0.46
B-FABP	0.76 ± 0.08*	0.24 ± 0.07*	308 ± 77**	3.45 ± 0.80

[a]Values represent the mean ± SE (n = 3–5). f_1 represents exchangeable domain; f_2 represents non-exchangeable domain; ΔP represents change in fluorescence polarization; SCP-2, sterol carrier protein-2. See Table 1 for other abbreviation. *$P < 0.005$ as compared to control based on the Student's t-test; **$P < 0.05$ as compared to control based on the Student's t-test.

metry in synaptic plasma membranes (53) and that the content of both H-FABP and B-FABP decreases dramatically in brains of aged mice (54). Many of the brain FABP also change quantitatively with brain differentiation and development to adulthood (5,7,42). The effects of these brain FABP on intermembrane sterol transfer were specific in contrast to other brain lipid-binding proteins, SCP-2 and acyl-CoA-binding protein, also found in synaptosomes. As shown herein, SCP-2 stimulated intermembrane sterol transfer while acyl CoA-binding protein was ineffective (not shown). Consequently, the two brain H-FABP have opposing effects to SCP-2 on intermembrane sterol trafficking, and the ratio of these proteins as well as their cellular and subcellular localization may be important to regulation of several functional processes in brain.

Finally, H-FABP and B-FABP appear to be differentially expressed in brain. Although H-FABP is also highly expressed in skeletal and vascular smooth muscle, in brain H-FABP is also localized to neurons. For example, H-FABP was enriched 3.5- and 7-fold in synaptosomal cytosol as compared to brain homogenate and synaptosomal nerve endings, respectively (54). Furthermore, immunohistochemical studies show cerebellar neuronal staining with antisera to H-FABP (9) while *in situ* hybridization histochemistry demonstrated H-FABP mRNA expression in hippocampal neuronal layers and in cerebral cortex neurons (7,55). In contrast, B-FABP was enriched only 2.6- and 6-fold in synaptosomal cytosol as compared to brain homogenate and synaptosomal nerve endings. This, taken together with immunohistochemical (1) and *in situ* hybridization (55) data, suggests that B-FABP is primarily a glial protein.

In summary, the data presented herein show that with the exception of the N-terminal amino acid, the native H-FABP from mouse brain is identical to that derived from mouse heart. Two true isoforms were observed, most likely due to an amino acid Asp98/Asn98 or Gln100/Glu100 exchange. However, the isoform pattern of H-FABP in brain was distinct from that previously reported for heart. These data as well as the results of intracellular localization, fatty acid binding, and intermembrane sterol exchange studies indicate that H-FABP and B-FABP may be functionally involved in membrane lipid trafficking and distribution in brain, albeit in different cell types.

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Metabolic Profile of Linoleic Acid in Stored Apples: Formation of 13(*R*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic Acid

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ABSTRACT: During our ongoing project on the biosynthesis of *R*-(+)-octane-1,3-diol the metabolism of linoleic acid was investigated in stored apples after injection of [1-^{14}C]-, [9,10,12,13-^{3}H]-,$^{13}C_{18}$- and unlabeled substrates. After different incubation periods the products were analyzed by gas chromatography–mass spectroscopy (MS), high-performance liquid chromatography–MS/MS, and HPLC-radiodetection. Water-soluble compounds and CO_2 were the major products whereas 13(*R*)-hydroxy- and 13-keto-9(*Z*),11(*E*)-octadecadienoic acid, 9(*S*)-hydroxy- and 9-keto-10(*E*),12(*Z*)-octadecadienoic acid, and the stereoisomers of the 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids were identified as the major metabolites found in the diethyl ether extracts. Hydroperoxides were not detected. The ratio of 9/13-hydroxy- and 9/13-keto-octadecadienoic acid was 1:4 and 1:10, respectively. Chiral phase HPLC of the methyl ester derivatives showed enantiomeric excesses of 75 % (*R*) and 65 % (*S*) for 13-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid and 9-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid, respectively. Enzymatically active homogenates from apples were able to convert unlabeled linoleic acid into the metabolites. Radiotracer experiments showed that the transformation products of linoleic acid were converted into (*R*)-octane-1,3-diol. 13(*R*)-Hydroxy-9(*Z*),11(*E*)-octadecadienoic acid is probably formed in stored apples from 13-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid. It is possible that the *S*-enantiomer of the hydroperoxide is primarily degraded by enzymatic side reactions, resulting in an enrichment of the *R*-enantiomer and thus leading to the formation of 13(*R*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid.

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Biosynthesis in fruits of aroma compounds, such as alcohols and esters, is directly associated with the metabolism of fatty acids and lipids (1). During our ongoing project on the biosynthesis of *R*-(+)-octane-1,3-diol in stored apples we observed that linoleic acid is the most efficient precursor for this antimicrobial diol (2).

Galliard (3) measured 880 and 947 mg of lipids, respectively, in 1 kg of pulp in pre- and postclimacteric Cox's Orange Pippin apples. Phospholipids constituted the largest category with amounts of 405 and 395 mg/kg fresh weight in pre- and postclimacteric apples, respectively. Recently, the lipid metabolism of ripening apples was investigated by Bartley (4). Fatty acids are generally esterified, and are found in very small amounts in the free state. The most abundant saturated and unsaturated fatty acids in apples are, respectively, hexadecanoic acid and linoleic acid, which represent 31 and 53% of total acids, respectively (5).

Previous experiments with homogenates of various plant materials with linoleic and linolenic acid have demonstrated that enzyme systems specifically degrading unsaturated fatty acids in the presence of oxygen are widely distributed in plant tissues (6). The key step is the dioxygenation of unsaturated fatty acids by lipoxygenase (7). The initial products of lipoxygenase action, i.e., hydroperoxides, are relatively unstable and cytotoxic. Fatty acid hydroperoxides are susceptible to nonenzymatic reactions such as isomerization, reduction, cleavage and radical polymerization and also to a variety of enzymatic processes catalyzing their further metabolism (8). Conversion of the hydroperoxide by, e.g., hydroperoxide lyases, hydroperoxide-dependent epoxygenases and allene oxide synthase results in the formation of compounds important for plant defense and as precursors of phytohormones (9).

The objective of the present study was the isolation and identification of the primary transformation products of linoleic acid in stored apples in order to understand the first steps of the biosynthesis of (*R*)-octane-1,3-diol.

MATERIALS AND METHODS

Materials. Linoleic acid and soybean lipoxygenase (LOX: Type I-S) were purchased from Sigma (Deisenhofen, Germany); linoleic acid-$^{13}C_{18}$ from Isotec (Miamisburg, OH), [1-^{14}C]linoleic acid (58 mCi/mmol) from DuPont (Boston, MA), [9,10,12,13-^{3}H]linoleic acid (60 Ci/mmol) from Biotrend (Köln, Germany), silica gel thin-layer chromatography (TLC) plates (0.25 mm, 60 F254) from Merck (Darmstadt, Germany). Solvents and reagents were of analytical or high-performance liquid chromatography (HPLC) grade. Fresh, ripe apple fruits (cv. Douce Moen) were kindly provided by Pernod Ricard, Créteil Cedex, France.

Synthesis of reference material. 13(*S*)-Hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HPOD) was obtained

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Abbreviations: ELSD, Evaporative light scattering detector; GC–MS, capillary gas chromatography–mass spectrometry; HODE, hydroxy-octadecadienoic acid; HPLC, high-performance liquid chromatography; HPLC–MS/MS, HPLC–tandem mass spectrometry; HPOD, hydroperoxy-octadecadienoic acid; KODE, keto-octadecadienoic acid; LC, liquid chromatography; LOX, lipoxygenase; RP18, octadecyl reversed phase; TLC, thin-layer chromatography; UV, ultraviolet.

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by incubating linoleic acid with soybean lipoxygenase (EC 1.13.11.12) (10–11) whereas 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid (9*S*-HPOD) was prepared using tomato lipoxygenase (12). Reduction of 13*S*-HPOD and 9*S*-HPOD with sodium borohydride afforded 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid (13*S*-HODE) and 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid (9*S*-HODE), respectively (13). 13-Keto-9(*Z*),11(*E*)-octadecadienoic acid (13-KODE) and 9-keto-10(*E*),12(*Z*)-octadecadienoic acid (9-KODE) were synthesized by the method of Andre and Funk (14). Reduction of 13-KODE and 9-KODE yielded racemic 13*R,S*-HODE and racemic 9*R,S*-HODE, respectively.

Application of fatty acids. Ethanolic solutions (100 µL) of [1-^{14}C]linoleic acid (5 µCi), [9,10,12,13-^{3}H]linoleic acid (50 µCi), linoleic acid $^{13}C_{18}$ (1 mg), and unlabeled linoleic acid (1 mg) were injected subepidermally with a syringe into ripe apples (*ca.* 40 g). Ten 10-µL aliquots were applied. The needle of the syringe was almost totally stuck into the apple, and the needle was carefully drawn back while the substrate was injected. The solution filled the space formed by the needle during the insertion into the apple. Apples were stored at 4°C while connected to a hood.

Isolation and identification of transformation products. Apples (*ca.* 40 g) were cut into small pieces and homogenized with 100 mL of water. After centrifugation (4000 × *g*, 15 min), the solid residue was extracted with 100 mL of water (3×). The supernatants were combined and passed through a conditioned XAD (polystyrene resin; Aldrich, Steinheim, Germany) column. After rinsing the column with 500 mL of water the products were eluted with 500 mL of diethyl ether and then 750 mL methanol. The diethyl ether extract was concentrated and was analyzed either by HPLC with ultraviolet (UV) and radiodetection or UV and evaporative light-scattering detection (ELSD). For the structural characterization two types of volatile derivatives were prepared: (i) samples were methylated by ethereal diazomethane solution and (ii) methyl esters were trimethylsilylated with a mixture of 1-(trimethylsilyl)-imidazole/pyridine (1:4 vol/vol) for at least 1 h at room temperature. After separation by RP18 (octadecyl reversed phase) chromatography, characterization of the stereoisomers of trihydroxy-octadecenoic acid was performed according to Hamberg (15,16). Separation of *cis*- and *trans*-1,4-diol isomers of methyl 9,10,13- and 9,12,13-trihydroxyoctadecenoates was achieved by TLC on silica gel and a solvent system consisting of diethyl ether/methanol 10:1 (15,16). Products were visualized by spraying with vanillin/H_2SO_4. For preparative TLC the products were applied as a band on the plate. After development a small strip of the aluminum TLC foil was cut off and sprayed with vanillin/H_2SO_4. The strip was used to localize the compounds on the plate. The bands were scraped off. Diastereomeric *cis*- and *trans*-1,4-diol isomers were silylated and separated by capillary gas chromatography–mass spectrometry (GC–MS) analysis on a DB 5 capillary column (15,16). Solid residues were dried at 80°C for 24 h. Aliquots of liquid samples were added to 10 mL of scintillation cocktail (Emulsifier-Safe: Packard, Groningen, The Netherlands). Solid samples were combusted in a biological oxidizer. The $^{14}CO_2$ formed was absorbed in 12 mL of the scintillation cocktail, Oxysolve 400 (Zinsser, Frankfurt, Germany). Recoveries of ^{14}C as $^{14}CO_2$ from test combustions fortified immediately before combustion with ^{14}C standards were greater than 90%. All measurements were carried out by means of liquid scintillation counting using corrections for chemiluminescence.

Stereochemical analysis of 13-HODE and 9-HODE. A mixture of ^{14}C- and a mixture of ^{3}H-labeled 13-HODE, 13-KODE, 9-HODE, and 9-KODE was obtained by semipreparative RP18-HPLC analysis, collecting the peaks eluting from 30–33 min (Fig. 1). The eluate was extracted (3×) with 3 mL of diethyl ether, and the combined organic phase was dried (Na_2SO_4) and concentrated. Individual compounds were separated by normal phase HPLC analysis (13). After methylation of 13-HODE and 9-HODE, separation of the enantiomers was achieved by chiral phase HPLC analysis. The enantiomers were collected and the radioactivity was determined by liquid scintillation counting.

Homogenate. Apples were homogenized in 600 mL 0.1 M acetate buffer pH 4.5 containing 2 mM ascorbic acid and 2 mM $Na_2S_2O_5$. An ethanolic solution (5 mL) of linoleic acid (150 mg) was added, and aliquots were withdrawn after 1, 2, 4, 6, 24, and 48 h. Samples were filtered and directly analyzed by HPLC analysis.

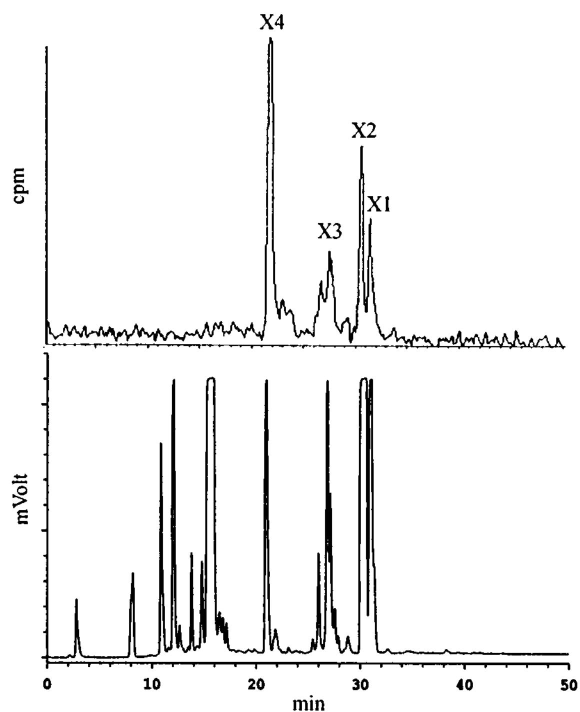


FIG. 1. Reverse-phase high-performance liquid chromatographic separation of the diethyl ether extract obtained by XAD (polystyrene resin) solid phase extraction from an apple incubated with [1-^{14}C]linoleic acid for 12 h. The upper panel shows the signal of the radiodetector and the lower panel depicts the evaporative light-scattering detector signal. Compounds eluting between 0 and 20 min are endogenous apple constituents.

GC–MS analysis. Analysis of the methylated and/or silylated products was performed by using a Fisons MD 800 Quadrupol mass spectrometer coupled to a Fisons GC 8000 with split injector (1:20) equipped with Fisons MassLab software (version 1.3). A J&W (Folsom, CA) DB5 fused-silica capillary column (30 m × 0.25 mm i.d.; film thickness, 0.25 µm), which was programmed from 60 to 300°C at 5°C/min, was used with helium gas at a flow rate of 3 mL/min. The MS operating parameters were: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature, 280 and 290°C, respectively.

HPLC analysis. Reverse-phase HPLC was carried out on a Eurospher 100 C-18 column (250 × 4 mm, 5 µm; Knauer, Berlin, Germany) using a flow rate of 1 mL/min, employing a Knauer HPLC pump MaxiStar coupled on-line to a Knauer multiwavelength UV/visible detector (205, 234, or 254 nm) and an ELSD (Sedere, Alfortville Cedex, France) at 40°C and 2.4 bar compressed air. Data acquisition was achieved using Eurochrom 2000 software (Knauer). Radio-HPLC was conducted using a Waters HPLC system equipped with a variable wavelength detector and a Canberra Packard A100 radioactivity detector with a 300 µL YSi scintillation tube. The following gradient was applied: solvent A (0.05% formic acid), solvent B (CH_3CN): 0–30 min, 5–80% B, 30–40 min, 80–100% B, 40–45 min, 100% B.

Normal phase HPLC analysis was performed on a Eurospher Si 100 column (250 × 4.6 mm, 5 µm, Knauer) using a mixture of isohexane/isopropanol/acetic acid (98:2:0.5 by vol) at a flow rate of 1 mL/min. Chiral phase HPLC analysis was carried out on a Chiralcel OB-H (250 × 4.6 mm, 5 µm) (Daicel) using a mixture of isohexane/isopropanol (100:7) at a flow rate of 0.5 mL/min.

Liquid chromatography (LC)–MS analysis. Analysis of the products and isolates was performed using a triple-stage quadrupole TSQ 7000 LC–MS/MS system with electrospray ionization (ESI) interface (Finnigan MAT, Bremen, Germany) in the positive mode. The temperature of the heated capillary was 220°C. ESI capillary voltage was set to 3.5 kV, resulting in 6.6 µA current. Nitrogen served both as sheath (70 psi) and auxiliary gas (10 L/min). Data acquisition and evaluation were carried out on a Personal DECstation 5000/33 (Digital Equipment, Unterföhring, Germany) and ICIS 8.1 software (Finnigan MAT).

Nuclear magnetic resonance (NMR). ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WM 400 spectrometer with $CDCl_3$ (Merck) as solvent.

RESULTS

Identification of transformation products. Linoleic acid-$^{13}C_{18}$, [1-^{14}C], and [9,10,12,13-^{3}H]linoleic acid were injected subepidermally into ripe apples cv. Douce Moen. The fruits were stored up to 4 d at 4°C, extracted with water, and the extract subjected to solid phase extraction. Tables 1 and 2 show the ^{14}C and ^{3}H recovery data, respectively, for the different fractions as a percentage of the applied radioactivity. A high amount of $^{14}CO_2$ is produced within 24 h, indicating a rapid degradation of linoleic acid by β-oxidation. The slight increase in $^{14}CO_2$ production after the first day is probably caused by the degradation of the oxyfunctionalized transformation products formed from linoleic acid. This hypothesis is supported by the fact that at day 4 the diethylether extract contained almost no ^{14}C radioactivity but still had ^{3}H activity. Therefore, linoleic acid has lost the labeled carbon by β-oxidation while degradation products carrying the tritium label are still available (Table 2).

TABLE 1
Distribution of Recovered ^{14}C (expressed as percentage of total uptake) in Different Fractions After the Application of [1-^{14}C]Linoleic Acid to Apples

	Days			
	0.5	1	2	4
XAD flow through	29.5	34.4	38.9	35.9
Diethyl ether extract	19.7	7.6	3.9	1.8
Methanol extract	1.9	1.9	2.1	1.8
Nonextractable residue[a]	18.0	14.8	19.9	19.8
CO_2 (calculated)	30.9	41.3	35.2	40.7

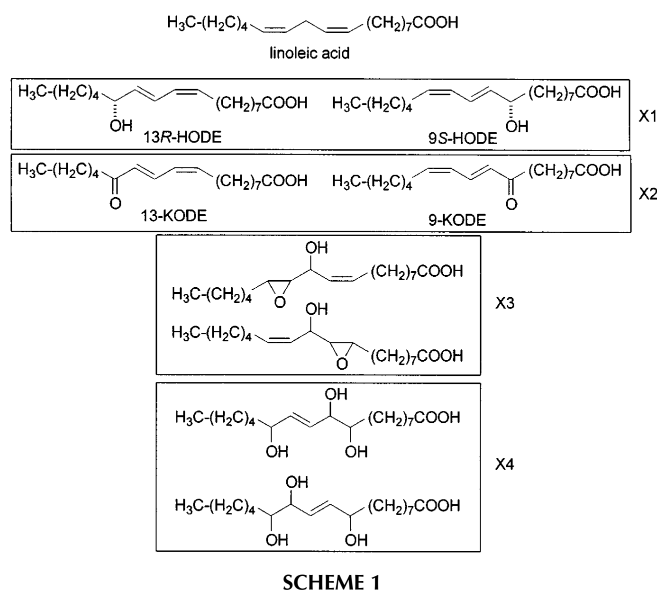
[a]Solid residues remaining after extraction with water. XAD, polystyrene resin.

TABLE 2
Distribution of Recovered ^{3}H (expressed as percentage of total uptake) in Different Fractions After the Application of [9,10,12,13-^{3}H]Linoleic Acid to Apples[a]

	Days			
	0.5	1	2	4
XAD flow through	35.2	38.3	40.3	45.0
Diethyl ether extract	35.6	32.8	27.1	26.6
Methanol extract	9.5	6.5	7.3	7.1
Nonextractable residue[a]	26.9	22.0	19.8	16.1
Sum	107.2	99.6	94.5	94.8

[a]For footnote and abbreviation see Table 1.

Analysis of the diethyl ether extracts obtained by XAD (polystyrene resin) solid phase extraction was performed by HPLC (Fig. 1). After 12 h applied linoleic acid was already completely degraded. Four new compounds (Fig. 1: X1–X4) were detected by radiodetection and ELSD. Product X1 was identified as a mixture of 13-KODE and 9-KODE (ratio 10:1) after methylation by GC–MS analysis and by comparison with the synthesized reference compounds (Scheme 1). LC–MS analysis of X1 showed characteristic ions at *m/z* 295 $[M + H]^+$, *m/z* 312 $[M + NH_4]^+$, and *m/z* 317 $[M + Na]^+$. GC–MS analysis of X1 formed after application of linoleic acid-$^{13}C_{18}$ showed the typical pattern for uniformly labeled 13-KODE and 9-KODE in addition to the unlabeled derivatives. Product X2 consisted of a mixture of 13-HODE and 9-HODE (ratio 4:1) (Scheme 1). The mass spectral data of derivatized X2 were in accordance with those for the reference material. Uniformly labeled and unlabeled 13-HODE and 9-HODE were detected after the application of linoleic acid-$^{13}C_{18}$. On the basis of the mass spectrum of its methyl ester trimethyl silyl (TMS) ether derivatives, X3 was tentatively



SCHEME 1

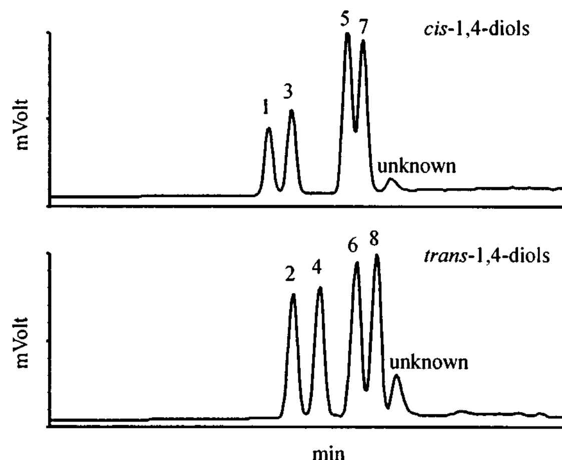


FIG. 2. Gas chromatographic separation of trimethyl silyl derivatives of trihydroxy-methyl esters obtained by incubation of linoleic acid with apples. Peak 1: 9,10,13-trihydroxy *threo/cis*-1,4-diol; peak 2: 9,10,13-trihydroxy *threo/trans*-1,4-diol; peak 3: 9,10,13-trihydroxy *erythro/cis*-1,4-diol; peak 4: 9,10,13-trihydroxy *erythro/trans*-1,4-diol; peak 5: 9,12,13-trihydroxy *threo/cis*-1,4-diol; peak 6: 9,12,13-trihydroxy *threo/trans*-1,4-diol; peak 7: 9,12,13-trihydroxy *erythro/cis*-1,4-diol; peak 8: 9,12,13-trihydroxy *erythro/trans*-1,4-diol.

assigned as a mixture of 11-hydroxy-12,13-epoxy-9-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-octadecenoic acid (17–19) (Scheme 1). The assumption is supported by the LC–MS analysis displaying ions of *m/z* 330 $[M + NH_4]^+$ and *m/z* 335 $[M + Na]^+$. Product X4 was separated by preparative RP18 chromatography, and nuclear magnetic resonance analysis revealed the occurrence of positional isomers of trihydroxy-octadecenoic acids (Scheme 1). LC–MS analysis displayed pseudomolecular ions at *m/z* 348 $[M + NH_4]^+$ and *m/z* 353 $[M + Na]^+$. Product X4 was methylated, and the derivatives were resolved on silica gel-coated TLC plates into *cis*-1,4- and *trans*-1,4-diol according to Hamberg (15). Each fraction was converted into the TMS derivative and analyzed by GC–MS (Fig. 2). A mixture of the positional isomers was obtained. Since all isomers showed almost the same intensity it was expected that the trihydroxy-octadecenoic acids are formed by chemical transformation during the long incubation period (16). Therefore, steric analysis of trihydroxy-octadecenoic acids was not performed. Many plants contain a hydroperoxide peroxygenase/epoxygenase system. In that case one positional isomer of the trihydroxy-octadecenoic acids is enriched (16). Hence, we have no indication for a peroxygenase pathway.

Steric analysis of 13-HODE and 9-HODE. Although lipoxygenase from apples has been investigated by several authors (3,20), the stereochemistry of the products formed by this enzyme is still unknown. Despite intensive LC–MS analysis according to Schneider *et al.* (11), linoleic acid hydroperoxides (HPOD) were not detected as primary products during the metabolism study in apples (Fig. 1). One reason might be the long incubation period of 12 h. However, high amounts of 13-HODE and 9-HODE, formed by enzymatic reduction of HPOD, have been identified. In order to quantify the enantiomers of 13-HODE and 9-HODE, ^{14}C- and ^{3}H-labeled alcohols were purified by semipreparative reverse-phase HPLC analysis followed by semipreparative normal phase HPLC analysis according to Wu *et al.* (13). Separation of the enantiomers was achieved on a Chiralcel OB-H HPLC column. Unlabeled synthesized 13*S*-HODE, 9*S*-HODE, 13*R*,*S*-HODE, and 9*R*,*S*-HODE were added as carrier. The individual enantiomers were collected and quantified by liquid scintillation counting. The enantiomeric ratios of 13-HODE and 9-HODE obtained in the experiments with the ^{14}C- and ^{3}H-labeled substrates were very similiar. 13-HODE and 9-HODE exhibited an enantiomeric excess of *ca.* 75% (*R*) and 65% (*S*), respectively (Fig. 3).

Homogenate. Apples were homogenized in acetate buffer of pH 4.5, and unlabeled linoleic acid was added. Although the solution was analyzed after 1 h of incubation, major products (9-HODE, 13-HODE, 9-KODE, 13-KODE) were detected by ELSD only after 2 h. Trihydroxy-octadecenoic acids were the main metabolites after 48 h.

DISCUSSION

Lipoxygenase from apples has always been studied in relation to its formation of C6 aldehydes such as hexanal and (3*Z*)- and (2*E*)-hexenals, important flavor compounds of apples. A lipoxygenase was extracted from apples and partially purified (21,22). The enzyme belongs to the LOX II type but shows a relatively high specificity toward the dioxygenation of position 13 (82% 13-HPOD:18% 9-HPOD) (21,22). Stereochemical analysis of the products has not been performed until now.

During our investigation on the metabolism of linoleic acid in stored apples we identified 13-HODE, 9-HODE, 13-KODE, and 9-KODE as major metabolites. At first glance the formation of these compounds can be explained by oxidation

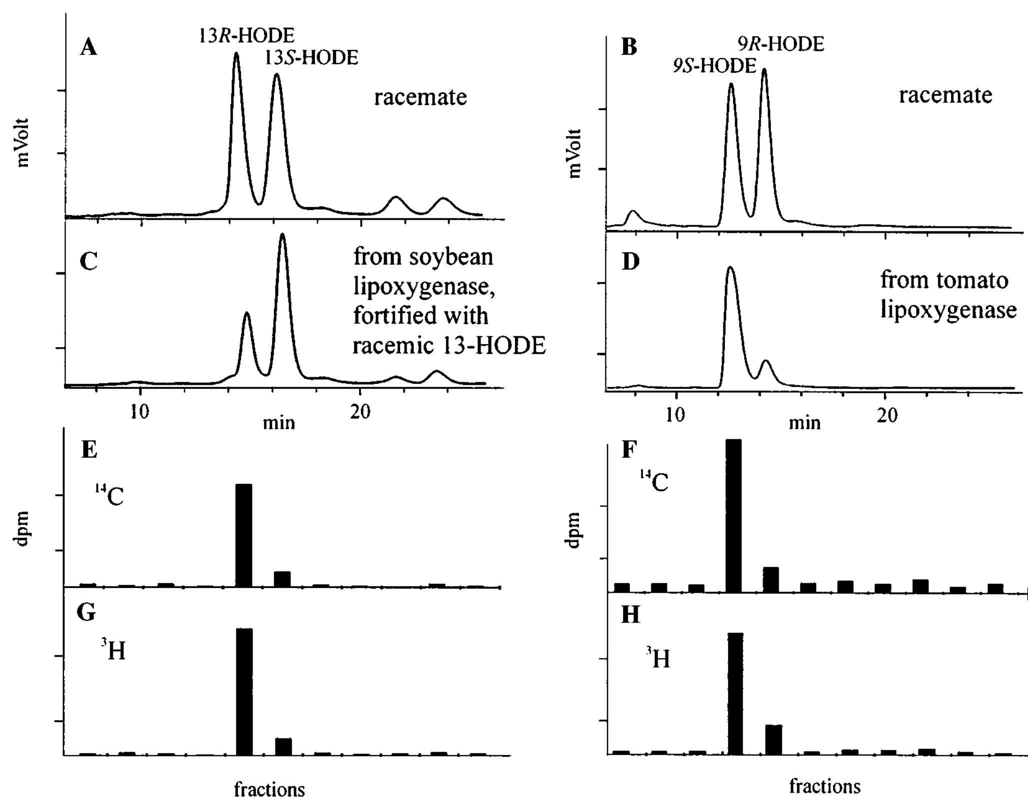


FIG. 3. Ultraviolet (UV) detection of the separation of racemic standards (A, B); UV detection of the separation of soybean lipoxygenase products, fortified with racemic 13-hydroxy-octadecadienoic acid (13-HODE) (C) as well as the separation of tomato lipoxygenase products (D). ^{14}C and ^{3}H in 13*R*- and 13*S*-HODE (E, G) as well as in 9*R*- and 9*S*-HODE (F, H) obtained from apple fruits incubated with labeled linoleic acid.

and reduction of the respective linoleic acid hydroperoxides (HPOD) formed by the action of LOX. Although we could not detect any hydroperoxides in apples we revealed the stereochemistry of the major metabolite 13-HODE to be *R*. Almost all plant LOX so far studied catalyze the formation of *S*-configured hydroperoxides. Only LOX from peas and the LOX type II enzymes from soybean seeds have been reported to form *R*-hydroperoxides (23). However, enzymes from several species of invertebrate, such as sea urchin, starfish and coral, express LOX which produce *R*-hydroperoxides (24,25). Recently, an arachidonic acid (8*R*)-LOX was cloned and sequenced (24), and the formation of (13*R*)-hydroperoxy-(9*Z*,11*E*)-octadecadienoic acid (13*R*-HPOD) by manganese LOX from the fungus *Gäumannomyces graminis* was demonstrated (25). In apples, 13*R*-HODE might be formed either by a (*R*)-LOX followed by enzymatic reduction of the hydroperoxide or by a bisallylic oxidation catalyzed by a cytochrome P450 monooxygenase followed by double-bond migration (26). As lyases degrade predominantly 13*S*-HPOD, 13*R*-HODE might also be generated by reduction of the remaining 13*R*-HPOD (27–29).

The linoleic acid metabolites 11-hydroxy-12,13-epoxy-9-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-octadecenoic acid, tentatively identified in this report, have been described as nonenzymatic degradation products of 13-HPOD (17,19,30), but they have also been reported to be free-radical decomposition products of 13-HPOD through formation of alkoxyl radicals (19). Nevertheless, the occurrence of the epoxy fatty acids gives a strong hint to the existence of hydroperoxides as their metabolic precursors in apples.

An indication for the nonenzymatic formation of the trihydroxy-octadecenoic acids is given by the observation that equal amounts of the stereoisomers were formed. This is not surprising, as apples have an internal pH of 3.5, sufficient to catalyze the rearrangement of unstable epoxides. In summary, the metabolic profile of linoleic acid in stored apples resembles the pattern obtained for the metabolism of linoleic acid in porcine leukocytes (31) and wheat (32) for which a predominant formation of LOX-derived products at position C-13 could be demonstrated.

Homogenates prepared from apples showed in principle the same product pattern after incubation with unlabeled linoleic acid as the intact apples. After prolonged incubation periods high amounts of trihydroxy acids accumulated, probably by chemical transformations, like the homolysis of hydroperoxides and autoxidation of linoleic acid. However, in apples the trihydroxy acids are probably further degraded by β-oxidation as can be seen by comparison of the recovery data for ^{14}C and ^{3}H in the diethyl ether extracts (Table 1 and 2). Radiotracer experiments showed that the transformation

products of linoleic acid were converted into (*R*)-octane-1,3-diol. However, the incorporation of the oxylipids into (*R*)-octane-1,3-diol was lower than the incorporation of linoleic acid (Beuerle, B., and Schwab, W., unpublished results).

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Metabolism of *Trans* Fatty Acids by Hepatocytes

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ABSTRACT: The present work was undertaken to study the metabolism of fatty acids with *trans* double bonds by rat hepatocytes. In liver mitochondria, elaidoyl-CoA was a poorer substrate for carnitine palmitoyltransferase I (CPT-I) than oleoyl-CoA. Likewise, incubation of hepatocytes with oleic acid produced a more pronounced stimulation of CPT-I than incubation with *trans* fatty acids. This was not due to a differential effect of *cis* and *trans* fatty acids on acetyl-CoA carboxylase (ACC) activity and malonyl-CoA levels. Elaidic acid was metabolized by hepatocytes at a higher rate than oleic acid. Surprisingly, compared to oleic acid, elaidic acid was a better substrate for mitochondrial and, especially, peroxisomal oxidation, but a poorer substrate for cellular and very low density lipoprotein triacylglycerol synthesis. Results thus show that *trans* fatty acids are preferentially oxidized by hepatic peroxisomes, and that the ACC/malonyl-CoA/CPT-I system for coordinate control of fatty acid metabolism is not responsible for the distinct hepatic utilization of *cis* and *trans* fatty acids.

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Trans fatty acids are geometrical isomers of unsaturated fatty acids that adopt a saturated fatty acid-like configuration. Human diets contain *trans* fatty acids derived from animal sources (e.g., dairy products and ruminant meats), but most are supplied by products containing industrially hydrogenated vegetable oils (e.g., margarines, shortenings, and baked goods) (1–3). A number of clinical studies have shown that, in contrast to *cis*-monounsaturated fatty acids (e.g., oleic acid), *trans*-monounsaturated fatty acids (e.g. elaidic acid) increase plasma levels of total cholesterol, total triacylglycerols, and low density lipoprotein (LDL) cholesterol, whereas they decrease plasma levels of high density lipoprotein (HDL) cholesterol (1–3). In addition, *trans* fatty acids may tend to increase the plasma concentration of the atherogenic lipoprotein (a) (1–3). Thus, intake of *trans* fatty acids may have an adverse effect on the plasma lipid profile, which represents an increased risk for cardiovascular diseases (1–4). On the basis of this evidence, claims have been made that the *trans* fatty acid content should be included on food labels (5). Other studies, however, have concluded that *trans* fatty acids do not significantly affect plasma cholesterol and triacylglycerol levels, and therefore the effect of dietary *trans* fatty acids on the plasma lipid profile remains controversial (1–4).

In spite of the important body of evidence showing that dietary *trans* fatty acids have a negative impact on plasma lipids, the mechanism responsible for this effect is as yet unknown. Since the liver is a major site of plasma lipoprotein generation, a possibility would be that inherent differences in the metabolism of *cis* and *trans* fatty acids occur in the liver. Indeed, it has been put forward that a distinct partitioning of *cis* fatty acids and saturated fatty acids between hepatic esterification and oxidation may be a factor responsible for the different effects of these two types of fatty acids on plasma lipid levels in the hamster (6). The present study was therefore designed to investigate the metabolic fate of *trans* fatty acids compared to *cis* fatty acids in hepatocytes. Special emphasis was put on two key enzymes of hepatic fatty acid metabolism, *viz.*, carnitine palmitoyltransferase I (CPT-I) and acetyl-CoA carboxylase (ACC), which catalyze the pace-setting steps of mitochondrial fatty acid oxidation and of fatty acid synthesis *de novo*, respectively (7–9).

EXPERIMENTAL PROCEDURES

Materials. [1-^{14}C]Elaidic acid and [1-^{14}C]oleic acid were supplied by ARC (St. Louis, MO). The various unlabeled fatty acids were supplied by Sigma (St. Louis, MO). Tetradecylglycidic acid (TDGA) was kindly donated by Dr. J.M. Lowenstein (Brandeis University, Waltham, MA).

Isolation and incubation of hepatocytes. Male Wistar rats (200–250 g) which had free access to food and water were used throughout this study. Hepatocytes were isolated and incubated as described in Reference 10.

Assay of CPT-I activity. CPT-I activity was determined as the incorporation of radiolabeled carnitine into acylcarnitine by two previously described procedures.

(*i*) CPT-I activity was determined in isolated mitochondria with elaidoyl-CoA or oleoyl-CoA as substrate (11). Mitochondrial preparations were obtained from rat hepatocytes and were practically devoid of peroxisomes, as judged from the low recovery (<5%) of catalase activity (11). The assay mixture consisted of 25 mM Tris-HCl, pH 7.4, 150 mM su-

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Abbreviations: ACC, acetyl-CoA carboxylase; CPT-I, carnitine palmitoyltransferase I; TDGA, tetradecylglycidic acid; VLDL, very low density lipoprotein.

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crose, 60 mM KCl, 1 mM EDTA, 1 mM dithioerythritol, 2 mg/mL defatted and dialyzed bovine serum albumin, 0.5 mM L-[*Me*-^{3}H]carnitine (1 Ci/mol) and varying concentrations of acyl-CoA. Reactions were carried out at 30°C for 2 min (the assay proceeds at a linear rate up to 4 min). Carnitine palmitoyltransferase (CPT) activity that was insensitive to 100 μM malonyl-CoA was always subtracted from the CPT activity experimentally determined. This malonyl-CoA-insensitive CPT activity routinely accounted for 5–10% of the total CPT activity experimentally determined.

(*ii*) CPT-I activity was determined in digitonin-permeabilized hepatocytes with palmitoyl-CoA as substrate exactly as described in Reference 12. Reactions were carried out at 37°C for 1 min (the assay proceeds at a linear rate up to 1.5 min). Evidence has been presented showing that the contribution of CPT-I to the total TDGA-sensitive CPT pool is very high (>85%); in contrast, microsomal CPT and peroxisomal CPT together only make a minor contribution (<15%) to the TDGA-sensitive CPT pool under the same assay conditions (12,13). Therefore, determination of CPT-I activity by the permeabilized-hepatocyte procedure is not prone to substantial error.

Assay of ACC activity. ACC activity was determined as the incorporation of radiolabeled acetyl- CoA into fatty acids in a reaction coupled to the fatty acid synthase reaction by two previously described procedures.

(*i*) ACC activity was determined in cytosolic fractions that were prepared from rat hepatocytes as described in References 14 and 15. The assay mixture consisted of 78 mM Hepes, pH 7.5, 125 mM mannitol, 2.5 mM 2-mercaptoethanol, 3 mM EDTA, 1 mM citrate, 5 mM $MgCl_2$, 2 mM ATP, 20 mM $KHCO_3$, 1 mg/mL defatted and dialyzed bovine serum albumin, 0.5 mM dithioerythritol, 0.5 mM NADPH, 0.062 mM butyryl-CoA, 0.062 mM [1-^{14}C]acetyl-CoA (4 Ci/mol), 3.2 mU fatty acid synthase, and, optionally, 150 μM acyl-CoA. Reactions were carried out at 37°C for 1 min (the assay proceeds at a linear rate up to 1.5 min). The rest of this procedure was performed as in Reference 16.

(*ii*) ACC activity was determined in digitonin-permeabilized hepatocytes exactly as described in Reference 16. Reactions were carried out at 37°C for 4 min (the assay proceeds at a linear rate up to 9 min). Although a large fraction (50–70%) of total hepatocellular ACC does not leak out from the hepatocytes under the permeabilization conditions used (15,16), evidence has been presented showing that ACC activity remaining inside the permeabilized hepatocytes is also measured (16).

Rates of fatty acid metabolism. (*i*) The rate of [^{14}C]fatty acid oxidation was determined as the formation of acid-soluble products (which mostly constitute ketone bodies) and CO_2 (17). Briefly, incubations were carried out at 37°C in the presence of 0.5 mM albumin-bound [1-^{14}C]fatty acid (0.1 Ci/mol) and stopped with 0.3 mL 2 M $HClO_4$ after 30 min (reactions proceed at a linear rate up to 45 min). At the same time, 0.15 mL of benzethonium hydroxide (1 M in methanol) was injected in a center well containing filter paper. Samples were allowed to equilibrate for an additional hour at 4°C, and the center well (with the CO_2 fixed as bicarbonate) was transferred to vials for radioactive counting. The cell precipitate was spun down, and supernatants were washed three times with light petroleum ether. Acid-soluble products were subsequently extracted from the samples as the aqueous phase of the Bligh and Dyer lipid extraction procedure (18). In some experiments, acetoacetate and 3-hydroxybutyrate produced from unlabeled fatty acids were quantified by standard spectrophotometric methods (19).

TDGA, a specific inhibitor of CPT-I and therefore of mitochondrial long-chain fatty acid oxidation, was used to determine separately the mitochondrial and peroxisomal oxidation of [^{14}C]fatty acids. Thus, the former was calculated as TDGA-sensitive oxidation, whereas the latter was measured as TDGA-insensitive oxidation (12). This method does not take into account the fact that peroxisomes degrade fatty acids only partially. However, the use of specific inhibitors of CPT-I to determine peroxisomal fatty acid oxidation, although it may yield overestimated values, is a widely valuated procedure (*cf.* 12,20).

(*ii*) The rate of [^{14}C]fatty acid incorporation into cellular and very low density lipoprotein (VLDL) lipids was monitored as described before (21). Briefly, incubations were carried out at 37°C in the presence of 0.5 mM albumin-bound [1-^{14}C]fatty acid (0.1 Ci/mol). Reactions were stopped after 60 min (reactions proceed at a linear rate up to 90 min for cellular lipid synthesis, and between 30 and 90 min for VLDL lipid secretion). Then, cells were transferred to centrifuge tubes and separated from the incubation medium, from which VLDL were isolated. Cellular and VLDL lipids were extracted (18) and subsequently resolved by thin-layer chromatography (21).

Malonyl-CoA concentration. Intracellular levels of malonyl-CoA were determined in neutralized perchloric acid cell extracts by a radioenzymatic method (21).

Statistical analysis. Results shown represent the means ± SD of the number of animals indicated in each case. Cell incubations and/or enzyme assays were always carried out in triplicate. Statistical analysis was performed by the Student *t* test.

RESULTS

Effect of trans *fatty acids on the ACC/malonyl-CoA/CPT-I system.* Experiments were conducted to ascertain the effect of elaidic acid (*trans*-9-octadecenoic acid) compared to that of its *cis* counterpart oleic acid (*cis*-9-octadecenoic acid) on CPT-I activity. In isolated hepatic mitochondria, elaidoyl-CoA was a poorer substrate for CPT-I than oleoyl-CoA over a broad range of acyl-CoA concentrations (Fig. 1A). Similar results were observed when bovine serum albumin was excluded from the assay mixture (Fig. 1B), indicating that the observed differences in CPT-I activity were not due to different binding affinities of the two acyl-CoA molecules for bovine serum albumin. When bovine serum albumin was absent from the assays, concentrations of the two acyl-CoA

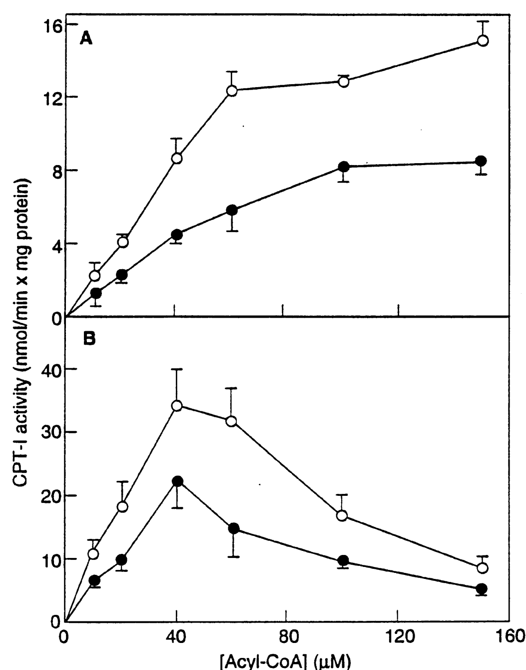

FIG. 1. Carnitine palmitoyltransferase I (CPT-I) activity in liver mitochondria with elaidoyl-CoA and oleoyl-CoA as substrates. Isolated liver mitochondria were incubated in the presence (Panel A) or absence (Panel B) of 2.0 mg/mL defatted and dialyzed bovine serum albumin, and CPT-I activity was determined with oleoyl-CoA (○) or elaidoyl-CoA (●) as substrate. Results correspond to four (Panel A) or three (Panel B) different experiments.

molecules higher than about 40 μM were inhibitory (Fig. 1B), possibly owing to detergent action.

CPT-I activity was subsequently determined in digitonin-permeabilized hepatocytes. As shown in Table 1, CPT-I activity increased when isolated hepatocytes were preincubated with a number of monounsaturated C_{18} nonesterified fatty acids. The maximal stimulation of CPT-I activity was evident after 10 min of cell exposure to the fatty acids. This maximal stimulation kept constant for up to at least 30 min (results not shown). Interestingly, CPT-I became much more activated by oleic acid than by the three *trans* acids tested, namely, elaidic acid, petroselaidic acid (*trans*-6-octadecenoic acid), and *trans*-vaccenic acid (*trans*-11-octadecenoic acid). Peroxisomal CPT activity was not affected by the different fatty acids tested (results not shown). Although a number of cellular modulators have been shown to exert stable short-term modifications of CPT-I activity (e.g., 7,11), the stimulatory effect of the C_{18} fatty acids on CPT-I was labile, since it did not survive extensive washing of the permeabilized cells prior to the assay of enzyme activity (results not shown).

The different stimulatory effects of *cis* and *trans* fatty acids on CPT-I might be due to their different effects on the activity of ACC, the enzyme that catalyzes the formation of malonyl-CoA, a well-established physiological inhibitor of CPT-I (7–9). However, when hepatocytes were incubated with either *cis* or *trans* fatty acids, a quantitatively similar decrease of ACC activity and intracellular malonyl-CoA levels ensued (Table 1). Likewise, elaidoyl-CoA and oleoyl-CoA exerted similar inhibitory effects on ACC activity as determined in a cytosolic fraction. Thus, ACC activity (in nmol/min × mg protein) was 1.25 ± 0.16 with no additions, 0.39 ± 0.05 with 150 μM oleoyl-CoA, and 0.44 ± 0.06 with 150 μM elaidoyl-CoA (n = 3). Hence, a malonyl-CoA-independent mechanism seems to be responsible for the differential stimulation of CPT-I by *cis* and *trans* fatty acids.

Metabolism of [^{14}C]elaidic acid in hepatocytes. The metabolism of [^{14}C]elaidic acid was subsequently studied in isolated hepatocytes. Although CPT-I is generally considered to play a key regulatory role in the control of fatty acid oxidation by hepatic mitochondria (7–9), Table 2 shows that the formation of acid-soluble products was surprisingly higher with [^{14}C]elaidic acid than with [^{14}C]oleic acid as substrate. CO_2 production was also higher from [^{14}C]elaidic acid than from [^{14}C]oleic acid (Table 2).

The contribution of the mitochondrial and the peroxisomal compartment to total hepatocellular fatty acid oxidation was determined. As shown in Table 2, [^{14}C]elaidic acid was a better substrate than [^{14}C]oleic acid for both mitochondrial and peroxisomal oxidation. Interestingly, the contribution of peroxisomal oxidation was significantly higher for [^{14}C]elaidic acid than for [^{14}C]oleic acid (Table 2). In order to corroborate that mitochondrial acid-soluble product formation actually reflects ketogenesis, the mass of ketone bodies (acetoacetate plus 3-hydroxybutyrate) was enzymatically determined. Sim-

TABLE 1
Effect of *Cis* and *Trans* Fatty Acids on ACC Activity, Malonyl-CoA Levels, and CPT-I Activity in Hepatocytes[a]

Addition	ACC activity (nmol/min × mg protein)	Malonyl-CoA (pmol/mg protein)	CPT-I activity (nmol/min × mg protein)
None	0.38 ± 0.04	64 ± 11	1.32 ± 0.22
Oleic acid	0.19 ± 0.03*	30 ± 7*	2.62 ± 0.16*
Petroselaidic acid	0.22 ± 0.02*	32 ± 4*	1.77 ± 0.11*/**
Elaidic acid	0.21 ± 0.02*	33 ± 6*	1.85 ± 0.21*/**
Trans-vaccenic acid	0.23 ± 0.02*	31 ± 7*	1.74 ± 0.10*/**

[a]Hepatocytes were preincubated for 10 min with 0.5 mM of the fatty acids indicated, and then aliquots were taken for determination of malonyl-CoA concentrations as well as of enzyme activities by the respective permeabilized-cell procedures. Results correspond to four different experiments. *P < 0.01 vs. the corresponding incubations with no additions. **P < 0.01 vs. the corresponding incubations with oleic acid. Abbreviations: ACC, acetyl-CoA carboxylase; CPT-I, carnitine palmitoyltransferase I.

TABLE 2
Metabolism of [^{14}C]Elaidic Acid and [^{14}C]Oleic Acid in Hepatocytes[a]

Parameter (nmol fatty acid into product/h × mg protein)	[^{14}C]Oleic acid	[^{14}C]Elaidic acid
Fatty acid oxidation		
Total	19.77 ± 3.81	33.86 ± 4.70*
CO_2	3.58 ± 0.33	6.01 ± 1.22*
Total acid-soluble products	16.19 ± 3.44	27.85 ± 3.41*
Mitochondrial acid-soluble products	12.41 ± 2.40	18.55 ± 1.97**
Peroxisomal acid-soluble products	3.78 ± 1.08	9.30 ± 1.44*
Ketone bodies	12.66 ± 2.13	18.48 ± 2.48**
Fatty acid esterification		
Total	10.97 ± 1.85	7.07 ± 0.62**
Triacylglycerols	7.92 ± 1.82	3.26 ± 0.96*
Phospholipids	2.14 ± 0.13	2.99 ± 0.45**
Cholesterol esters	0.22 ± 0.12	0.13 ± 0.08
VLDL lipid output		
Total	1.69 ± 0.51	1.64 ± 0.51
Triacylglycerols	0.97 ± 0.21	0.59 ± 0.20**
Phospholipids	0.31 ± 0.17	0.39 ± 0.19
Cholesterol esters	0.09 ± 0.06	0.07 ± 0.03
Total [^{14}C]fatty acid metabolized	32.43 ± 3.77	42.57 ± 3.79*

[a]Hepatocytes were incubated with 0.5 mM [^{14}C]oleic acid or [^{14}C]elaidic acid except for the determination of ketone bodies, for which incubations contained 0.5 mM of the unlabeled fatty acids. In the latter case endogenous ketone body production was subtracted. The rates of oxidation, esterification, and VLDL lipid production were determined. Results correspond to three different experiments. Compared to the corresponding incubations with oleic acid: *$P < 0.01$; **$P < 0.05$. VLDL, very low density lipoprotein.

ilar results were obtained as with acid-soluble product formation, i.e., elaidic acid was observed to be a better substrate for ketogenesis than oleic acid (Table 2).

Since the fatty acid concentration may influence the relative contributions of mitochondria and peroxisomes to hepatic fatty acid oxidation (22), experiments were subsequently performed with different doses of the fatty acids. Figure 2 shows that mitochondrial and—especially—peroxisomal oxidation of [^{14}C]elaidic acid was higher than that of [^{14}C]oleic acid in the range of 0.2–0.8 mM fatty acid.

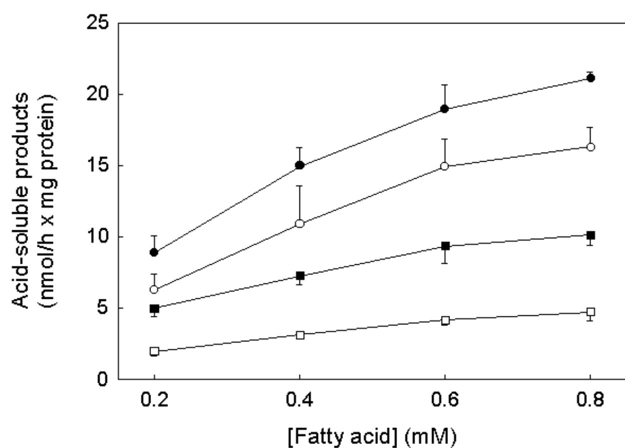

FIG. 2. Mitochondrial and peroxisomal oxidation of [^{14}C]elaidic acid and [^{14}C]oleic acid in hepatocytes. Cells were incubated with varying concentrations of [^{14}C]oleic acid (○,□) or [^{14}C]elaidic acid (●,■), and the formation of mitochondrial (○,●) and peroxisomal (□,■) acid-soluble products was quantified. Results correspond to three different experiments.

The incorporation of [^{14}C]elaidic acid into both cellular and VLDL triacylglycerols was lower than that of [^{14}C]oleic acid (Table 2). In contrast, an increase in the incorporation of [^{14}C]elaidic acid into phospholipids was evident (Table 2). Table 2 also shows that the total amount of [^{14}C]elaidic acid metabolized by hepatocytes was higher than that of [^{14}C]oleic acid, indicating that factors in addition to the modulation of the balance between oxidation and esterification may be involved in the different utilization of *cis* and *trans* fatty acids by the liver.

DISCUSSION

Liver peroxisomes exhibit a preference for oxidizing trans *fatty acids rather than* cis *fatty acids*. Although there is general agreement that the fatty acids that are more abundant in a normal diet are preferentially oxidized in mitochondria rather than in peroxisomes, some types of fatty acids (e.g., very long chain, branched-chain, and dicarboxylic fatty acids) are mostly oxidized in peroxisomes (22,23). The present report shows that the rate of peroxisomal oxidation of elaidic acid is about 2.5-fold that of oleic acid. Since the entry of long-chain acyl-CoA into peroxisomes is independent of a CPT system (22,23), this preferential utilization of trans fatty acids by peroxisomes may reflect a substrate specificity of the peroxisomal β-oxidation equipment.

Elaidic acid was shown to be a poorer substrate than oleic acid for the synthesis of both cellular and VLDL triacylglyc-

erols in rat hepatocytes. If these observations could be extrapolated to the *in vivo* situation, other mechanisms should be responsible for the putative hyperlipidemic effect of *trans* fatty acids. In addition, a preferential incorporation of elaidic acid into hepatic phospholipids was observed, in line with data obtained by Fukuda *et al.* (24) on the metabolism of *trans*, *trans*-9,12-octadecadienoic acid in the perfused rat liver. The regulation of liver phospholipid biosynthesis involves a number of mechanisms distinct from those acting in liver triacylglycerol formation (25,26). However, the reason for the diversion of *trans* fatty acids into phospholipid synthesis is as yet unknown.

The ACC/malonyl-CoA/CPT-I system is not responsible for the distinct oxidation of cis *and* trans *fatty acids by liver mitochondria*. Short-term changes in CPT-I activity are usually accompanied by parallel changes in the rate of long-chain fatty acid oxidation (7). In addition, under pathophysiological situations in which long-chain fatty acid oxidation is induced, CPT-I activity increases, and vice versa (8,9). These short- and long-term changes in CPT-I activity usually (though not always) occur in concert with reciprocal changes in ACC activity and malonyl-CoA levels (7–9). The ACC/malonyl-CoA/CPT-I system is therefore considered to play a central role in the control of the flux through the fatty acid-oxidative pathway (7–9). However, the ACC/malonyl-CoA/CPT-I system is not responsible for the distinct hepatic utilization of *cis* and *trans* fatty acids. This assumption is mainly based on the observation that incubation of hepatocytes with oleic acid produces a much more pronounced stimulation of CPT-I than incubation with various *trans* fatty acids, in spite of the quantitatively similar depressive effect of oleic acid and the *trans* fatty acids on ACC activity and malonyl-CoA levels. In addition, although in isolated hepatocytes mitochondrial oxidation is more active toward elaidic acid than toward oleic acid, elaidoyl-CoA is a poorer substrate than oleoyl-CoA for CPT-I in isolated liver mitochondria. Our data therefore indicate that one or more steps involved in the intramitochondrial oxidation of fatty acids exert(s) preference for elaidic acid over oleic acid. This is also suggested by the observation that the total utilization of elaidic acid by hepatocytes is higher than that of oleic acid, i.e., the increased mitochondrial oxidation of elaidic acid is not simply due to a preferential shift of this fatty acid from esterification to oxidation. Hence, enhanced intramitochondrial utilization of elaidic acid would overcome the lower entry of this fatty acid into mitochondria through the CPT-I system.

The reason for which oleic acid is more potent activator of CPT-I than *trans* fatty acids is not obvious. Data clearly indicate that this effect is independent of malonyl-CoA. Recent evidence from our laboratories has put forward a novel malonyl-CoA-independent mechanism of acute control of hepatic CPT-I, which involves modulation of the interactions between CPT-I and cytoskeletal components (13,27,28). However, changes in CPT-I activity mediated by that mechanism survive extensive washing of the permeabilized hepatocytes prior to determination of enzyme activity, and this is not the case for the fatty acids used in the present study. It should be kept in mind that in our experiments hepatocytes were preincubated with the C_{18} fatty acids and then CPT-I activity was determined by the permeabilized-cell procedure with palmitoyl-CoA as substrate. This supports the notion that long-chain fatty acids exert a labile, malonyl-CoA-independent allosteric stimulation of CPT-I. The existence of an allosteric site for long-chain fatty acyl-CoA in liver CPT-I, although previously suggested (29), has still to be demonstrated.

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Synthesis of a Novel Lipopeptide with α-Melanocyte-Stimulating Hormone Peptide Ligand and Its Effect on Liposome Stability

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ABSTRACT: Introduction of liposomes into target cells is important for drug delivery systems. For this purpose, the surface of the liposome is equipped with ligand peptides, which may bind to specific receptors on the cell membrane. An artificial novel lipopeptide (MSH-C4A2) containing the α-melanocyte-stimulating hormone (α-MSH) sequence and two long alkyl chains was designed and synthesized, and the liposome, composed of egg phosphatidylcholine (EPC) and MSH-C4A2, was prepared. The stability of the liposome was estimated by measuring calcein leakage from the liposome inner phase. The stability of the liposome decreased upon addition of MSH-A4C2, which seemed to be attributable to the amphiphilic property of the peptide moiety (α-MSH) of MSH-A2C4. The stability was, however, recovered fairly well upon addition of cholesterol (Ch) or phosphatidylglycerol (PG). It was concluded therefore that the ternary system, MSH-C4A2/Ch/EPC or MSH-C4A2/PG/EPC, is suitable for preparing the functional liposome.

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Liposome has been extensively studied as a carrier of drugs (1), proteins (2), and nucleic acids (3,4) for drug delivery systems (DDS). Since liposome is usually entrapped by reticuloendothelial systems by nonspecific endocytosis or phagocytosis (5), the liposome cannot remain in the organs long enough to be introduced into the target cell. To solve this problem, the specificity toward target cells should be improved. One promising method may be to equip the surface of liposome with ligand peptides which can bind to the specific receptors on cell membrane. In this study we have designed an artificial novel lipopeptide with α-melanocyte-stimulating hormone (α-MSH) and have prepared the liposomes including the lipopeptide in the bilayer membrane. This type of covalently bound lipopeptide has two advantages. One is that we can use the solid phase synthesis method, which makes procedures such as a deprotection step easier, and the other is that we can attach the peptide moiety onto the liposome surface more efficiently than by incorporating it after liposomes have been formed. The α-MSH peptide has 13 amino acid residues that can bind to the α-MSH receptor found on the melanocyte and melanoma cell surface (6). As the first step of the liposome study toward DDS, we have estimated its stability by measuring calcein leakage from the inner aqueous phase of liposome. This estimation is important for developing DDS because we have no knowledge of the stability of the present novel liposome; the efficiency of targeting toward cells would be decreased if the liposome membranes are not stable and active substances leak from the inner phase. We have been also interested in the captured volume of liposomes, which is ordinarily used as an index of the efficiency of liposome formation (7–9).

EXPERIMENTAL PROCEDURES

Materials. t-Butoxylcarbonyl-aspartic acid (Boc-Asp) and *N*-α-9-fluorenylmethoxycarbonyl (Fmoc) amino acids were purchased from Watanabe Chemical Ind., Ltd. (Hiroshima, Japan). TGR resin was commercially available from Calbiochem Novabiochem Corp. (La Jolla, CA). Egg phosphatidylcholine hydrogenated (EPC) and 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] sodium salt (phosphatidylglycerol: PG) were obtained from Avanti Polar Lipids, Inc. (Alabaster, AL). 5-Cholesten-3β-ol (Ch) was purchased from Sigma Chemical Co. (St. Louis, MO). Calcein was obtained from Kanto Chemical Co., Inc. (Tokyo, Japan). All other reagents were of the highest grade commercially available.

Instruments. Proton nuclear magnetic resonance (NMR) spectra (270 MHz) were recorded on a Jeol JNM EX-270 FT spectrometer. Time-of-flight (TOF) mass spectra were acquired on a Kratos Analytical Kompact Maldi III spectrome-

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Abbreviations: Ch, 5-cholesten-3β-ol (cholesterol); DDS, drug delivery system; DMAP, 4-dimethylaminopyridine; DMF, dimethylformamide; EPC, egg phosphatidylcholine hydrogenated; FAB, fast-atom bombardment; Fmoc, *N*-α-9-fluorenylmethoxycarbonyl; HOBt, 1-hydroxybenzotriazole; MS, mass spectrometry; α-MSH, α-melanocyte-stimulating hormone; NMR, nuclear magnetic resonance; PG, 1,2-dipalmitoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] sodium salt (phosphatidylglycerol); TBTU, 2-(1H-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate; TFA, trifluoroacetic acid; WSCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride.

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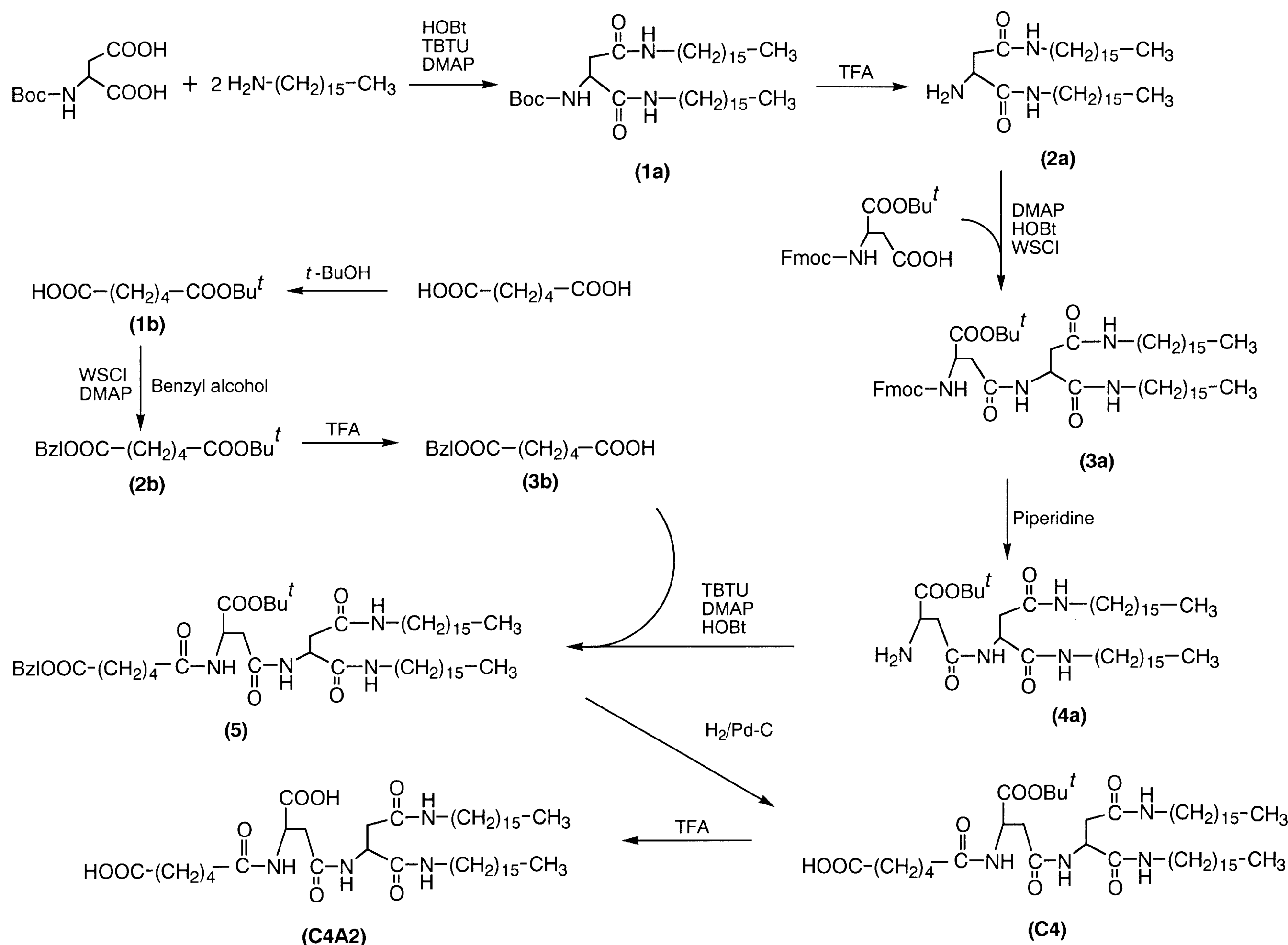
ter, and fast-atom bombardment (FAB) mass spectra were measured on a Jeol JMS-SX102A mass spectrometer. Peptides were synthesized with a PerSeptive Pioneer (Framingham, MA) peptide synthesizer. Measurement of fluorescence was carried out on a Jasco FP-777 spectrometer.

Synthesis of lipid. The synthetic route for the following compounds is shown in Scheme 1.

*(i) **1a***: *t*-Butoxylcarbonyl-aspartic acid (3.00 g, 12.9 mmol) was dissolved in a mixture of dimethylformamide (DMF) (15 mL) and $CHCl_3$ (15 mL). 1-Hydroxybenzotriazole (HOBt: 4.74 g, 31.0 mmol) (10) in DMF (30 ml) and 2-(1H-benzotriazole-1-yl)-1,1,3,3-trimethyluronium tetrafluoroborate (TBTU) (9.94 g, 31.0 mL) (11,12) in DMF (60 mL) were added to the above mixture. Hexadodecylamine (6.23 g, 25.8 mmol) was dissolved in $CHCl_3$ (15 mL), into which 4-dimethylaminopyridine (DMAP: 3.78 g, 31.0 mmol) in $CHCl_3$ (20 mL) was added. This solution was further added to the above DMF solution and stirred for 3 h at room temperature. The reaction mixture was washed with water (*ca.* 150 mL × 3), and the $CHCl_3$ phase was dried over Na_2SO_4. The Na_2SO_4 was filtered off, and the filtrate was condensed. The resultant residue was purified by column chromatography on silica gel (i.d., 7 × 11 cm; eluant, 3% $MeOH/CHCl_3$) to form a white solid. Yield: 7.16 g (10.5 mmol, 81.4%). ^1H NMR ($CDCl_3$) δ 6.94 (*s* br, 1H, N*H*CH_2), 6.21 (*d*, 1H, N*H*CH, J = 6.6 Hz), 5.95 (*s* br, 1H, N*H*CH_2), 4.39 (*ddd*, 1H, NHC*H*, J = 4.0, 6.6 and 6.6 Hz), 3.1–3.3 (*m*, 4H, $2NHCH_2$), 2.83 (*dd*, 1H, CH_AH_BCO, J = 4.0 and 14.8 Hz), 2.48 (*dd*, 1H, CH_AH_BCO, J = 6.6 and 14.8 Hz), 1.4–1.55 (*m*, 4H, $2NHCH_2CH_2$), 1.45 [*s*, 9H, $C(CH_3)_3$], 1.25 [*s* br, 52H, $2CH_3(CH_2)_{13}$], 0.88 [*t*, 6H, $2CH_3(CH_2)_{13}$, J = 6.5 Hz]. Anal. calcd. for $C_{41}H_{81}N_3O_4$: C, 72.41; H, 12.00; N, 6.18. Found: C, 72.58; H, 12.06; N, 6.07. FAB mass spectrometry (MS) calcd.: 680.6 (MH^+), found: 681.

*(ii) **2a***: Compound **1a** (7.03 g, 10.6 mmol) was dissolved in $CHCl_3$ (90 mL) and trifluoroacetic acid (TFA)/$CHCl_3$ (3:7, vol/vol, 27 mL) (13), and the solution was stirred for 4 h at room temperature. Then 5 N NaOH aqueous solution (22 mL) was added to the above solution to neutralize it. The reaction mixture was then washed with water (*ca.* 100 mL × 3), and the $CHCl_3$ layer was dried over Na_2SO_4. After filtering off the Na_2SO_4, the filtrate was condensed and purified by column chromatography on silica gel (i.d., 7 × 12 cm; eluant: 3% $MeOH/CHCl_3$) to form a white solid. Yield: 5.84 g



SCHEME 1

(10.1 mmol, 95.3%). ^{1}H NMR ($CDCl_3$) δ 7.48 (*t*, 1H, N*H*CH_2, *J* = 5.0 Hz), 6.12 (*t*, 1H, N*H*CH_2, *J* = 5.0 Hz), 3.65 (*dd*, 1H, NH_2C*H*, *J* = 5.0 and 6.5 Hz), 3.15–3.3 (*m*, 4H, 2NHCH_2), 2.59 (*dd*, 1H, C$H_A$$H_B$CO, *J* = 5.0 and 14.5 Hz), 2.56 (*dd*, 1H, $CH_A$$H_B$CO, *J* = 6.5 and 14.5 Hz), 1.86 (*s* br, 2H, NH_2), 1.4–1.55 (*m*, 4H, 2NHCH_2CH_2), 1.26 [*s* br, 52H, 2(CH_2)$_{13}$$CH_3$], 0.88 (*t*, 6H, 2$CH_2CH_3$, *J* = 6.4 Hz). Anal. calcd. for $C_{36}H_{73}N_3O_2$: C, 74.55; H, 12.69; N, 7.25. Found: C, 74.49; H, 13.01; N, 7.16. FAB MS calcd.: 580.6 (MH^+), found: 581.

*(iii) **3a***: Compound **2a** (0.94 g, 1.63 mmol) was dissolved in $CHCl_3$ (80 mL), into which DMAP (0.53 g, 2.93 mmol) in $CHCl_3$ (2 mL) was added. Fmoc-Asp-OBu^t (1.00 g, 2.44 mmol) (14) was dissolved in $CHCl_3$ (5 mL), and HOBt (0.45 g, 2.93 mmol) in DMF (2 mL) was added to this $CHCl_3$ solution. The mixture was further added to the **2a**-DMAP solution, into which 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride (WSCI) (0.56 g, 2.93 mmol) (15) in $CHCl_3$ (3 mL) was added, and the mixture was stirred for 3 h at room temperature. The reaction mixture was washed with water (*ca*. 100 mL × 3), and the $CHCl_3$ layer was dried over Na_2SO_4. The Na_2SO_4 was filtered off, the filtrate was condensed, and the residue was purified by silica gel column chromatography (i.d., 7 × 20 cm; eluant, 3–5% MeOH/$CHCl_3$ vol/vol) to form a white solid. Yield: 1.50 g (1.54 mmol, 94.5%). ^{1}H NMR ($CDCl_3$) δ 7.75 (*d*, 2H, fluorene, *J* = 7.6 Hz), 7.60 (*d*, 2H, fluorene, *J* = 7.6 Hz), 7.35–7.45 (*m*, 3H, N*H*CH and fluorene), 7.30 (*t*, 2H, fluorene, *J* = 7.6 Hz), 7.21 (*t*, 1H, N*H*CH_2, *J* = 5.8 Hz), 5.84 (*s* br, 2H, N*H*CH_2 and N*H*CH), 4.63 (*ddd*, 1H, NHC*H*, *J* = 3.0, 7.3 and 7.3 Hz), 4.45–4.6 (*m*, 1H, NHC*H*), 4.35–4.45 (*m*, 2H, CH_2O), 4.22 (*t*, 1H, C*H*CH_2O, *J* = 6.8 Hz), 3.1–3.3 (*m*, 4H, NHCH_2), 2.81 (*dd*, 1H, C$H_A$$H_B$CO, *J* = 3.0 and 14.8 Hz), 2.72 (*dd*, 1H, $CH_A$$H_B$CO, *J* = 5.0 and 16.2 Hz), 2.63 (*dd*, 1H, C$H_A$$H_B$CO, *J* = 7.3 and 16.2 Hz), 2.40 (*dd*, 1H, $CH_A$$H_B$CO, *J* = 7.3, and 14.8 Hz), 1.47 [*s* br, 13H, 2NHCH_2CH_2 and C(CH_3)$_3$], 1.26 [*s* br, 52H, 2CH_3(CH_2)$_{13}$], 0.88 [*t*, 6H, 2CH_3(CH_2)$_{13}$, *J* = 6.6 Hz]. Anal. calcd. for $C_{59}H_{96}N_4O_7$: C, 72.80; H, 9.96; N, 5.76. Found: C, 72.86; H, 10.22; N, 5.70. FAB MS calcd.: 973.7 (MH^+), found: 974.

*(iv) **4a***: Compound **3a** (0.291 g, 0.299 mmol) was dissolved in $CHCl_3$ (50 mL), and piperidine (3 mL) (16) was added, which was then stirred for 3 h at room temperature. The reaction mixture was purified by silica gel column chromatography (i.d., 7 × 14 cm; eluant, 3–5% MeOH/$CHCl_3$ vol/vol) to form a white solid. Yield: 0.209 g (0.278 mmol, 93.0%). ^{1}H NMR ($CDCl_3$) δ 8.12 (*d*, 1H, N*H*CH, *J* = 7.9 Hz), 7.47 (*t*, 1H, N*H*CH_2, *J* = 5.6 Hz), 6.06 (*t*, 1H, N*H*CH_2, *J* = 5.8 Hz), 4.69 (*ddd*, 1H, NHC*H*, *J* = 3.6, 6.3, and 7.9 Hz), 3.75 (*dd*, 1H, NH_2C*H*, *J* = 5.0 and 8.3 Hz), 3.1–3.3 (*m*, 4H, 2NHCH_2), 2.89 (*dd*, 1H, C$H_A$$H_B$CO, *J* = 3.6 and 14.8 Hz), 2.67 (*dd*, 1H, $CH_A$$H_B$CO, *J* = 5.0 and 15.0 Hz), 2.48 (*dd*, 1H, C$H_A$$H_B$CO, *J* = 8.3 and 15.0 Hz), 2.46 (*dd*, 1H, $CH_A$$H_B$CO, *J* = 6.3 and 14.8 Hz), 1.72 (*s*, 2H, NH_2), 1.47 [*s* br, 13H, C(CH_3)$_3$ and 2NHCH_2CH_2], 1.25 [*s* br, 52H, 2CH_3(CH_2)$_{13}$], 0.88 [*t*, 6H, 2CH_3(CH_2)$_{13}$, *J* = 6.6 Hz]. Anal. calcd. for $C_{44}H_{86}N_4O_5$: C, 70.35; H, 11.54; N, 7.46; found: C, 70.23; H, 11.99; N, 7.27. FAB MS calcd.: 751.7 (MH^+), found: 752.

*(v) **1b***: 2-Methyl-2-propanol (10 mL, 106 mmol), WSCI (1.30 g, 6.8 mmol) in $CHCl_3$ (10 mL), and DMAP (0.83 g, 6.8 mmol) in $CHCl_3$ (10 mL) were added to adipic acid (1.0 g, 6.8 mmol), and the mixture was stirred for 9 h at room temperature. It was then diluted with diethyl ether (100 mL) and washed with 0.05 N HCl (3 times) and water (3 times). The $CHCl_3$ layer was dried over Na_2SO_4 and condensed. Purification of the residue by silica gel column chromatography (i.d., 7 × 11 cm; eluant, AcOEt/hexane 4:6 vol/vol) gave liquid product. Yield: 0.4 g (1.97 mmol, 29%). ^{1}H NMR ($CDCl_3$) δ 2.38 (*t*, 2H, CH_2CO, *J* = 7.1 Hz), 2.24 (*t*, 2H, CH_2CO, *J* = 6.9 Hz), 1.6–1.7 (*m*, 4H, COCH_2CH_2C$H_2$$CH_2$CO), 1.44 [*s*, 9H, C($CH_3$)$_3$]. Anal. calcd. for $C_{10}H_{18}O_4$: C, 59.39; H, 8.97. Found: C, 59.40; H, 9.14. FAB MS calcd.: 203.1 (MH^+), found: 203.

*(vi) **2b***: CH_2Cl_2 (5 mL), benzyl alcohol (97.3 mg, 0.90 mmol) in CH_2Cl_2 (3 mL), WSCI (229 mg, 1.2 mmol) in CH_2Cl_2 (2 mL), and DMAP (101 mg, 0.83 ml) in CH_2Cl_2 (3 mL) were added to **1b** (166.2 mg, 0.82 mmol), and the reaction mixture was stirred for 2 h at room temperature. It was washed with water and the CH_2Cl_2 layer was dried over Na_2SO_4. The Na_2SO_4 was filtered off, and the filtrate was condensed and the residue was purified by silica gel column chromatography (i.d., 3 × 8 cm; eluant, AcOEt/hexane 1:9 vol/vol) to form a liquid product. Yield: 156 mg (0.53 mmol, 65.0%). ^{1}H NMR ($CDCl_3$) δ 7.35 (*m*, 5H, C_6H_5), 5.11 (*s*, 2H, PhCH_2), 2.38 (*t*, 2H, OCCH_2, *J* = 6.9 Hz), 2.22 (*t*, 2H, OCCH_2, *J* = 6.9 Hz), 1.6–1.8 [*m*, 4H, CH_2(CH_2)$_2$$CH_2$], 1.43 [*s*, 9H, C($CH_3$)$_3$]. Anal. calcd. for $C_{17}H_{24}$: C, 69.84; H, 8.27. Found: C, 69.59; H, 8.14.

*(vii) **3b***: To **2b** (64.2 mg, 0.22 mmol) was added $CHCl_3$ (5 mL) and TFA (1 mL), and the mixture was stirred for 3 h at room temperature. It was washed with water, and the $CHCl_3$ layer was dried over Na_2SO_4. The Na_2SO_4 was filtered off, the filtrate was condensed, and the residue was purified by column chromatography on silica gel (i.d., 3 × 8 cm; eluant, AcOEt/hexane 4:6 vol/vol) to form a liquid product. Yield: 44.5 mg (0.19 mmol, 85.6%). ^{1}H NMR ($CDCl_3$) δ 7.35 (*m*, 5H, C_6H_5), 5.12 (*s*, 2H, PhCH_2), 2.3–2.5 (*m*, 4H, OCCH_2), 1.6–1.8 [*m*, 4H, CH_2(CH_2)$_2$$CH_2$]. Anal. calcd. for $C_{13}H_{16}O_4 \cdot 0.2H_2O$: C, 65.10; H, 6.89. Found: C, 65.38; H, 7.13.

*(viii) **5***: Compound **4a** (0.114 g, 0.152 mmol) was dissolved in $CHCl_3$ (30 mL), into which TBTU (0.0964 g, 0.300 mmol) in DMF (3 mL) and DMAP (0.0246 g, 0.201 mmol) in $CHCl_3$ (1 mL) were added. **3b** (0.0418 g, 0.201 mmol) was dissolved in $CHCl_3$ (1 mL), into which HOBt (0.0460 g, 0.3 mmol) in DMF (1 mL) was added. The mixture was stirred for 4.5 h at room temperature and washed with water three times. The $CHCl_3$ layer was dried over Na_2SO_4, the filtrate was condensed, and the residue was purified by column chromatography on silica gel (i.d., 7 × 12 cm; eluant, 3–5% MeOH/$CHCl_3$) to form a white solid. Yield: 133 mg (0.143 mmol, 93.1%). ^{1}H NMR ($CDCl_3$) δ 7.3–7.45 (*m*, 7H, N*H*CH,

N*H*CH_2, and phenyl), 6.65 (*d*, 1H, N*H*CH, J = 8.1 Hz), 6.01 (*t*, 1H, N*H*CH_2, J = 4.3 Hz), 5.11 (*s*, 2H, PhCH_2), 4.74 (*dt*, 1H, NHC*H*, J = 5.3 and 8.1 Hz), 4.62 (*ddd*, 1H, NHC*H*, J = 3.4, 6.9, and 6.9 Hz), 3.1–3.3 (*m*, 4H, 2NHCH_2), 2.81 (*dd*, 1H, CH_AH_BCO, J = 3.4 and 15.1 Hz), 2.73 (*d*, 2H, CH_2CO, J = 5.3 Hz), 2.40 (*dd*, 1H, CH_AH_BCO, J = 6.9 and 15.1 Hz), 2.38 (*t*, 2H, COCH_2CH_2, J = 6.9 Hz), 2.24 (*t*, 2H, COCH_2CH_2, J = 6.8 Hz), 1.68 (*s* br, 4H, 2COCH_2CH_2), 1.45 [*s* br, 13H, $C(CH_3)_3$ and 2NHCH_2CH_2], 1.25 [*s*, 52H, $2CH_3(CH_2)_{13}$], 0.88 (*t*, 6H, $2CH_3(CH_2)_{13}$, J = 6.6 Hz). Anal. Calcd. for $C_{57}H_{100}O_8N_4$: C, 70.62; H, 10.40; N, 5.78. Found: C, 70.61; H, 10.97; N, 5.59. FAB MS calcd.: 969.8 (MH^+), found: 970.

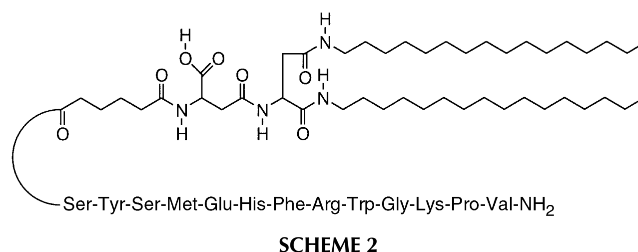
(ix) ***C4***: Compound **5** (0.133 g, 0.137 mL) was dissolved in CH_2Cl_2 (20 mL) and 2-propanol (10 mL). Then 10% Palladium/Aktivkohle (0.040 g; Merck, Schucherdt, Germany) was added to the reaction mixture and hydrogen gas was introduced for 1 h (17). After the reaction, Palladium/ Aktivkohle was removed by a polytetrafluoroethylene membrane. Purification of the reaction mixture by silica gel column chromatography (i.d., 3 × 2 cm; eluant, 8% MeOH/$CHCl_3$) gave a white solid. Yield: 99.3 mg (0.113 mmol, 82.4%). ^{1}H NMR ($CDCl_3$) δ 7.39 (*d*, 1H, N*H*CH, J = 6.9 Hz), 7.33 (*t*, 1H, N*H*CH_2, J = 5.8 Hz), 6.65 (*d*, 1H, N*H*CH, J = 7.9 Hz), 5.99 (*s* br, 1H, N*H*CH_2), 4.75 (*dt*, 1H, NHC*H*, J = 5.3 and 7.9 Hz), 4.63 (*ddd*, 1H, NHC*H*, J = 3.3, 6.9, and 6.9 Hz), 3.1–3.3 (*m*, 4H, 2NHCH_2), 2.09 (*dd*, 1H, CH_AH_BCO, J = 3.3 and 15.2 Hz), 2.74 (*d*, 2H, CH_2CO, J = 5.3 Hz), 2.41 (*dd*, 1H, CH_AH_BCO, J = 6.9 and 15.2 Hz), 2.33 (*t*, 2H, COCH_2CH_2, J = 6.8 Hz), 2.34 (*t*, 2H, COCH_2CH_2, J = 5.4 Hz), 1.66 (*s* br, 4H, 2COCH_2CH_2), 1.46 [*s* br, 13H, $C(CH_3)_3$ and 2NHCH_2CH_2], 1.25 [*s* br, 52H, $2CH_3(CH_2)_{13}$], 0.88 [*t*, 6H, $2CH_3(CH_2)_{13}$, J = 6.3 Hz]. FAB MS calcd.: 879.7 (MH^+), found: 880.

(x) ***C4A2***: Compound **C4** (64.2 mg, 0.073 mmol) was dissolved in $CHCl_3$ (1 mL). After adding TFA (1 mL), the mixture was reacted for 5 h at room temperature. After the solvent was evaporated and the residue was dried *in vacuo* for 12 h at 70°C, a white solid was obtained. Yield: 60 mg (0.072 mmol, 99%). ^{1}H NMR ($CDCl_3$ and CD_3OD) δ 4.7–4.8 (*m*, 1H, CH), 4.6–4.7 (*m*, 1H, CH), 3.1–3.2 (*m*, 4H, 2NHCH_2), 2.79 (*dd*, 1H, CH_ACH_B, J = 5.9 and 15.7 Hz), 2.75 (*dd*, 1H, CH_ACH_B, J = 6.6 and 15.7 Hz), 2.63 (*dd*, 1H, CH_ACH_B, J = 5.9 and 15.2 Hz), 2.54 (*dd*, 1H, CH_ACH_B, J = 6.3 and 15.2 Hz), 2.33 (*t*, 2H, CH_2CH_2CO, J = 6.9 Hz), 2.27 (*t*, 2H, CH_2CH_2CO, J = 7.3 Hz), 1.6–1.7 (*m*, 4H, 2CH_2CH_2CO), 1.4–1.55 (*m*, 4H, 2NHCH_2CH_2), 1.2 [*s* br, 52H, $2(CH_2)_{13}CH_3$], 0.89 (t, 6H, $2CH_2CH_3$, J = 6.6 Hz). Anal. calcd. for $C_{44}H_{82}N_4O_8$·0.5CF_3COOH: C, 64.13; H, 9.91; N, 6.37. Found: C, 63.83; H, 9.83; N, 6.13. FAB MS calcd.: 823 (MH^+), found: 824.

Synthesis of α-MSH. Peptide α-MSH was synthesized in an ordinary Fmoc method (18) by a peptide synthesizer (PerSeptive Biosystems, Pioneer Peptide Synthesis System). The major conditions were as follows: TGR resin, 1 g (0.2 mmol); Fmoc amino acid, 0.8 mmol in DMF; coupling reagents, HOBt (0.5 M)/TBTU (0.5 M)/diisopropylethylamine (1.0 M) in DMF; deblocking reagent, 20% piperidine in DMF; washing solvent, DMF; condensation reaction time, 20 min; deblocking reaction time, 5 min. The peptide was cleaved from the resin by the reaction in TFA (2.5 mL), 1,2-ethanedithiol (EDT) (62.5 μL), triisopropylsilane (62.5 μL), and H_2O (125 μL) for 5 h at room temperature. After filtering off the resin, ethyl ether was added to the filtrate and a white precipitate formed. After centrifugation (4°C, 3500 rpm, 20 min), the supernatant was removed, ethyl ether was added again, and the same procedure was repeated. The white precipitate obtained was dissolved in water and purified by high-performance liquid chromatography (HPLC) (YMC pack ODS column; i.d., 10 × 250 mm; eluant, 0.08% aq. TFA/MeCN = 100:0 → 50:50 during 30 min; flow rate, 2 mL/min). The retention time of the separated peak was 14.9 min. The purified peptide was finally obtained after lyophilization. Yield: 49.9%. FAB MS calcd.: 1623 (MH^+), found: 1624.

Synthesis of MSH-C4A2 (Scheme 2). The peptide (α-MSH sequence) on the resin was reacted with **C4**: *viz*., the peptide on resin, **C4**, HOBt, and *N,N′*-diisopropylcarbodiimide (molar ratio 1:1:10:10) were mixed in *N*-methylpyrrolidione (NMP) and reacted for 12 h at 60°C. After filtering off the resin, washing it with *N*-methylpyrrolidione (2 mL × 10) and MeOH (2 mL × 10) and drying it for 10 min, TFA (2.5 mL), 1,2-ethanedithiol (62.5 μL), triisopropylsilane (62.5 μL), and H_2O (125 μL) were added to the resin and reacted for 5 h at room temperature in order to obtain the free lipopeptide. The resin was filtered off, and ethyl ether was added to the filtrate to obtain a white precipitate, which was then centrifuged (4°C, 3500 rpm, 20 min). After removing the supernatant, diethyl ether was added again, and the centrifugation was repeated. The white precipitate was dissolved in 20% acetate solution and purified by high-performance liquid chromatography (YMC pack C4; i.d., 10 × 250 mm; eluant:, 0.08% aq. TFA/MeCN = 100:0 → 50:50 during 30 min; flow rate, 2 mL/min). The retention time of the separated peak was 11.7–12.3 min. The final product was obtained after lyophilization. Yield: 12.6%. Time-of-flight MS calcd.: 2410 (MH^+ – 17), found: 2410.

Preparation of lipid thin film. (i) EPC. Solution **A** was prepared by dissolving EPC (18.35 mg) in $CHCl_3$ (5 mL). One hundred microliters of solution **A** was put into a 10-mL test tube. Lipid thin film was prepared by evaporating the solvent by a N_2 gas stream as the test tube was rotated. The



SCHEME 2

resultant EPC thin film was dried in a desiccator under vacuum for 2 h.

(ii) EPC/MSH-C4A2. Solution **B** was prepared by dissolving MSH-C4A2 (1.455 mg) in 20% acetic acid (250 µL). Solution **A** (100 µL) and solution **B** (10 µL or 20 µL) were put into a 10-mL test tube. After adding MeOH to prevent phase separation, lipid thin film was prepared in a similar manner as EPC lipid thin film. The film was then dried in a desiccator overnight, since it was somewhat difficult to remove the solvent when the synthetic lipids were contained. The molar ratios of EPC/MSH-C4A2 were 20:1 and 10:1, respectively.

(iii) EPC/Ch. Solution **C** was prepared by dissolving Ch (1.16 mg) in MeOH/$CHCl_3$ (20:80 vol/vol, 200 µL). Solution **A** (100 µL) and solution **C** (10 µL) were put into a 10-mL test tube. Lipid thin film was prepared in a similar manner, which was then dried in a desiccator for 2 h. The molar ratio of EPC/Ch was 10:3.

(iv) EPC/MSH-C4A2/Ch. Solution **A** (100 µL), solution **B** (20 µL) and solution **C** (10 µL) were put into a 10-mL test tube. MeOH was added to make the solution homogeneous. After lipid thin film was prepared in a similar manner, it was dried in a desiccator overnight. The molar ratio of EPC/MSH-C4A2/Ch was 10:1:3.

(v) EPC/PG. Solution **D** was prepared by dissolving PG (1.841 mg) in MeOH/$CHCl_3$ (20:80 vol/vol, 200 µL). Solution **A** (100 µL) and solution **D** (10 µL) were put into a 10-mL test tube. Lipid thin film was prepared in a similar manner and dried in a desiccator for 2 h. The molar ratio of EPC/PG was 10:3.

(vi) EPC/MSH-C4A2/PG. Solution **A** (100 µL), solution **B** (20 µL), and solution **D** (10 µL) were put into a 10-mL test tube. MeOH was added to make the solution homogeneous. Lipid thin film was prepared in a similar manner and dried in a desiccator overnight. The molar ratio of EPC/MSH-C4A2/PG was 10:1:3.

(vii) EPC/MSH-C4A2/Ch/PG. Solution **A** (100 µL), solution **B** (20 µL), solution **C** (10 µL), and solution **D** (10 µL) were put into a 10-mL test tube. MeOH was added to make the solution homogeneous. After lipid thin film was prepared in a similar manner, it was dried in a desiccator overnight. The molar ratio of EPC/MSH-C4A2/Ch/PG was 10:1:3:3.

(viii) EPC/C4A2. Solution **E** was prepared by dissolving C4A2 (1.4 mg) in MeOH/$CHCl_3$ (20:80 vol/vol, 3.4 mL). Solution **A** (100 µL) and solution **E** (100 µL) were put into a 10-mL test tube. Lipid thin film was prepared in a similar manner and dried in a desiccator overnight. The molar ratio of EPC/C4 was 10:1.

Preparation of liposomes. One milliter of calcein solution (20 mM, pH 7.15, containing 120 mM NaCl) was put into a test tube containing lipid thin film, and the solution was stirred for 5 min. After the freeze-thaw procedure (19) was repeated five times, liposomes with homogeneous size (<0.1 µm) were obtained by the extrusion method.

Measurement of calcein leakage from liposome. Stability of the liposomes was estimated by using calcein as a fluorescence probe in a manner similar to that of using carboxyfluorescein (20). Liposomes and calcein were separated by gel filtration (Sephadex G-75; i.d., 10 × 285 mm) eluted with HEPES buffer (10 mM HEPES, 150 mM NaCl, pH 7.2). Two milliters of liposome solution including calcein were obtained. After the liposome solution had been put in a water bath (37°C) for 5 min, 24 µL of the solution was transferred to a cuvette and diluted by HEPES buffer to the total volume of 2.4 mL. When calcein molecules are located inside liposomes, fluorescence is depressed by self-quenching of calcein. If calcein passes through liposome membranes and reaches the bulk phase, however, the fluorescence increases because the self-quenching disappears by dilution. The time course of calcein leakage from liposomes was measured on a fluorescence spectrometer (λ_{ex} = 490 nm, λ_{em} = 520 nm) (21). Calcein leakage (%) was calculated by Equation 1,

$$\text{leakage (\%)} = \frac{F(t) - F(0)}{F(\text{triton}) - F(0)} \times 100 \quad [1]$$

where $F(0)$, $F(t)$, and F(triton) are the fluorescence intensity at time 0, t, and after collapsing liposomes by triton X-100 (final concentration was 0.3%), respectively. Here it was assumed that after adding triton X-100 liposomes were completely collapsed and all calcein molecules were in the bulk phase. In the measurement using α-MSH peptide as a free form, 1 mL of α-MSH solution (Hank's buffer) was added to the calcein-included liposome solution, whose final molar ratio was 10:1 (EPC/MSH).

Calculation of captured volume ratio. Captured volume ratio, which is the quantity giving the relative size of liposomes, was calculated by Equation 2,

$$\text{captured vol ratio (\%)} = \frac{F(\text{triton:MSH-C4A2 / EPC})}{F(\text{triton:EPC})} \times 100 \quad [2]$$

where F(triton:MSH-C4A2/EPC) and F(triton:EPC) are the fluorescence intensities, respectively, in MSH-C4A2/EPC liposome and in EPC liposome after addition of triton X-100.

RESULTS

Calcein leakage from liposome. The degree of calcein leakage through liposome membranes was used as an index of the stability of liposomes perturbed by lipopeptide MSH-C4A2. Figure 1 shows the time course of calcein leakage in different concentrations of MSH-C4A2. It is apparent that MSH-C4A2 makes liposome unstable; that is, calcein leakage was increased by 2.9 times for 5 mol% MSH-C4A2 and by 3.8 times for 10 mol% MSH-C4A2 compared to the control experiment. In order to determine which part of the MSH-C4A2 molecule primarily contributes to the instability of liposome, we compared calcein leakage among liposomes of MSH-C4A2 free (control), 10 mol% MSH-C4A2, 10 mol% MSH, and 10 mol% C4A2 (Fig. 2). α-MSH and C4A2 were used to estimate the individual contribution from the peptide part and the lipid part. As mentioned already MSH-C4A2 increased the calcein leakage from 1.6 to 6.3%. Similarly to MSH-C4A2, free MSH obviously made liposomes unstable,

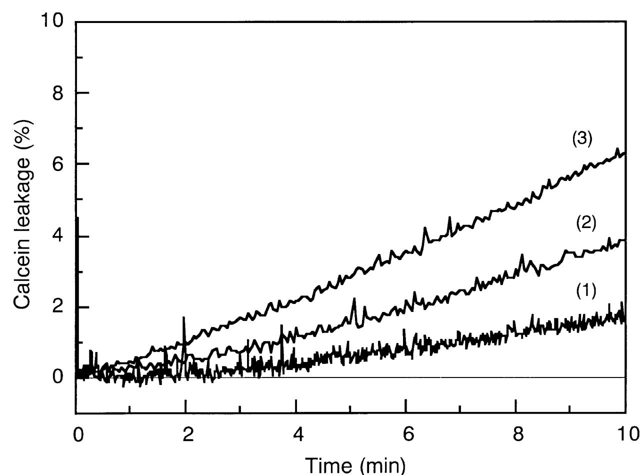


FIG. 1. Time course of calcein leakage from liposomes containing (1) 0% MSH-C4A2 (control), (2) 5 mol% MSH-C4A2, and (3) 10 mol% MSH-C4A2. MSH-C4A2, melanocyte-stimulating hormone .

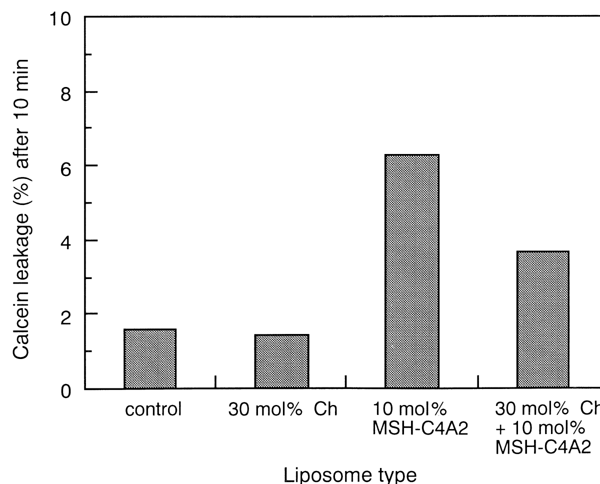


FIG. 3. Effect of cholesterol (Ch) on calcein leakage from MSH-C4A2/EPC liposome; control liposome is composed of only EPC. For abbreviations see Figures 1 and 2.

since calcein leakage increased from 1.6 to 7.7%. Contrary to MSH-C4A2 and MSH, C4A2, which corresponds to the lipid skeleton of MSH-C4A2, had little effect on liposome stability (*viz*., calcein leakage increased by only 0.4%).

Effect of Ch and PG. Some kinds of lipids like Ch and PG are known to have special functions to stabilize lipid membranes (22,23). To recover the stability of MSH-C4A2-liposome, we added Ch and/or PG to liposome membranes. In Figure 3 the calcein leakage after 10 min is compared in four systems: control, Ch (30 mol%), MSH-C4A2 (10 mol%), and Ch (30 mol%) + MSH-C4A2 (10 mol%). When Ch alone was present in liposome membrane, the stability increased a little. When MSH-C4A2 alone was present, the stability was much reduced (*viz*., calcein leakage increased). It should be noted, however, that the stability recovered considerably (*viz*., the calcein leakage was reduced by 42%) when both Ch and MSH-C4A2 were introduced to liposome membranes. Another promising stabilizer is PG (24), whose effect on calcein leakage is shown in Figure 4. Although PG (30 mol%) did not have a large effect on the control liposome, it largely stabilized MSH-C4A2 (10 mol%) liposome (*viz*., calcein leakage was reduced by 37%). It was expected then that addition of both Ch and PG should reduce calcein leakage to a greater extent than when either of them was present individually. Contrary to our expectation, however, calcein leakage was at a level similar to that of the Ch or PG system (Fig. 5).

Captured volume ratio. Captured volume ratio was obtained from the measurement of calcein leakage (data not shown). The ratio is reduced to 72 and 32% as the content of MSH-C4A2 increases to 5 and 10 mol%, respectively, which means that MSH-C4A2 inhibits liposome formation or reduces the liposome size (7–9).

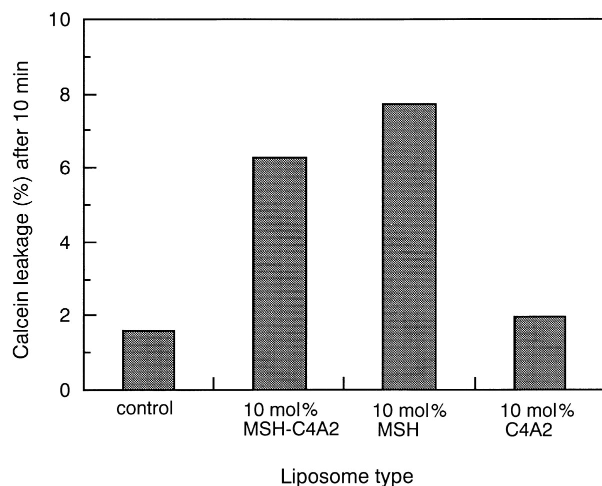


FIG. 2. Effect of MSH peptide ligand moiety and lipid skeleton part on calcein leakage from MSH-C4A2/EPC liposome; control liposome is composed of only EPC. EPC, egg phosphatidylcholine hydrogenated; for other abbreviations see Figure 1.

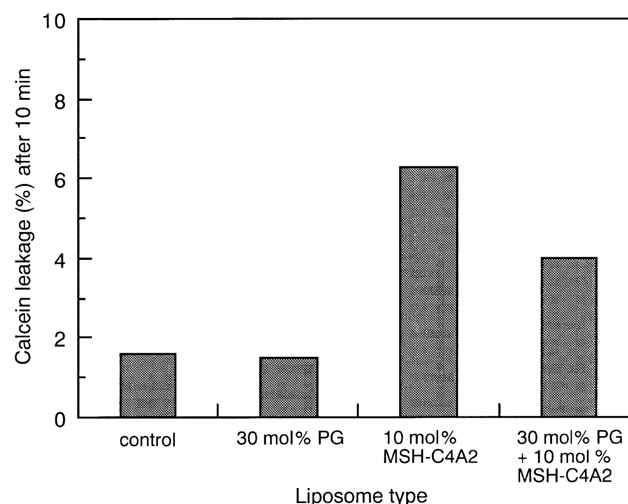


FIG. 4. Effect of phosphatidylglycerol (PG) on calcein leakage from MSH-C4A2/EPC liposome; control liposome is composed of only EPC. For abbreviations see Figures 1 and 2.

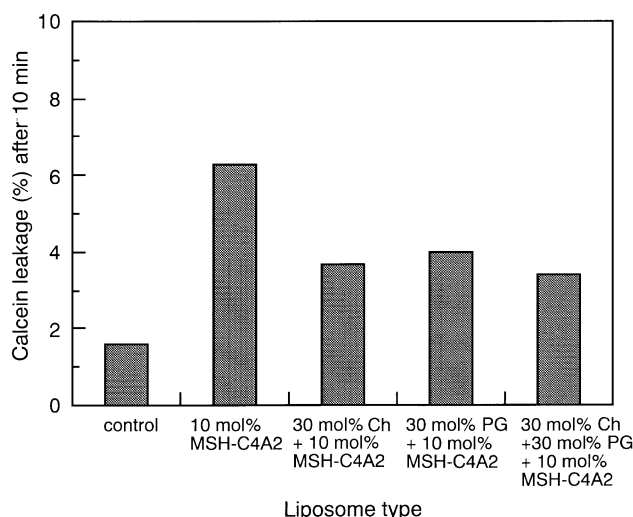


FIG. 5. Effect of coexistence of Ch and PG on calcein leakage from MSH-C4A2/EPC liposome; control liposome is composed of only EPC. For abbreviations see Figures 1–4.

DISCUSSION

The results of calcein leakage shown in Figure 1 suggest that the peptide ligand (α-MSH sequence) of MSH-C4A2 greatly influences the stability of liposome. It is predicted from the amino acid sequence that α-MSH should have an amphiphilic property. Amphiphilic peptides generally have a tendency to make liposome unstable (25). As free α-MSH peptide probably forms an α-helix, it may act as an amphiphilic reagent with its hydrophobic side being embedded in liposome membranes. With respect to the effect of charge of the membrane components on liposomes, the expected charges in the neutral pH region are +2 for α-MSH, −2 for calcein, −1 for PG, −2 for C4A2, +2 for MSH-C4A2, and 0 for EPC. Since the degree of the calcein leakage was similar in the control experiment (Fig. 2), 10 mol% C4A2 (Fig. 2), 30 mol% Ch (Fig. 3), and 30 mol% PG (Fig. 4), we concluded that the anionic charge does not affect liposome stability.

One Ch molecule and two phospholipid molecules can form a cluster in lipid membranes in the range of 30–50 mol% of Ch, and thus the membranes become more stable (26,27). The stabilization of MSH-C4A2 liposome by 30 mol% Ch observed in Figure 3 must be accounted for by the foregoing mechanism. Although the detailed mechanism of the stabilization by PG is unknown, there may be some electrostatic repulsion between calcein with a −2 charge and PG with a −1 charge, leading to the suppression of calcein leakage from liposomes. The effects of both Ch and PG were much smaller when they were present in liposome without MSH-C4A2 (*viz*., control liposome). Probably the membrane of the control liposome was stable enough even if Ch and PG were not present, so the effect of Ch and PG was not noticeable.

The decrease in the captured volume ratio may be due to the inhibition of liposome formation by MSH-C4A2. Since MSH-C4A2 is a lipid bearing a peptide moiety, its properties related to molecular size and hydrophilic-hydrophobic balance must be different from those of EPC. MSH-C4A2 may affect the orientation of EPC by intermolecular interactions such as steric hindrance. Since liposome with larger captured volume is capable of holding more bioactive compounds in its inner phase, such a liposome is appropriate for DDS. As captured volume primarily depends on the method of preparing liposome (9,19), the freeze-thaw method used here seems to be the best in terms of the efficiency of capturing bioactive compounds.

We have designed and synthesized the novel lipopeptide MSH-C4A2. Although the liposome composed of EPC is not so stable in the presence of MSH-C4A2, its stability is largely restored by addition of the third component, Ch or PG. The test of whether the liposome containing MSH-C4A2 can bind to melanoma cells will be performed by flow cytometry.

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Anomalous Enantioselectivity in the Sharpless Asymmetric Dihydroxylation Reaction of 24-Nor-5β-cholest-23-ene-3α,7α,12α-triol: Synthesis of Substrates for Studies of Cholesterol Side-Chain Oxidation[1]

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ABSTRACT: Recently we described a block in bile acid synthesis in cerebrotendinous xanthomatosis (CTX), a lipid storage disease related to an inborn error of bile acid metabolism. In this disease a defect in hepatic microsomal (24*S*) hydroxylation blocks the transformation of 5β-cholestane-3α,7α,12α,25-tetrol into (24*S*) 5β-cholestane-3α,7α,12α,24,25-pentol and cholic acid. Mitochondrial cholesterol 27-hydroxylation has also been reported to be abnormal in CTX subjects, but the relative importance of the enzymatic defect in this alternative microsomal pathway (namely, the 24*S* hydroxylation of 5β-cholestane-3α,7α,12α,25-tetrol relative to the abnormality in mitochondrial 27-hydroxylase) has not been established in CTX. To delineate the sequence of side-chain hydroxylations and the enzymatic block in bile acid synthesis, we synthesized the (23*R* and 23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols utilizing a modified Sharpless asymmetric dihydroxylation reaction on 24-nor-5β-cholest-23-ene-3α,7α,12α-triol, a C_{26} analog of the naturally occurring C_{27} bile alcohol, 5β-cholest-24-ene-3α,7α,12α-triol. Stereospecific conversion of the unsaturated 24-nor triol to the corresponding chiral compounds (23*R* and 23*S*), 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols, was quantitative. However, conversion of the unsaturated 24-nor triol to the chiral nor-pentols had absolute stereochemistry opposite to the products predicted by the Sharpless steric model. The absolute configurations and enantiomeric excess of the C_{26} nor-pentols and the C_{27} pentols (synthesized from 5β-cholest-24-ene-3α,7α,12α-triol for comparison) were confirmed by nuclear magnetic resonance and lanthanide-induced circular dichroism Cotton effect measurements. These results may contribute to a better understanding of the role of the 24*S*-hydroxylation vs. 27-hydroxylation step in cholic acid biosynthesis.

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In normal human physiology, biosynthesis of the primary bile acids, cholic and chenodeoxycholic acids, from cholesterol involves conversion of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α-diol, respectively, to 27-hydroxylated products (1–3). This reaction is catalyzed by a mitochondrial cytochrome P_{450} enzyme, 27-hydroxylase (previously termed “26-hydroxylase”) (4,5). An alternative biosynthetic pathway which was discovered in our laboratory and which does not involve mitochondrial or peroxisomal enzymes proceeds *via* microsomal 25-hydroxylation of 5β-cholestane-3α,7α,12α-

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Abbreviations: AD, asymmetric dihydroxylation; CD, circular dichroism; CTX, cerebrotendinous xanthomatosis; DEPT, distortionless enhancement polarization transfer; EI–MS, electron ionization mass spectra; $Eu(fod)_3$, tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octane-4,6-dionato)europium(III); FAB–MS, fast atom bombardment–mass spectrometry; GLC, gas–liquid chromatography; NMR, nuclear magnetic resonance; PHAL, phthalazine; TLC, thin-layer chromatography.

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triol to give 5β-cholestane-3α,7α,12α,25-tetrol (6) followed by stereospecific 24*S*-hydroxylation to yield 5β-cholestane-3α,7α,12α,24*S*,25-pentol. The pentol is then oxidized to 3α,7α,12α-trihydroxy-5β-cholestan-24-one, which is degraded by cytosolic enzymes to give cholic acid and acetone (7–9). Although this "alternative" 25-hydroxylation pathway is responsible for less than 5% of the cholic acid produced in normal individuals, it becomes a major pathway in cerebrotendinous xanthomatosis (CTX), an autosomal recessive inborn error of bile acid metabolism (7,10,11). CTX is characterized by the accumulation of the cholesterol metabolite, 5α-cholestanol, in various tissues including the nervous system, arteries, and tendons, leading to the diverse clinical manifestations of this disease (7–12).

Further studies by Skrede *et al.* (13) and Cali and Russel (4) demonstrated that a major enzymatic defect in CTX is the presence of an abnormal sterol 27-hydroxylase (EC 1.14.13.15). The molecular genetics in North African Jewish families exhibiting CTX indicated three separate gene mutations, of which the most recently described is a missense mutation that results in a methionine for threonine substitution (15–17).

To investigate further the sequence of side-chain hydroxylations and the quantitative importance of this metabolic defect in CTX, we developed methods for efficient and stereospecific large-scale syntheses of (23*R* and 23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols as model substrates. We utilized a nonenzymatic method for enantioselective hydroxylations recently developed by Sharpless *et al.* (17–19) utilizing a modified phthalazine (PHAL) class of cinchona alkaloid-based chiral ligands. In this report, we describe methods for synthesis, purification, and identification of these isomeric (23*R* and 23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols. These experiments will be important not only in yielding stereoisomeric compounds of interest in our current work but also in serving as model substrates for the synthesis of other isomers of potentially important biologic interest.

EXPERIMENTAL PROCEDURES AND RESULTS

Melting points. Melting points were determined on a model MP-12600 Thermolyne apparatus (Thermolyne Corp., Dubuque, IA) and are uncorrected.

Thin-layer chromatography (TLC). All tri- and tetrahydroxy bile alcohols and their corresponding isomers were separated on silica gel G plates (Uniplates; Analtech, Newark, NJ, 0.25-mm thickness) in the solvent system $CHCl_3/(CH_3)_2CO/CH_3OH$, 70:50:10 (by vol). For pentahydroxy sterols and their corresponding isomeric analogs, the solvent system $CHCl_3/(CH_3)_2CO/CH_3OH$, 70:50:15 (by vol) was used (9,20,21). The spots were detected by spraying the plates with phosphomolybdic acid (3.5% in isopropanol), sulfuric acid (10%), and heating for 1 min at 110°C.

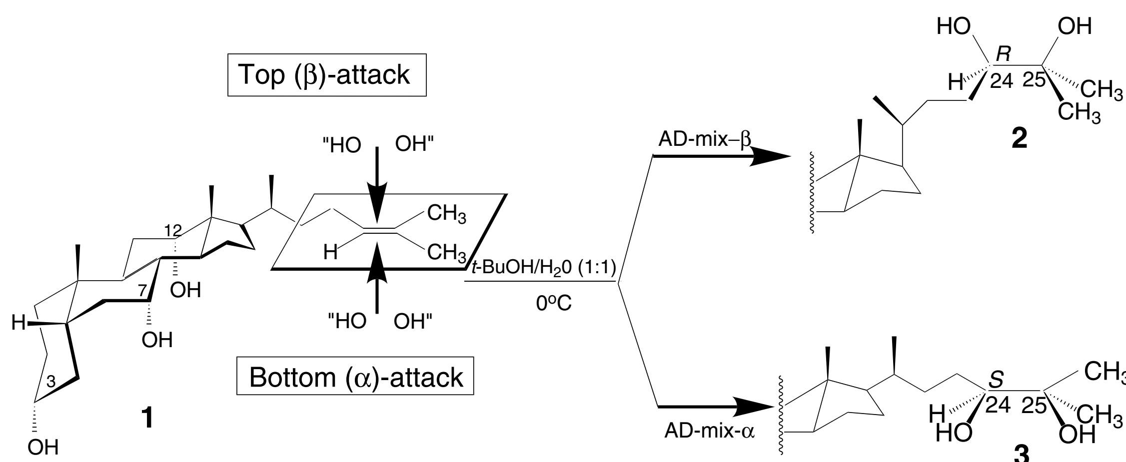
(^1H and ^{13}C) Nuclear magnetic resonance (NMR) spectrometry. NMR spectra (^1H and ^{13}C) were recorded on a Varian XL-400 (400 MHz) spectrometer equipped with Fourier transform mode. All NMR spectra were taken in (CD_3OD) solution or $CDCl_3$with Me_4Si as the internal standard. In addition, the nature of each carbon in 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols was deduced through distortionless enhancement by polarization transfer (DEPT) spectra (in $CDCl_3$) performed by using a polarization pulse of 90 and 135°, respectively, obtaining in the first case only signals for CH groups, and in the second case positive signals for CH and CH_3 and negative signals for CH_2 groups. All these data were recorded on an XL-400 spectrometer at 100 MHz for ^{13}C nuclei (9,20–23).

Optical measurements. Circular dichroism (CD) measurements were carried out on a Jasco-500A spectropolarimeter at 24°C, under a stream of high-purity dry N_2. The coefficient of dichroic absorption, $\Delta\varepsilon$, was calculated from the molar ellipticity (θ) by the following equation: molar ellipticity (θ) = 3300$\Delta\varepsilon$. Both molar ellipticity (θ) and $\Delta\varepsilon$ are expressed in degree $\times$ $cm^2 \times dmol^{-1}$ (9,24–26).

Positive ion fast atom bombardment–mass spectrometry (FAB–MS). Direct probe mass spectrometric analysis of the C_{26} bile alcohols (24-nor-5β-cholestane-3α,7α,12α,23,25-pentols) and the C_{27} series of bile alcohols (25-tetrol/pentol) was performed by FAB–MS using a ZAB-IF mass spectrometer (VG, Manchester, United Kingdom) equipped with a standard FAB source as described previously (9,27,28). The FAB technique has emerged as a simple and extremely versatile ionization process that is particularly well suited to biomedical applications. Although negative ion FAB–MS is much more satisfactory than the positive ion mode, this was not available at that time. In the FAB mode (positive ions) the sample was dissolved in thioglycerol, and a small aliquot (1–2 μL) placed on the mass spectrometer direct probe. After insertion into the mass spectrometer, the sample was bombarded with a neutral atom beam of xenon having approximately 6 kV of translational energy.

Electron ionization mass spectra (EI–MS). EI–MS of the bile alcohols were obtained with a Varian MAT-5 and Varian MAT-III gas chromatograph–mass spectrometer as described previously (29).

Gas–liquid chromatography (GLC). Capillary GLC analysis of bile alcohols (as their trimethylsilyl derivatives) was performed on Hewlett-Packard model No. 4890 (equipped with a flame-ionization detector) and a split column injector using a CPsil 5 (CB) WCOT capillary column (25 m × 0.22 mm with 0.13-mm film thickness) (23). Helium was used as a carrier gas at a flow rate of 20.2 mL/min (135 kPa).



SCHEME 1

Preparation of asymmetric dihydroxylation (AD)-mix-α and AD-mix-β. This procedure has been adequately described by Sharpless and associates (17–20,30–42). AD-mix-β consists of a mixture of potassium ferricyanide, potassium carbonate, and a catalytic quantity of potassium osmate blended with the chiral dihydroquinidine ligand, $(DHQD)_2$-PHAL and AD-mix-α has the chiral dihydroquinine-derived ligand, $(DHQ)_2$-PHAL.

*(i) Synthesis of 5β-cholestane-3α,7α,12α, 24*S *(or 24β),25-pentol (Scheme 1, compound* **3***)* via *asymmetric dihydroxylation of 5β-cholest-Δ^{24}-3α,7α,12α-triol (Scheme 1, compound* **1***).* To a well-stirred mixture of AD-mix-α (1.8 g) in 20 mL of 1:1 *tert*-butanol/water (10 mL each) and $MeSO_2NH_2$ (0.19 g, 2 mmol) at 0°C was added 5β-cholest-Δ^{24}-3α,7α,12α-triol (0.509 g, 1.22 mmol) (20,29,43). The mixture was stirred at 0°C overnight, then 6 g of solid Na_2SO_3 was added and stirred at room temperature for 1 h. The *tert*-butanol layer was separated, and the aqueous layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water and saturated NaCl solution, dried over Na_2SO_4, concentrated under a stream of N_2 at room temperature, and the residue subjected to flash column chromatography (EtOAc/$CHCl_3$, 90:10 vol/vol) to give pure 5β-cholestane-3α,7α,12α, 24*S* (or 24β),25-pentol (**3**, Scheme 1) (455 mg, 89%). ^{1}H NMR (400 MHz, $CDCl_3$ + CD_3OD): δ 0.70 (*s*, 18-CH_3), 0.90 (*s*, 19-CH_3), 1.0 (*d*, 21-CH_3, 6 Hz), 1.2/1.17 (26 CH_3 + 27 CH_3), 3.32 (*dd*, 24-H, 10 Hz), 3.45 (*m*, H-3), 3.86 (*br, s*, H-7), 4.0 (*br, s*, H-12) (Table 1). GLC: relative retention times (24*S*) pentol, 2.70 min, and retention time of 5α-cholestane, 12.90 min. The CD spectrum of the 24*S*,25-pentol in the presence of $Eu(fod)_3$ showed $\Delta\varepsilon_{307}$ = +9.4 degrees × cm^2 × $dmol^{-1}$ (first Cotton effect) and $\Delta\varepsilon_{284}$ = −5.9 degrees × cm^2 × $dmol^{-1}$ (second Cotton effect) (20,44,45). These positive Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of the two hydroxyl groups (20,44,45) having a 1,2-glycol system in the side chain, and thus the chirality at C-24 hydroxyl group in this pentol was assigned as *S* (20,21).

By using a similar protocol, 5β-cholestane-3α,7α,12α, 24*R* (or 24α),25-pentol (Scheme 1, compound **2**) was synthesized from AD-mix-β: ^{1}H NMR for 24*R*,25-pentol (400 MHz, $CDCl_3$ + CD_3OD): δ 0.70 (*s*, 18-CH_3), 0.90 (*s*, 19-CH_3), 1.0 (*d*, 21-CH_3, 6 Hz), 1.2/1.17 (26-CH_3 + 27-CH_3), 3.25 (*t*, 24*H*), 3.41 (*m*, H-3), 3.83 (*s*, H-7), 4.0 (*br, s*, H-12) (Table 1). GLC: relative retention time (24*R*) pentol, 2.66

TABLE 1
^{1}H- and ^{13}C Nuclear Magnetic Resonance Data for (24*R*) and (24*S*) Pentols and (23*R* and 23*S*) 24-Nor-5β-cholestane-3α,7α,12α,23,25-pentols (in CD_3OD and $CDCl_3$ + CD_3OD solution)

Types	^{1}H	^{13}C
5β-Cholestane-3α,7α,12α,24*R*,25-pentol	3.4 (*t*) C-*24H*	78.6
5β-Cholestane-3α,7α,12α,24*S*,25-pentol	3.2 (*d*) C-*24H*	79.6
(23*R*) 24-Nor-5β-cholestane-3α,7α,12α,23,25-pentol	3.45 (*d*) C-*23H*	73.9
(23*S*) 24-Nor-5β-cholestane-3α,7α,12α,23,25-pentol	3.48 (*d*) C-*23H*	73.9

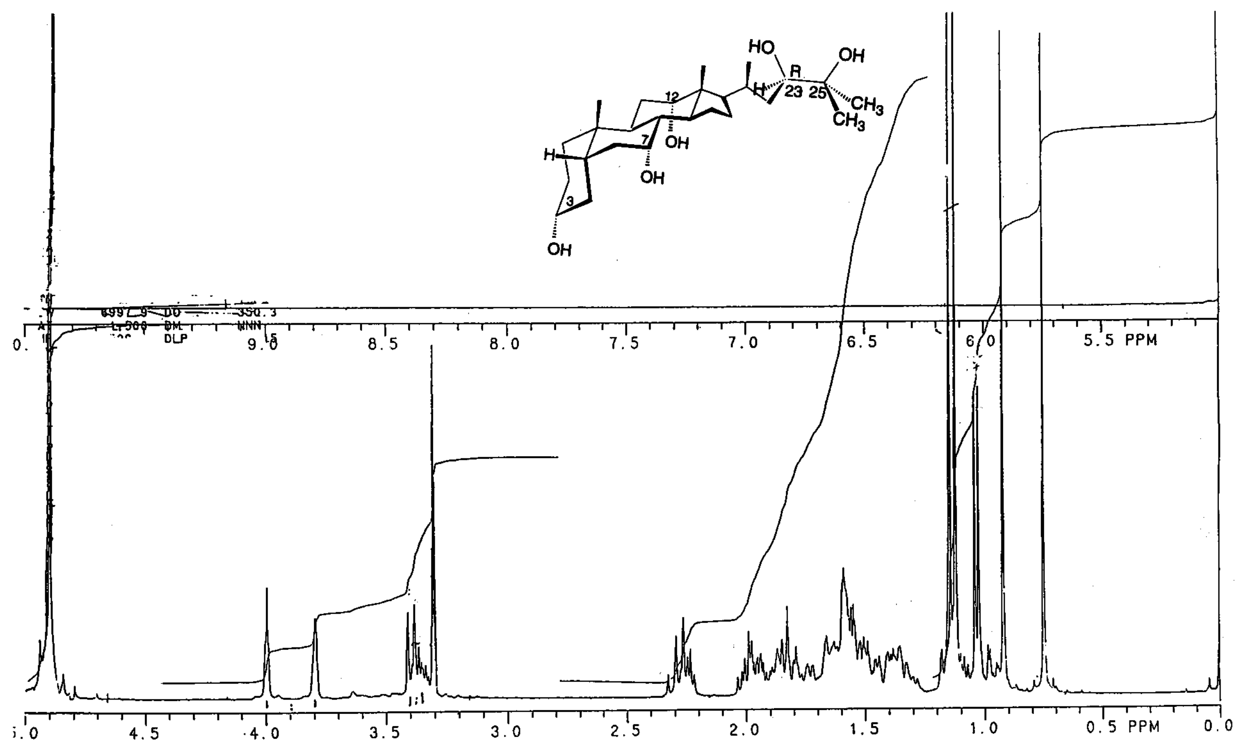


FIG. 1. [1]H nuclear magnetic resonance (NMR) spectrum of 24-nor-5β-cholestane-3α,7α,12α-23*R*,25-pentol.

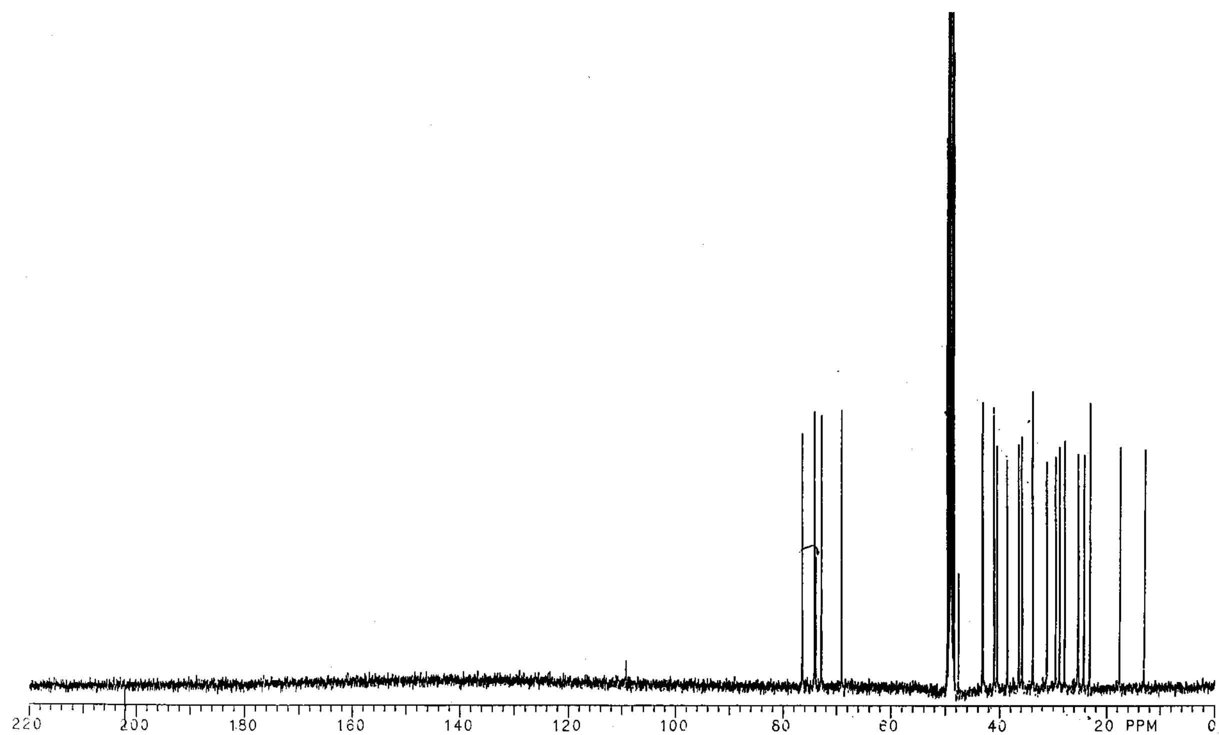
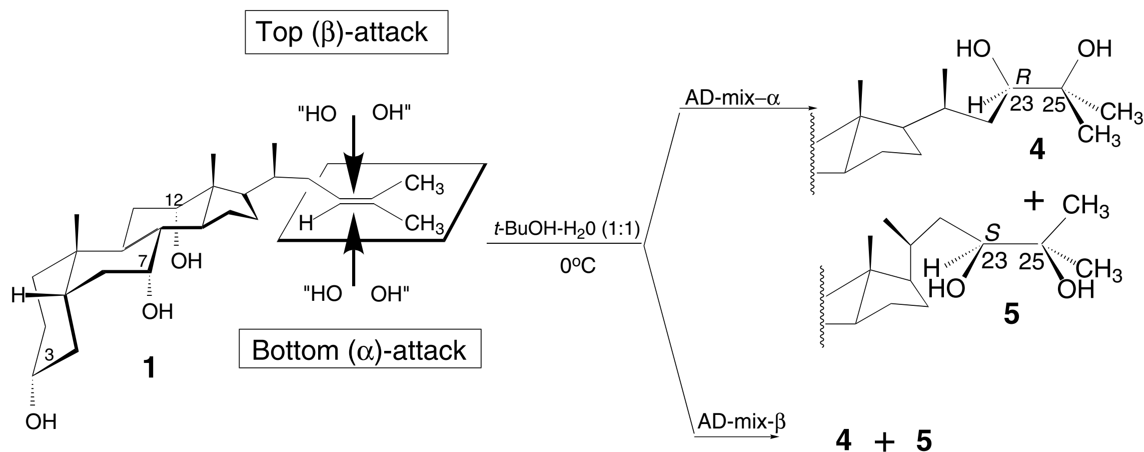


FIG. 2. ^{13}C NMR ([1]H-decoupled) spectrum of 24-nor-5β-cholestane-3α,7α,12α,23*R*,25-pentol. See Figure 1 for abbreviation.



SCHEME 2

min, and retention time of 5α-cholestane 12.90 min. The CD spectrum of the 24*R*,25-pentol in the presence of Eu(fod)$_3$ showed $\Delta\varepsilon_{307} = -5.83$ degrees × cm^2 × dmol^{-1} (first Cotton effect) and $\Delta\varepsilon_{284} = +409$ degrees × cm^2 × dmol^{-1} (second Cotton effect) (20,44). These negative Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of the two hydroxyl groups having a 1,2-glycol system in the side chain and thus the chirality at the C-24 hydroxyl group in this pentol was assigned as *R* as described previously (20,21).

The FAB mass spectra of both *R* and *S* isomers provided protonated and sodium-adduct molecular ions at *m/z* 453 and 475, respectively, and a weak signal at *m/z* 905 which corresponded to $[2M + H]^+$. In addition, the mass spectra displayed a number of fragment ions representing successive losses of

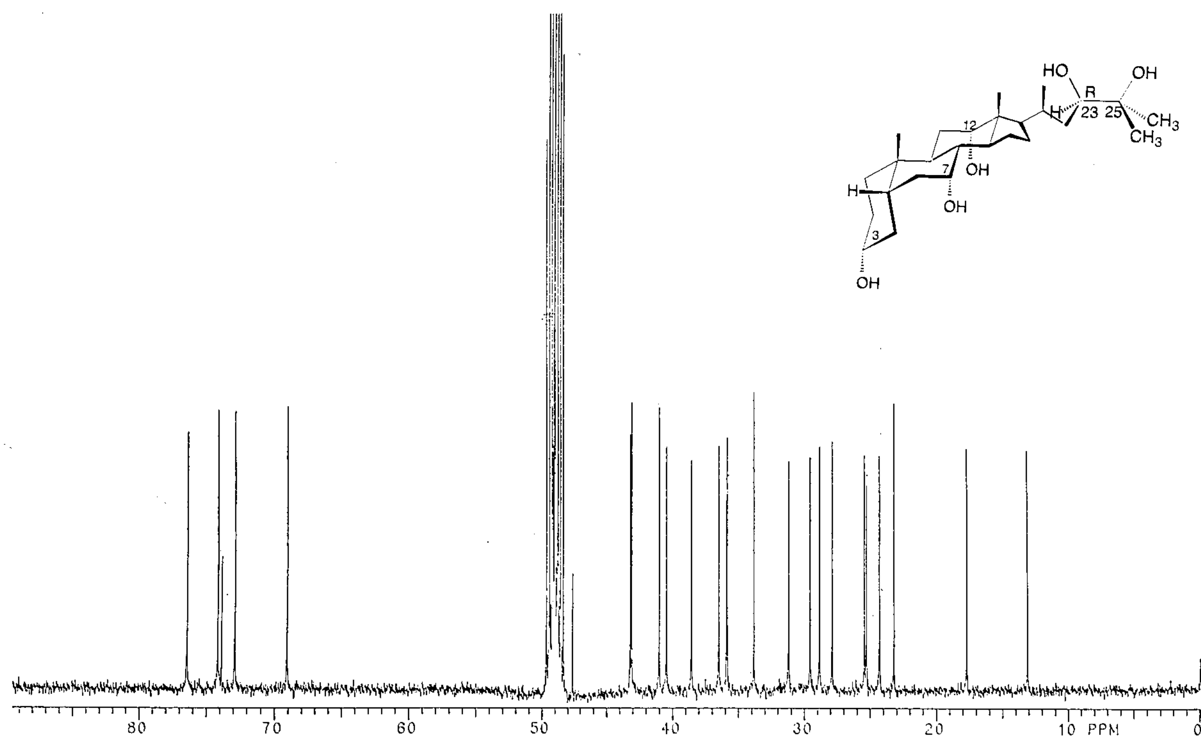


FIG. 3. ^{13}C NMR [^{1}H-decoupled, expanded version of Figure 2 distortionless enhancement polarization transfer (DEPT)] spectrum of 24-nor-5β-cholestane-3α,7α,12α-23*R*,25-pentol. See Figure 1 for abbreviation.

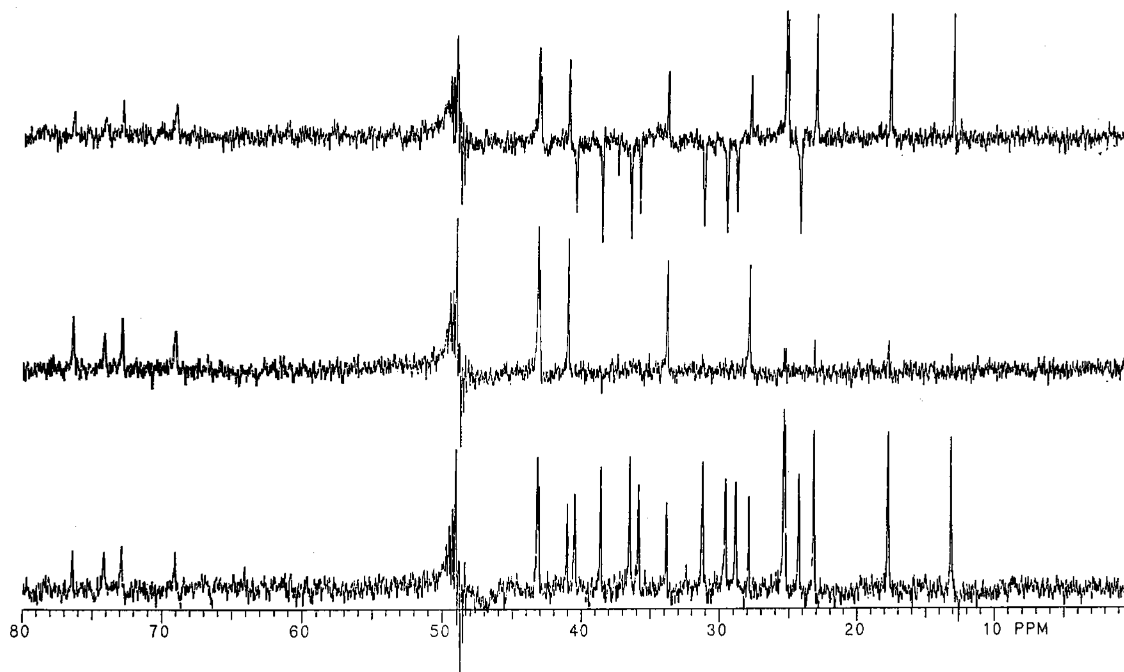


FIG. 4. ^{13}C NMR (^{1}H-decoupled, DEPT) spectrum of 24-nor-5β-cholestane-3α,7α,12α-23*R*,25-pentol. See Figures 1 and 3 for abbreviations.

H_2O molecules from the protonated molecular ions at *m/z* 435 $[M + H - H_2O]^+$, 417 $[M + H - 2H_2O]^+$, 399 $[M + H - 3H_2O]^+$, 381 $[M + H - 4H_2O]^+$, and 363 $[M + H - 5H_2O]^+$.

*(ii) Synthesis of 24-nor-5β-cholestane-3α,7α,12α, 23*R*,25-pentol (Scheme 2, compound* **4***)* via *asymmetric dihydroxylation of 24-nor-5β-cholest-Δ^{23}-3α,7α,12α-triol (Scheme 2, compound* **1***).* To a well-stirred mixture of AD-mix-α (450 mg) in 10 mL of 1:1 *tert*-butanol/water (5 mL each) and $MeSO_2NH_2$ (0.19 g, 2 mmol) at 0°C was added 24-nor-5β-cholest-Δ^{23}-3α,7α,12α-triol (100.0 mg, 0.25 mmol); TLC, $R_f = 0.69$, solvent system chloroform/acetone/methanol, 70:50:15 (by vol) (46,47). The mixture was stirred at 0°C overnight, then 0.5 g of solid Na_2SO_3 was added and stirred at room temperature for 1 h. The *tert*-butanol layer was separated, and the aqueous layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, concentrated, and the residue subjected to flash column chromatography (ethyl acetate/chloroform, 90:10, vol/vol) to give pure 24-nor-5β-cholestane-3α,7α,12α, 23*R*,25-pentol (**4**, Scheme 2) (55.0 mg, 55%). TLC: $R_f = 0.32$ (upper spot, solvent system: chloroform/acetone/methanol, 70:50:15, by vol). ^{1}H NMR, 23,25-pentol (400 MHz, CD_3OD): δ 0.75 (*s*, 18-CH_3), 0.92 (*s*, 19-CH_3), 1.03 (*d*, 21-CH_3, 6 Hz), 1.12/1.14 (26-CH_3 + 27-CH_3), 3.45 (*dd*, 10 Hz, 23-H), 3.32 (*m*, H-3), 3.80 (*br, s*, H-7), 4.0 (*br, s*, H-12) (Fig. 1, Table 1). ^{13}C NMR (^{1}H-decoupled spectrum, Fig. 2; ^{1}H-decoupled spectrum, expanded version, Fig. 3; and ^{1}H-decoupled, DEPT spectrum, Fig. 4).

The CD spectrum of the 24-nor-5β-cholestane-23,25-pentol in the presence of $Eu(fod)_3$ showed $\Delta\varepsilon_{308} = -8.48$ degrees × cm^2 × $dmol^{-1}$ (first Cotton effect) and $\Delta\varepsilon_{284} = +5.90$ degrees × cm^2 × $dmol^{-1}$ (second Cotton effect) (Fig. 5). These negative Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of the two hydroxyl groups (20,44,45) having a 1,2-glycol system in the side chain and thus the chirality at the C-23 hydroxyl group in 24-nor-5β-cholestane3α,7α,12α,23,25-pentol (Scheme 2, compound **4**) was assigned as *R* (Fig. 5).

Further elution of the flash chromatography column with ethyl acetate/chloroform (90:10, vol/vol) gave pure 24-nor-5β-cholestane-23,25-pentol (Scheme 2, compound **5**, lower isomer, 35 mg). TLC analysis: $R_f = 0.24$ (solvent system: chloroform/acetone/methanol, 70:50:15, by vol). ^{1}H NMR showed an isomer of 23,25-pentol (400 MHz, CD_3OD): δ 0.75 (*s*, 18-CH_3), 0.92 (*s*, 19-CH_3), 1.03 (*d*, 21-CH_3, 6 Hz), 1.12/1.14 (26-CH_3 + 27-CH_3), 3.45 (*d*, 23-H), 3.35 (*m*, H-3), 3.80 (*s*, H-7), 4.0 (*br, s*, H-12) (Table 1). The CD spectrum of this 23,25-pentol in the presence of $Eu(fod)_3$ showed $\Delta\varepsilon_{307} = +2.73$ degrees × cm^2 × $dmol^{-1}$ (first Cotton effect) and $\Delta\varepsilon_{285} = -1.36$ degrees × cm^2 × $dmol^{-1}$ (second Cotton effect) (20). These positive Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of

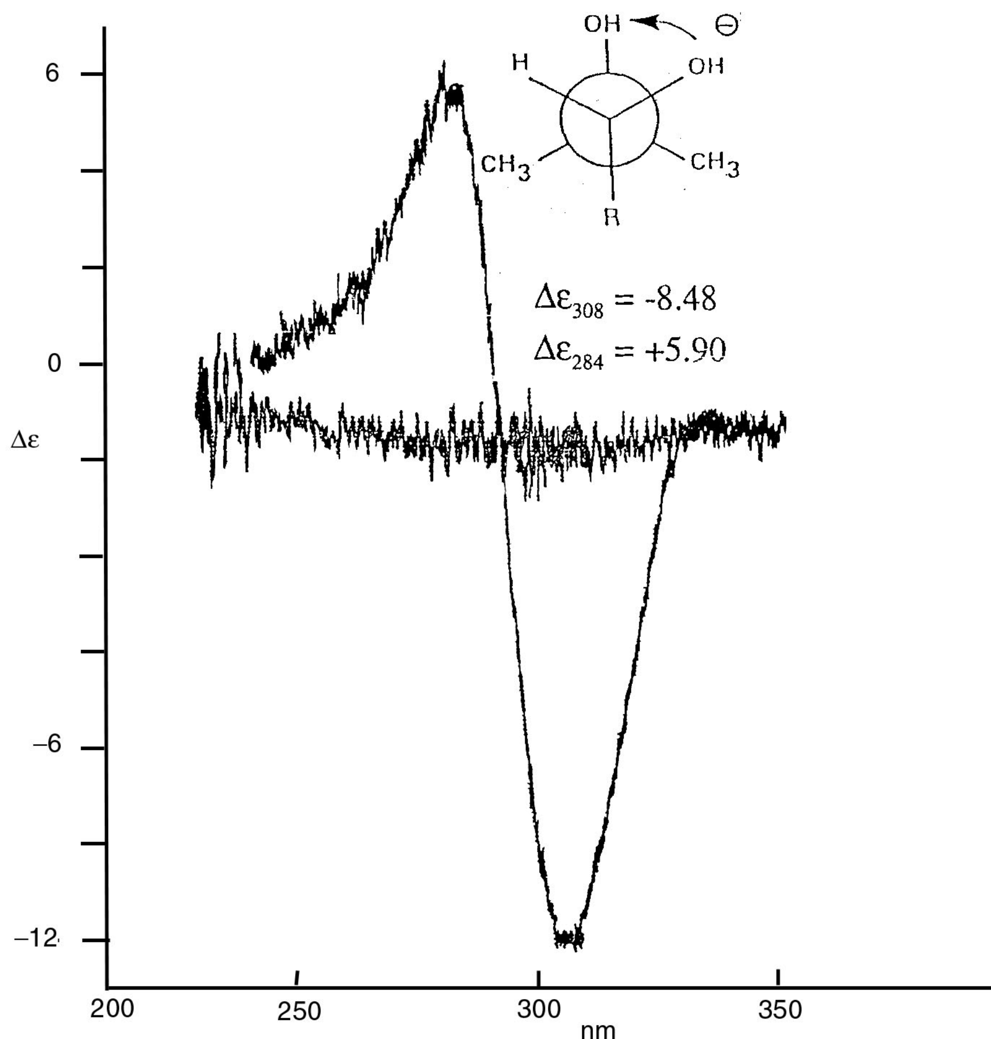


FIG. 5. Circular dichroism of 2×10^{-4} M 24-nor-5β-cholestane-3α,7α,12α-23*R*,25-pentol and 2×10^{-4} M tris-1,1,1,2,2,3,3-heptafluoro-7,7-dimethyl-octane-4,6-dionato) europium(III) in dry $CHCl_3$ at ambient temperature within 30 min after mixing.

the two hydroxyl groups having a 1,2-glycol system in the side chain and thus the chirality at the C-23 hydroxyl group in this pentol was assigned as *S* (44,45). The isomeric mixture in 24-nor-5β-cholestane-3α,7α,12α,23 (*R* and *S*),25-pentols was present in the ratio of (55:35, *R/S*).

The FAB mass spectra of both *R* and *S* isomers provided protonated and sodium-adduct molecular ions at *m/z* 439 and 461, respectively, and a weak signal at *m/z* 877 which corresponded to $[2M + H]^+$. In addition, the mass spectra displayed a number of fragment ions representing successive losses of H_2O molecules from the protonated molecular ions at *m/z* 421 $[M + H - H_2O]^+$, 403 $[M + H - 2H_2O]^+$, 385 $[M + H - 3H_2O]^+$, 367 $[M + H - 4H_2O]^+$, and 349 $[M + H - 5H_2O]^+$.

EI–MS displayed a number of fragment ions representing successive losses of H_2O and CH_3 molecules from the parent molecular ions at *m/z* 402 $[M - 2H_2O]^+$, 387 $[M - 2H_2O - CH_3]^+$, 384 $[M - 3H_2O]^+$, 271 $[M - 2H_2O - \text{side chain}]^+$, and 253 $[M - \text{side chain} - 3H_2O]^+$.

*(iii) Synthesis of (23*R *and 23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols (Scheme 2, compounds* **4** *and* **5***)* via *asymmetric dihydroxylation of 24-nor-5β-cholest-Δ*^{23}*-3α,7α,12α-triol (Scheme 2, compound* **1***).* To a well-stirred mixture of AD-mix-β (450 mg) in 10 mL of 1:1 *tert*-butanol/water (5 mL each) plus $MeSO_2NH_2$ (0.19 g, 2 mmol) at 0°C was added 24-nor-5β-cholest-Δ^{23}-3α,7α,12α-triol (100.0 mg, 0.25 mmol) (46,47). The mixture was stirred at 0°C overnight, then 0.5 g of solid Na_2SO_3 was added and stirred at room temperature for 1 h. The *tert*-butanol layer was separated, and the aqueous layer was extracted with EtOAc (3 × 25 mL). The combined organic layers were washed with water and brine, dried over Na_2SO_4, concentrated, and the residue subjected to flash column chromatography (ethyl ac-

etate/chloroform, 90:10) to give pure 24-nor-5β-cholestane-23*R*,25-pentol (80.0 mg, 80%). TLC: $R_f = 0.31$ (upper isomer, solvent system: chloroform/acetone/methanol, 70:50:15 (by vol). ^{1}H NMR, 23*R*,25-pentol (400 MHz, CD_3OD): δ 0.75 (*s*, 18-CH_3), 0.92 (*s*, 19-CH_3), 1.03 (*d*, 21-CH_3, 6 Hz), 1.12/1.14 (26-CH_3 + 27-CH_3), 3.45 (*dd*, 10 Hz, 23H), 3.32 (*m*, H-3), 3.80 (*br, s*, H-7), 4.0 (*br, s*, H-12). The CD spectrum of the 24-nor-5β-cholestane-23*R*,25-pentol in the presence of $Eu(fod)_3$ showed $\Delta\varepsilon_{308} = -7.65$ degrees × cm^2 × dmol^{-1} (first Cotton effect) and $\Delta\varepsilon_{284} = +5.40$ degrees × cm^2 × dmol^{-1} (second Cotton effect) (20,44). These negative Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of the two hydroxyl groups (20,44,45) having a 1,2-glycol system in the side chain and thus the chirality at the C-23 hydroxyl group in this pentol was assigned as *R* (Scheme 2, compound **4**). TLC analysis using silica gel G plates and a solvent system of chloroform/acetone/methanol 70:50:15 (by vol) revealed a component with $R_f = 0.21$ (lower spot on TLC, minor isomer, 20%). TLC and ^{1}H NMR showed an isomer of 23,25-pentol (400 MHz, $CDCl_3$ + CD_3OD): δ 0.70 (*s*, 18-CH_3), 0.90 (*s*, 19-CH_3), 1.0 (*d*, 21-CH_3, 6 Hz), 1.2/1.17 (26-CH_3 + 27-CH_3), 3.48 (*d*, 23-H), 3.41(*m*, H-3), 3.86 (*s*, H-7), 4.0 (*br, s*, H-12). The CD spectrum of this 23,25-pentol in the presence of $Eu(fod)_3$ showed $\Delta\varepsilon_{308} = +5.8$ degrees × cm^2 × dmol^{-1} (first Cotton effect) and $\Delta\varepsilon_{285} = -5.3$ degrees × cm^2 × dmol^{-1} (second Cotton effect) (44). These positive Cotton effects measured at its maximum value, around 310 nm, were found to correlate with the chirality of the two hydroxyl groups having a 1,2-glycol system in the side chain and thus the chirality at the C-23 hydroxyl group in this pentol was assigned as *S* (Scheme 2, compound **5**) (9,20,45).

DISCUSSION

A defect in mitochondrial cytochrome P_{450} enzyme 27-hydroxylase, in which the conversion of 5β-cholestane-3α,7α,12α-triol to the 5β-cholestane-3α,7α,12α,26 (or 27)-tetrol is blocked, is considered by many to be the major inherited abnormality in the formation of cholic acid in CTX (4,11,13–16). Recent results from our laboratory have described an alternative microsomal pathway for the cleavage of the side chain in this triol starting with microsomal 25-hydroxylation followed by 24-hydroxylation to yield 5β-cholestane-3α,7α,12α,24*S*,25-pentol (6–12,48). This pathway is involved in synthesis of cholic acid but not in the chenodeoxycholic acid pathway (4,8,9,12,13).

Despite the interest of many laboratories in this pathway, which involves neither mitochondrial nor peroxisomal enzymes, its importance relative to the 27-hydroxylase block in CTX is still controversial (4,8,11,12,43,48–50).

In this report, we synthesized both the C_{27}- and the C_{26}-analogs of naturally occurring C_{27}-bile alcohols by means of the new Sharpless' chiral ligands dihydroquinidine $(DHQD)_2$-PHAL and dihydroquinine $(DHQ)_2$-PHAL (17–20, 30–35,39,51,52). With the proper choice of the chiral reagent, this reaction permitted the preparation of the enantiomerically pure (24*R* and 24*S*) 5β-cholestane-3α,7α,12α,24,25-pentols (Scheme 1, compounds **2** and **3**) from the prochiral substrate, 5β-cholest-Δ^{24}-3α,7α,12α-triol (Scheme 1, compound **1**). The absolute stereochemistry (24*R*/24*S*) of 5β-cholestane-3α,7α,12α,24,25-pentols was established *via* NMR and lanthanide-induced CD Cotton effect measurements, and thus the AD reaction in the 5β-cholestane-24,25-pentols was confirmed as predicted by the face selection rule of Sharpless and coworkers (17–21). Moreover, we observed an anomalous enantioselectivity in the Sharpless catalytic AD reaction (36–42) of 24-nor-5β-cholest-Δ^{23}-3α,7α,12α-triol (Scheme 2, compound **1**) to yield the isomeric (23*R*) and (23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols in high yield (80–87%) and with moderate to good stereoselectivity (30–80%) (Scheme 2, compounds **4** and **5**).

The absolute configuration at C-23 of 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols was further established by lanthanide-induced CD analysis experiments (20,21). Our results in the nor series show that the stereoselective nature of the AD reaction is only partial and that the preexisting stereogenic center at C-20 affects the stereochemical outcome of the reaction. Indeed, the absolute configuration of the nor-pentols is dictated by the overwhelming asymmetric induction of the stereogenic center at C-20 and not by the chiral ligands used. However, one cannot predict the interplay of the steric or polar hydrogen-bond effects of the molecule over the sterically demanding osmium-ligand complexes. In this case, both chiral ligands afforded the expected pentols, but an anomalous enantioselectivity of the AD reaction was observed (30–42).

Using AD-mix-α, we obtained 65% *R* isomer and 35% *S* isomer (Scheme 2, compounds **4** and **5**), and with AD-mix-β, 80% *R* isomer and 20% *S* isomer. These results clearly indicate that the Sharpless AD reaction of nor compounds is not controlled by the chiral reagent and that maybe some asymmetric induction of C-20 exerts a directional effect. Clearly, all these results emphasize the need for exercising great caution when applying the Sharpless face-selection rule to the 24-nor-series of steroids (30–42).

The configuration at C-23 of 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols was established by NMR experiments and verified by CD analysis as previously described for (24*R* and 24*S*) 5β-cholestane-3α,7α,12α,24,25-pentols (20,21). The results of ^{1}H and ^{13}C NMR studies are summarized in Table 1. In (24*R*) 5β-cholestane-3α,7α,12α,24,25-pentol, the C-*24H* showed a triplet at δ 3.4 and in (24*S*)-pentol showed a doublet at δ 3.2. The ^{13}C NMR spectra for (24*R*

and 24*S*) 5β-cholestanepentols (in CD_3OD) exhibited 30 signals between 140 and 10 ppm and analyzed by DEPT-135 spectra in CD_3OD which allowed the identification of seven quaternary carbons, ten CH, nine CH_2, and five CH_3 (20,21). In 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols the C-*23H* in both (23*R* and 23*S*) pentols showed a doublet at δ 3.45 and 3.48. A standard ^{13}C NMR DEPT spectrum in CD_3OD was used to delineate the multiplicity of each carbon resonance in 24-nor-5β-cholestane-3α,7α,12α,23*R*,25-pentol. A complete analysis with respect to quaternary carbons and CH, CH_2, and CH_3 groups using spectral editing with the DEPT pulse sequence (signals between 80 and 10 ppm) is shown in Figure 4. However, these ^{13}C data could not differentiate the isomeric pairs of (23*R* and 23*S*) 24-nor-5β-cholestane-3α,7α,12α,23,25-pentols. Therefore, the signal assignments were further confirmed by comparison of the expanded version of ^{13}C NMR spectra of 23*R* pentol with that of the ^{13}C NMR DEPT spectra of model compounds, namely 5β-cholestane-3α,7α,24β (24β carbon absorptions, 79.66 ppm)25-tetrol and 5β-cholestane-3α,7α,24α (24α carbon absorptions, 78.86 ppm)25-tetrol as described previously (20,21). Since these sharp signals were unequivocally identified and separated completely from each other, the shielding data provided a straightforward identification of each isomer, as well as an estimate of purity.

FAB–MS data of (23*R* and 23*S*) 24-nor-5β-cholestanepentols and (24*R*)- and (24*S*)-pentols in the presence of NaCl (9,23,27,28) provided very intense molecular ions, 461 = $[M + Na]^+$ and 475 = $[M + Na]^+$, respectively, as base peaks in the spectra of these molecules. Since the fragmentation pattern was similar for both samples as discussed above, these data could differentiate neither 24*S*-pentol from the 24*R*-pentol nor 23*R*-pentol from the 23*S*-pentols.

The synthesis and characterization of 24-norpentols described in these studies will be helpful in studying the stereospecificity of the hepatic enzymes that promote oxidation of the cholesterol side chain, and in determining whether these compounds that contain one less carbon atom in their side chains are capable of undergoing the same reaction sequences as the normally occurring C_{27} bile alcohols.

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METHOD

Improved Separation of Conjugated Fatty Acid Methyl Esters by Silver Ion–High-Performance Liquid Chromatography

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ABSTRACT: Operating from one to six silver ion–high-performance liquid chromatography (Ag$^+$–HPLC) columns in series progressively improved the resolution of the methyl esters of conjugated linoleic acid (CLA) isomeric mixtures from natural and commercial products. In natural products, the 8 *trans*, 10 *cis*-octadecadienoic (18:2) acid was resolved from the more abundant 7 *trans*, 9 *cis*-18:2, and the 10 *trans*, 12 *cis*-18:2 was separated from the major 9 *cis*, 11 *trans*-18:2 peak. In addition, both 11 *trans*, 13 *cis*-18:2 and 11 *cis*, 13 *trans*-18:2 isomers were found in natural products and were separated; the presence of the latter, 11 *cis*, 13 *trans*-18:2, was established in commercial CLA preparations. Three Ag$^+$–HPLC columns in series appeared to be the best compromise to obtain satisfactory resolution of most CLA isomers found in natural products. A single Ag$^+$–HPLC column in series with one of several normal-phase columns did not improve the resolution of CLA isomers as compared to that of the former alone. The 20:2 conjugated fatty acid isomers 11 *cis*, 13 *trans*-20:2 and 12 *trans*, 14 *cis*-20:2, which were synthesized by alkali isomerization from 11 *cis*, 14 *cis*-20:2, eluted in the same region of the Ag$^+$–HPLC chromatogram just before the corresponding geometric CLA isomers. Therefore, CLA isomers will require isolation based on chain length prior to Ag$^+$–HPLC separation. The positions of conjugated double bonds in 20:2 and 18:2 isomers were established by gas chromatography–electron ionization mass spectrometry as their 4,4-dimethyloxazoline derivatives. The double-bond geometry was determined by gas chromatography–direct deposition–Fourier transform infrared spectroscopy and by the Ag$^+$–HPLC relative elution order.

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Recently we reported for the first time the separation of a complex mixture of conjugated linoleic acid (CLA) isomers by silver ion–high-performance liquid chromatography (Ag$^+$–HPLC) as their methyl esters using 0.1% acetonitrile in hexane as the mobile phase (1). With this method, a commercial CLA mixture was separated into three groups of *trans,trans*, *cis/trans*, and *cis,cis* octadecadienoic acid (18:2) isomers. Each of the three groups of geometric isomers was shown to contain four major positional CLA isomers 8,10-18:2, 9,11-18:2, 10,12-18:2, and 11,13-18:2. Application of Ag$^+$–HPLC to the separation of natural products revealed additional CLA peaks, including several that were not resolved. For instance, 7 *trans*, 9 *cis*-18:2 was reported for the first time to be present in cow and human milk, cheese, beef and human adipose tissue, but it coeluted with 8 *trans*, 10 *cis*-18:2 (2). Furthermore, it was reported recently that mouse liver microsomes elongated 9 *cis*, 11 *trans*-18:2 to 11 *cis*, 13 *trans*-20:2 (3). The longer-chain 20:2 conjugated fatty acids (CFA) were synthesized by alkali isomerization of 11 *cis*, 14 *cis*-20:2, but no supporting evidence was provided (3). Therefore, it was not known where longer-chain CFA such as 20:2 eluted relative to CLA on Ag$^+$–HPLC columns. Based on the elution order of saturated fatty acid methyl esters (FAME) or their triacylglycerols by Ag$^+$–HPLC using a similar acetonitrile in hexane mobile phase, an inverse relationship of retention volume and chain length was expected (4).

In the present communication, we report the markedly improved resolution of the CLA isomers, and the separation of the 18:2 from the 20:2 CFA isomers, by using two to six Ag$^+$–HPLC columns in series. This resulted in the separation of several previously coeluting pairs of CLA isomers found in foods and biological systems.

MATERIALS AND METHODS

The polyunsaturated fatty acids (PUFA) linoleic acid (9 *cis*, 12 *cis*-18:2) and 11 *cis*, 14 *cis*-eicosadienoic acid (11 *cis*, 14 *cis*-20:2) were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). CLA standards (9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2) were obtained from Matreya, Inc. (Pleasant Gap, PA). A 10% solution of trimethylsilyldiazomethane in hexane was obtained from TCI America (Portland, OR). 2-Amino-2-methyl-1-propanol (95%) was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). All solvents and chemicals were reagent grade.

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Abbreviations: CFA, conjugated fatty acids; *cis/trans*, refers to all the CLA isomers having either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; GC–DD–FTIR, gas chromatography–direct deposition–Fourier transform infrared; GC–EIMS, gas chromatography–electron ionization mass spectrometry; Ag$^+$–HPLC, silver ion–high-performance liquid chromatography; PUFA, polyunsaturated fatty acids.

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The two PUFA 9 *cis*, 12 *cis*-18:2 and 11 *cis*, 14 *cis*-20:2 were isomerized according to published procedures (5,6). Briefly, 20 mL of ethylene glycol was placed in a three-necked round-bottom flask (100 mL) equipped with a magnetic stirrer, a condenser, and a thermostatically controlled heater, and heated to 110°C. Argon was passed through the reaction flask throughout the heating process. KOH (5 g) was carefully added to the flask, and the temperature was raised to 160°C after the KOH had dissolved. At this point, a PUFA was added and heated for 40 min at 160 ± 5°C. The flask was allowed to cool to room temperature, and the liquid was transferred into a 500-mL separatory funnel containing 100 mL of 6 N HCl; final pH 1–2. The liquid was extracted with two portions of 40 mL petroleum ether/diethyl ether (1:1). The combined organic phase was washed with 80 mL distilled water and dried over Na_2SO_4.

The 4,4-dimethyloxazoline (DMOX) derivatives of the CFA were prepared directly from the free fatty acids by reaction with a threefold excess of 2-amino-2-methyl-1-propanol at 170°C for 0.5 h in a 1-mL vial (7). The DMOX derivatives were analyzed by gas chromatography–electron ionization mass spectrometry (GC–EIMS) (1). The CFA were methylated with trimethylsilyldiazomethane according to Hashimoto *et al.* (8). The FAME were then analyzed by GC using a 100-m CP-Sil 88 column (9). FAME were also measured by GC–direct deposition–Fourier transform infrared (GC–DD–FTIR) (10). A Bio-Rad (Cambridge, MA) Tracer™ GC–FTIR 60A spectrometer system was used. This system was used with a 50-m CP-Sil 88 capillary column as described earlier (11).

Ag^+–HPLC separation of the CFA methyl esters was carried out using an HPLC (Waters 510 solvent delivery system; Waters Associates, Milford, MA), equipped with a 100-µL injection loop (Waters 600E, System Controller), a photodiode array detector (Waters 996) operated at 233 nm, and an operating system (Waters Millennium™ version 2.15). Two to six ChromSpher 5 Lipids analytical silver-impregnated columns (each 4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size; Chrompack, Bridgewater, NJ) were used in series. The mobile phase was 0.1% acetonitrile in hexane and operated isocratically at a flow rate of 1.0 mL/min; a flow rate of 2.0 mL/min sharpened all the peaks but led to less resolution in the *trans,trans* and *cis/trans* regions. The flow was initiated 0.5 h prior to sample injection. Typical injection volumes were 5–15 µL. The column head pressure increased from about 350 psi to about 750, 1050, 1300, and 2000 psi as the number of columns in series increased from one to two, three, four, and six, respectively. The silica LC-Si, phenyl LC-DP, and diol LC-Diol columns, each 4.6 mm i.d. × 250 mm stainless steel, 5 µm particle size, were obtained from Supelco Inc. (Bellefonte, PA).

RESULTS AND DISCUSSION

The separation of CLA isomers progressively improved by increasing the number of Ag^+–HPLC columns connected in series (up to six) as demonstrated with a beef lipid mixture in Figure 1. This is the first chromatographic separation of peaks attributed to the minor CLA isomers 8 *trans*, 10 *cis*-18:2 and 10 *trans*, 12 *cis*-18:2 in natural products, which were previously unresolved (2). The use of three columns in series was sufficient to begin resolving 8 *trans*, 10 *cis*-18:2 from 7 *trans*, 9 *cis*-18:2 and 10 *trans*, 12 *cis*-18:2 from 9 *cis*, 11 *trans*-18:2. The presence of these minor CLA isomers was previously confirmed in cheese lipids by GC–EIMS even though they had not been resolved with a single Ag^+–HPLC column (2,12). Further examples of three-column separation of total CLA methyl esters are presented for cheese (Fig. 2A) and cow milk (Fig. 2B). In a recent publication, the presence of the 10 *trans*, 12 *cis*-18:2 isomer in cheese was indirectly deduced by using partial hydrazine reduction and GC techniques (13).

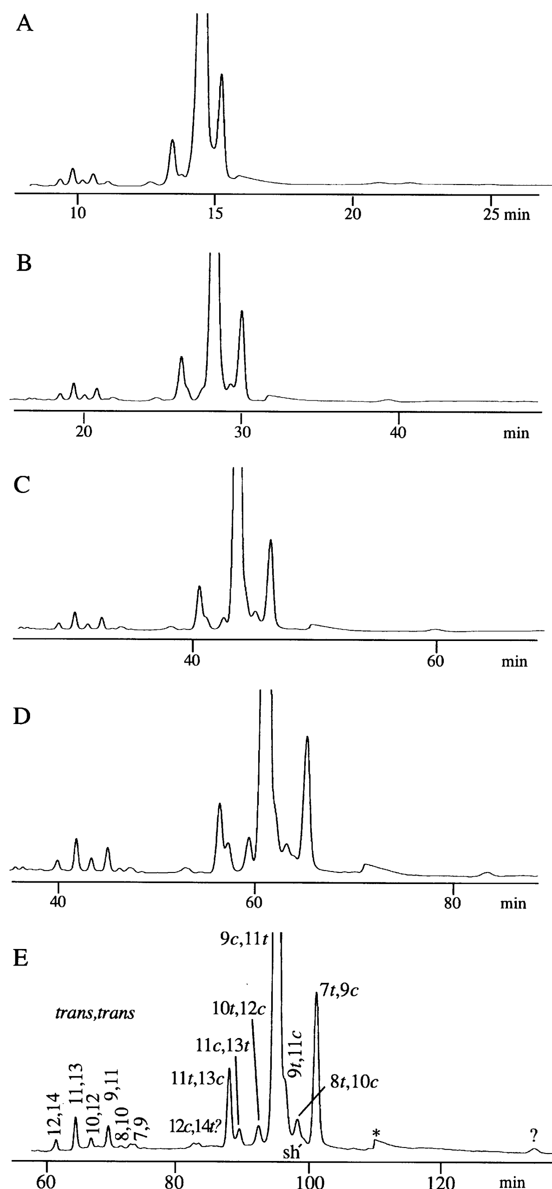

FIG. 1. Separation of total beef conjugated fatty acids using one (A), two (B), three (C), four (D), and six (E) silver ion–high-performance liquid chromatography (Ag^+–HPLC) columns in series. The asterisk (*) denotes an ultraviolet absorption at 233 nm due to methyl oleate. A shoulder after the 8*t*,10*c* peak was abbreviated "sh."

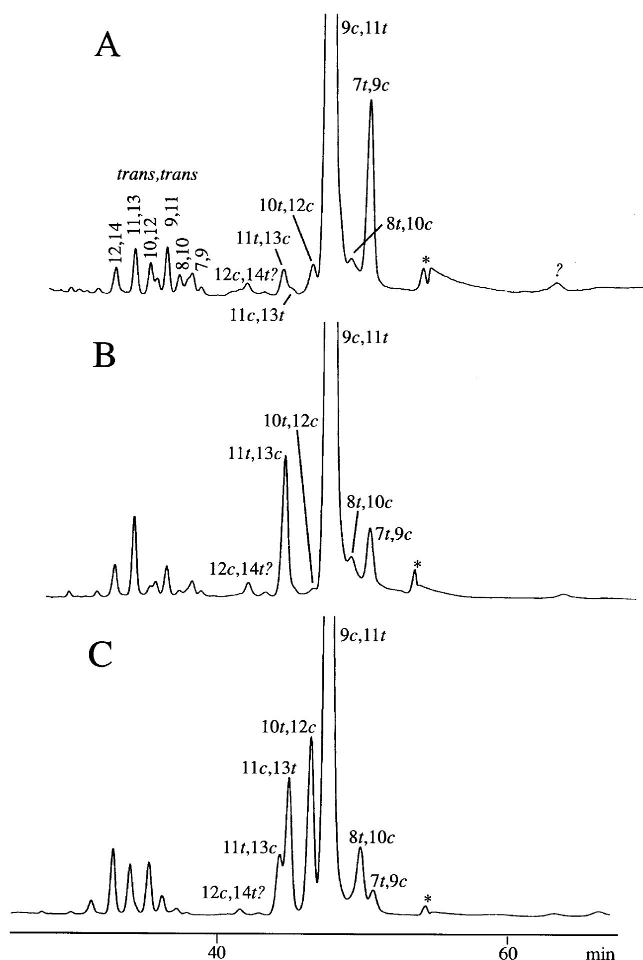

FIG. 2. Ag^+–HPLC profiles of cheese (A) and cow milk (B) using three silver-ion columns in series. Profile for coinjected mixtures of cow milk (C) with a commercial preparation from Nu-Chek-Prep (Elysian, MN). The extra peaks in the *trans,trans* region have not been identified. For asterisks and abbreviation see Figure 1.

Furthermore, the use of two to six Ag^+–HPLC columns in series clearly resolved the two 11,13-18:2 geometric isomers (Fig. 1B–E). The identification of these two isomers was achieved by coinjection of each of the natural product mixtures investigated with a known Nu-Chek-Prep commercial preparation of CLA; the resulting chromatogram for the mixture of cow milk plus Nu-Chek-Prep is shown in Figure 2C. The geometric configuration of the 11,13-18:2 isomer in the commercial CLA preparation was previously established as 11 *cis*, 13 *trans*-18:2 based on its chromatographic elution order by GC and Ag^+–HPLC (2,12,14). Therefore, the first-eluting major 11,13-18:2 isomer in cow milk (Fig. 2B) was tentatively assigned to be 11 *trans*, 13 *cis*- 18:2. The 11 *trans*, 13 *cis*-18:2 isomer was also found to be the most abundant 11,13-18:2 isomer in beef (Fig. 1), cheese (Fig. 2A), and human adipose tissue (data not shown), unlike the human milk that we examined which showed about equal amounts of the 11,13-18:2 pair of isomers.

A shoulder on the tail of the 9 *cis*, 11 *trans*-18:2 peak, tentatively attributed to the 9 *trans*, 11 *cis*-18:2 isomer, was observed only when the separation was carried out with six columns connected in series (Fig. 1E). A similar partial resolution of the *cis/trans* 9,11-18:2 pair of isomers was recently reported by using two Ag^+–HPLC columns in series under different experimental conditions, namely, 0.5% acetonitrile in hexane and detection at 206 nm (4); 9 *trans*, 11 *cis*-18:2 eluted as a shoulder on the leading edge of the 9 *cis*, 11 *trans*-18:2 peak in that case (15). The presumed reversal of the elution sequence of this pair of *cis/trans* geometric isomers may be an effect of mobile phase composition and is being investigated further.

The relative proportion of the different minor CLA isomers varied slightly in the different biological matrices investigated. For example, the 7 *trans*, 9 *cis*-18:2 was more abundant than 11 *trans*, 13 *cis*-18:2, except for cow milk, whereas 11 *trans*, 13 *cis*-18:2 was more abundant than 11 *cis*, 13 *trans*-18:2, except for human milk. These findings probably are not general characteristics but may be attributed to the limited number of test samples investigated. Also unclear is the dependence on diet of these variations in CLA distribution in human tissue lipids.

In using the previously identified Nu-Chek-Prep commercial mixture as reference (1), the separation of CLA mixtures was further investigated by joining several normal-phase HPLC columns, such as silica, phenyl, and diol columns, before or after a silver-ion column in series. In general, a degradation in resolution was obtained. The best combination of all of these columns was achieved by using a silica column followed by a silver-ion column (Fig. 3A). However, this result did not match the excellent separation obtained by using even two Ag^+–HPLC columns in series (Fig. 3B).

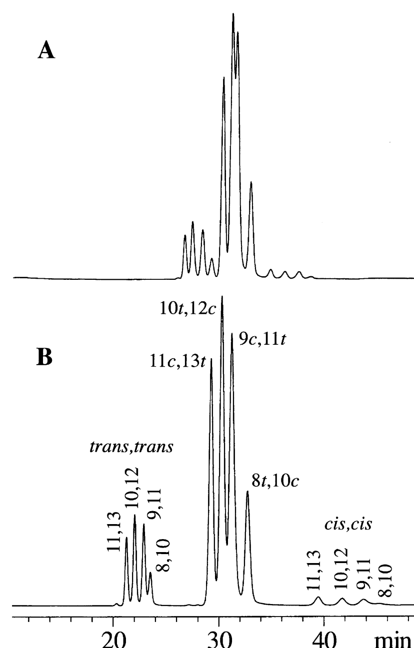

FIG. 3. Separation of a commercial conjugated linoleic acid preparation (Nu-Chek-Prep, Elysian, MN), using a combination of a silica column followed by a silver-ion column (A), and two silver-ion columns in series (B).

The resolution of synthetic C_{18} and C_{20} CFA mixtures was also enhanced with multiple Ag^+–HPLC columns. The isomerized FAME products of either 9 *cis*, 12 *cis*-18:2 (Fig. 4A) or 11 *cis*, 14 *cis*-20:2 (Fig. 4B) gave rise to two peaks in each of the *trans,trans*, *cis/trans*, and *cis,cis* regions of the Ag^+–HPLC chromatogram. The major CFA peaks were due to the *cis/trans* isomers. In using two Ag^+–HPLC columns in series, the retention times (volumes) for these two *cis/trans* pairs of CFA were close, as seen from the analysis of a mixture of these CFA mixtures (Fig. 4C). In each case, the three pairs of C_{20} CFA eluted before the corresponding C_{18} CFA in the *trans,trans*, *cis/trans*, and *cis,cis* regions, respectively. By comparison, using a single Ag^+–HPLC column was inadequate (Fig. 4D). A similar partial resolution was previously reported (16) for some positional C_{18} and C_{20} monounsaturated fatty acid isomers, using a single Ag^+–HPLC column. Furthermore, the inverse relationship between elution order and chain length was similar to that reported for saturated FAME and their triacylglycerols (4). These conjugated C_{18} and C_{20} products were also analyzed by GC. The two *cis/trans* C_{18} CFA isomers eluted at the same GC retention times as those of the standards 9 *cis*, 11 *trans*-18:2 and 10 *trans*, 12 *cis*-18:2 on the 100-m CP-Sil 88 column. The two *cis/trans* C_{20} CFA isomers eluted shortly after arachidonic acid. Their equivalent chain lengths were 22.48 and 22.57 for 11,13-20:2 and 12,14-20:2, respectively; the equivalent chain length of arachidonic acid on this GC column was 22.40.

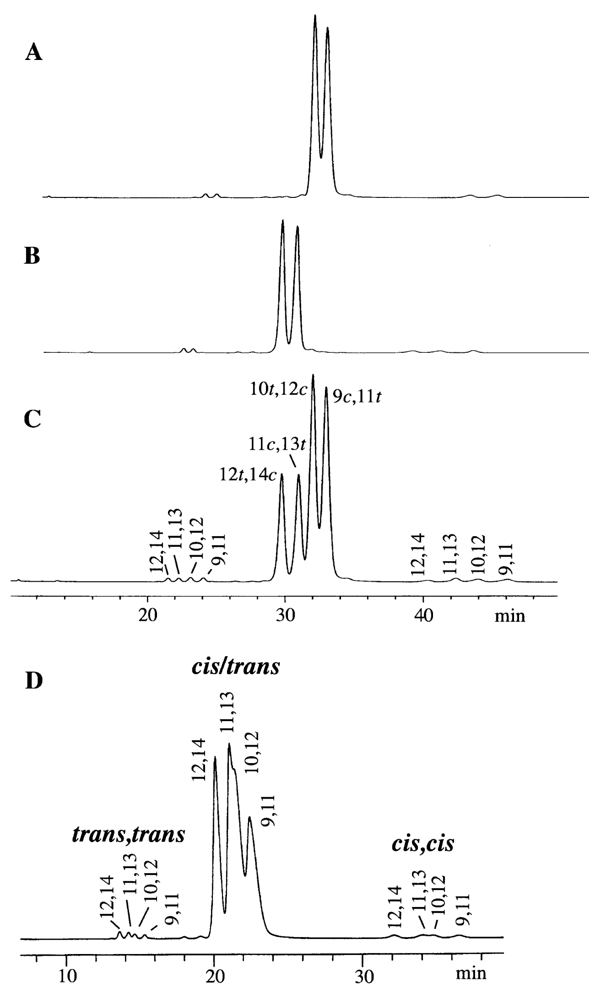

FIG. 4. Ag^+–HPLC separation using two silver-ion columns in series for (A) a CLA mixture obtained by alkali isomerization of linoleic acid (9 *cis*, 12 *cis*-18:2), (B) a conjugated fatty acid mixture obtained by alkali isomerization of 11 *cis*, 14 *cis*-20:2, and (C) coinjection of mixtures (A) and (B). Chromatogram (D) shows the separation obtained by analyzing a mixture of (A) and (B) on a single Ag^+–HPLC column. Different amounts of conjugated 18:2 and 20:2 mixtures were analyzed in (C) and (D). For abbreviations see Figures 1–3.

The isomerization products of 9 *cis*, 12 *cis*-18:2 were identified as 9,11-18:2 and 10,12-18:2 (data not shown), and those of 11 *cis*, 14 *cis*-20:2 were found to be 11,13-20:2 and 12,14-20:2 by GC-EIMS as the DMOX derivatives (Fig. 5). The characteristic fragment ions due to the double bond occurred at *m/z* 224, 236, 250, and 262 for 11,13-20:2 (Fig. 5A), and at *m/z* 238, 250, 264, and 276 for 12,14-20:2 (Fig. 5B). Favorable allylic cleavages occurred at *m/z* 210 and 290 for 11,13-20:2 and *m/z* 224 and 304 for 12,14-20:2. In addition, the ion that was 14 mass units greater than the higher- mass allylic ion was also abundant, i.e., *m/z* 304 and 318, for 11,13-20:2 and 12,14-20:2, respectively.

The *cis/trans* geometries of the C_{18} and C_{20} CFA methyl ester isomers were established by GC–DD–FTIR (Fig. 6). These compounds exhibited characteristic =C-H stretching vibrations at 3020 and 3002 cm^{-1} and deformation bands at 985 and 946 cm^{-1}, as previously described for *cis/trans* conjugated dienes (10). The relative intensities of the C-H stretching vibrations for *cis/trans* C_{18} and C_{20} CFA methyl esters were compared in Figure 7 after normalization relative to the ester carbonyl band. The results showed that, whereas the asymmetric C-H stretch (2952 cm^{-1}) for the CH_3 group in these two CFA isomers was similar, the asymmetric (2924 cm^{-1}) and symmetric (2853 cm^{-1}) CH_2 stretching vibration bands were more intense in C_{20} than in C_{18} CFA. These data were consistent with the formation of 11 *cis*, 13 *trans*-20:2 and 12 *trans*, 14 *cis*-20:2, respectively.

The results of the present study demonstrated the improved resolution of CLA isomers observed by using two or more Ag^+–HPLC columns in series. It would appear that the use of three Ag^+–HPLC columns was the best compromise to achieve, in a timely manner, resolution of most CLA isomers in biological matrices. More than three columns should only be used to resolve specific critical pairs of isomers. Furthermore, this method resolved the 11,13-18:2 pair of geometric CLA isomers. No improvements or advantages were gained by combining a silver-ion column with any of the normal-phase columns, such as silica, phenol, or diol.

In addition, the results show that the CFA with different chain lengths eluted in the same region of the Ag^+–HPLC chromatogram. Therefore, for future investigations of CLA metabolites a prior isolation based on chain length, such as reversed-phase HPLC (17,18), may be required.

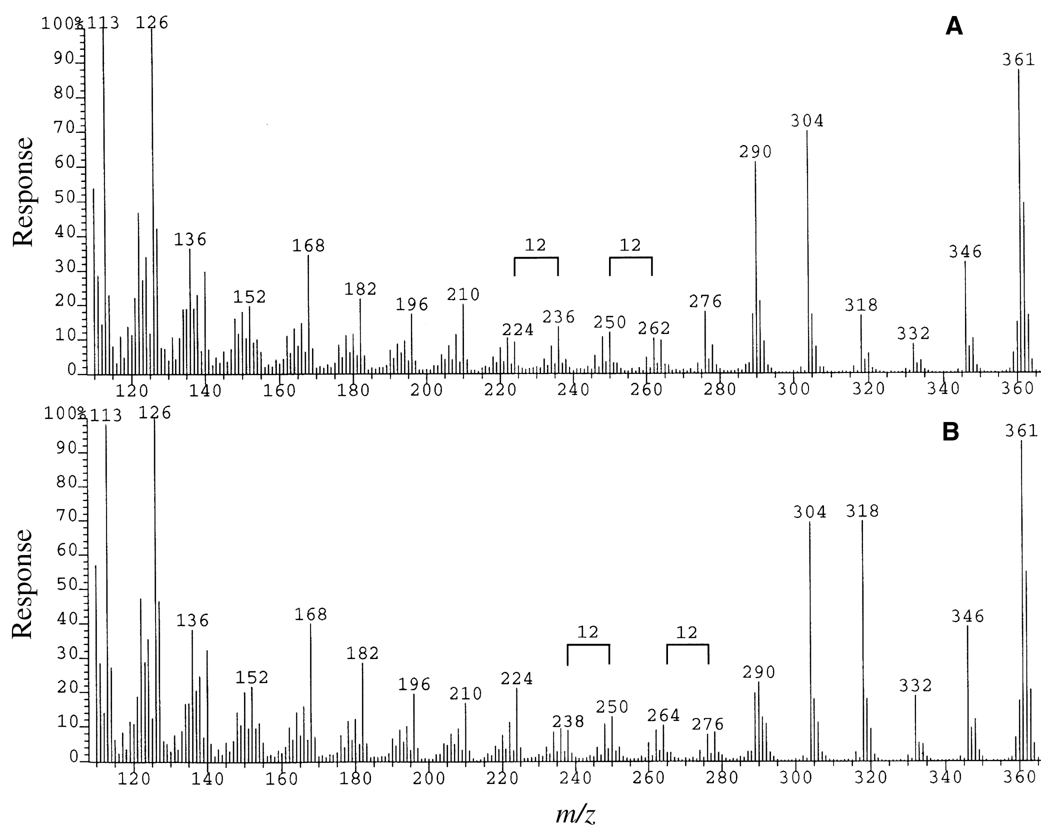

FIG. 5. Gas chromatography–electron ionization mass spectrometry of the 4,4-dimethyloxazoline derivative of isomerized 11 *cis*, 14 *cis*-20:2. The two major *cis/trans* isomers were 11,13-20:2 (A) and 12,14-20:2 (B).

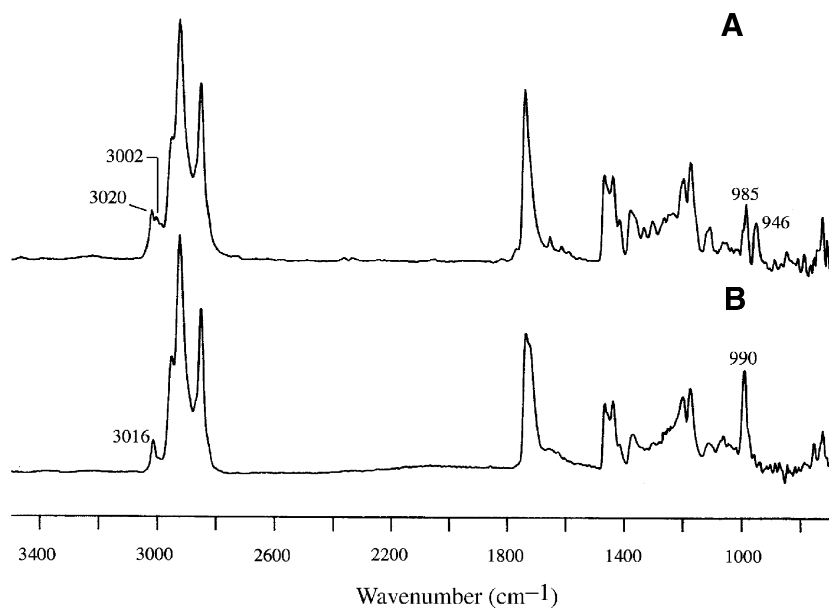

FIG. 6. Characteristic gas chromatography–direct deposition–Fourier transform infrared spectral regions, indicating *cis/trans* (A) and *trans,trans* (B) conjugated double bonds in 20:2 fatty acid methyl esters.

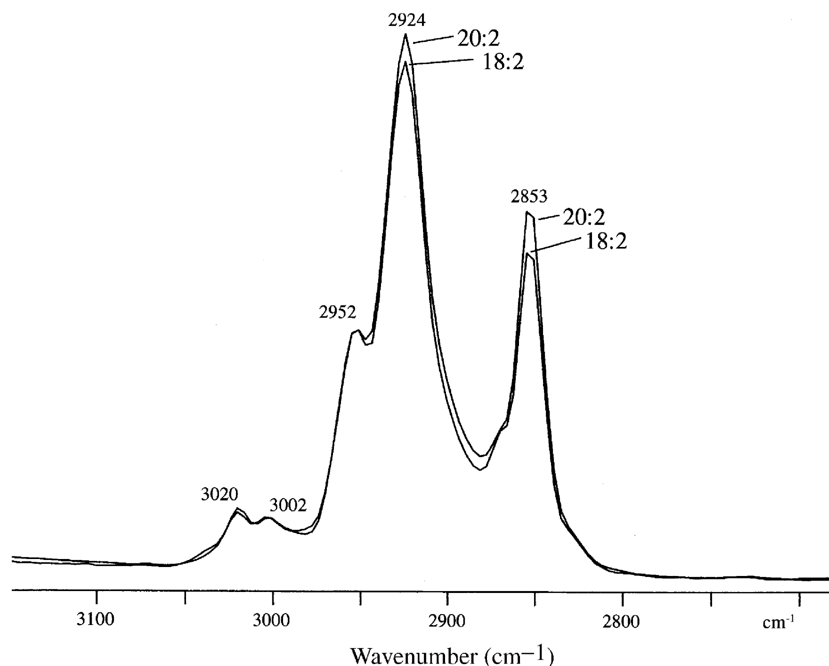

FIG. 7. Comparison of the relative intensity of C-H stretching vibrations for *cis/trans* C_{18} and C_{20} conjugated fatty acid methyl esters after normalization relative to the ester carbonyl band: asymmetric C-H stretch for the CH_3 group at 2952 cm^{-1}, asymmetric (2924 cm^{-1}) and symmetric (2853 cm^{-1}) C-H stretch for the CH_2 groups.

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Simultaneous Determination of *RRR*- and *SRR*-α-Tocopherols and Their Quinones in Rat Plasma and Tissues by Using Chiral High-Performance Liquid Chromatography

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ABSTRACT: We established a method to simultaneously determine *RRR*- and *SRR*-α-tocopherol (α-Toc) and their quinones in biological samples by chiral-phase high-performance liquid chromatography (HPLC). α-Toc had a shorter retention time than α-tocopherylquinones (α-TQ), and 2-*ambo*-α-Toc was completely separated into two peaks; the first peak was *RRR*-α-Toc and the second *SRR*-isomer by chiral HPLC connected Chiralcel OD-H column and Sumichiral OA4100 column. In contrast, of the two peaks of α-TQ, the first was the *SRR*-isomer. We also investigated differences in the distribution of *RRR*- and *SRR*-α-TQ in rat tissues after oral administration of 2-*ambo*-α-Toc by the above HPLC method. Rats deficient in vitamin E were divided into two groups, control and experimental, and tissues were collected at 3, 6, and 24 h after oral 2-*ambo*-α-Toc administration. The concentrations of *RRR*- and *SRR*-α-Toc and their quinones in plasma and each tissue were determined. The concentration of *SRR*-α-TQ in plasma and adrenal glands was not significantly different from *RRR*-α-TQ. However, the concentration of *SRR*-α-TQ in liver up to 6 h after oral administration was higher than that of *RRR*-α-TQ, and *SRR*- and *RRR*-α-TQ levels were similar at 24 h after oral administration. Therefore, we may assume that the formation of α-TQ *in vivo* was not different between *RRR*- and *SRR*-isomer and that it was not affected by the presence of α-Toc stereoisomers.

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Vitamin E, especially α-tocopherol (α-Toc), is known to be a major antioxidant protecting cellular membranes (1). There are two types of α-Toc, natural and synthetic. Synthetic α-Toc (all-*rac*-α-Toc), which is used in many types of medical preparations and food additives, is composed of equal amounts of the eight stereoisomers of α-Toc arising from the three asymmetric carbons (2-position in chroman ring and 4′ and 8′ positions in side chain). Among the eight stereoisomers, only the 2*R*,4′*R*,8′*R* (*RRR*) stereoisomer occurs naturally. Biological activities of the eight stereoisomers of α-Toc were determined by the rat fetal resorption–gestation assay, as follows: *RRR* = 100%, *RRS* = 90%, *RSS* = 73%, *SSS* = 60%, *RSR* = 57%, *SRS* = 37%, *SRR* = 31%, and *SSR* = 21% (2). 2-*ambo*-α-Toc is composed of equal amounts of *RRR*- and *SRR*-α-Toc.

In 1987, Ingold *et al.* (3) synthesized deuterium-substituted *RRR*- and *SRR*-α-Toc, in which the substituted numbers of deuterium were varied for purposes of differentiation, and elucidated their absorption, transportation, and biodiscrimination of *RRR*- and *SRR*-α-Toc. In their study, the ratio of *RRR*-α-Toc to *SRR*-α-Toc in every tissue except the liver was higher than 1.0, showing that biodiscrimination may occur in the liver. Traber *et al.* (4) had elucidated the differentiation of *RRR*- and *SRR*-α-Toc in the lipoprotein subfraction of human blood by using the same methodology and concluded that biodiscrimination of the α-Toc isomers might occur when very low density lipoprotein (VLDL) is excreted to transport lipids with α-Toc from liver (4). Furthermore, we have examined the distribution and biodiscrimination of α-Toc stereoisomers (especially 2*R*- and 2*S*-isomers) in rats and humans (5–10) by a chiral high-performance liquid chromatography (HPLC) method (11), and we found that the ratios of α-Toc stereoisomers in the lymph of rats dosed with all-*rac*-α-Toc were the same as the ratio of α-Toc stereoisomers administered: 2*R*-isomers constituted approximately 50% of α-Toc. The uptake of α-Toc stereoisomers into small intestinal cells was measured by using Caco-2 cell lines. The concentration of *RRR*-α-Toc in Caco-2 cells was not significantly different from that of *SRR*-α-Toc. This finding suggested that the discrimination of α-Toc stereoisomers does not occur during absorption in small intestine and indicated the contribution of the liver to this discrimination (8). Moreover, we had investigated changes in the concentrations of the stereoisomers of α-Toc in serum and lipoproteins in healthy humans who were dosed orally with synthetic α-Toc acetate. α-Toc stereoisomers detected in the serum of all subjects administered 100 mg all-*rac*-α-Toc acetate/day were all 2*R*-isomers; no 2*S*-isomers were found (9). Weiser *et al.* (12) reported the

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Abbreviations: HPLC, high-performance liquid chromatography; PMC, 2,2,5,7,8-pentamethyl-6-hydroxychroman; TLC, thin-layer chromatography; α-Toc or γ-Toc, α- or γ-tocopherol; α-TQ, α-tocopherylquinone; α-TTP, α-tocopherol transfer protein; VLDL, very low density lipoprotein.

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measurements of all eight stereoisomers of α-Toc in rat plasma and tissues and concluded that the configuration at C-2 of the α-Toc molecule is of major importance with regard to uptake and storage of α-Toc stereoisomers, whereas the chiral centers of the phytyl side chain have less influence. Furthermore, Hosomi *et al.* (10) examined the structural characteristics of vitamin E analogs required for recognition by α-Toc transfer protein (α-TTP). Relative affinities (*RRR*-α-Toc = 100%) calculated from the degree of competition were as follows: γ-Toc, 9%; *SRR*-α-Toc, 11%; and so on. From this result, they proposed that the affinities of vitamin E analogs for α-TTP, which may determine their plasma levels, are a major determinant of their biological activity.

According to these reports, orally administered α-Toc stereoisomers (especially *RRR*- and *SRR*-isomer) are absorbed equally from the small intestine without discrimination. After uptake into intestinal cells, α-Toc stereoisomers are secreted into chylomicrons. Chylomicron remnants are catabolized during the circulation by the lipoprotein lipase. After uptake of chylomicron remnants by the liver, α-Toc stereoisomers are discriminated by α-TTP in liver, which transports *RRR*-α-Toc preferentially over *SRR*-α-Toc into VLDL during assembly. VLDL rich in *RRR*-α-Toc is then circulated in plasma and transported to each tissue. On the other hand, *SRR*-α-Toc remained in liver. Kayden and Traber (13) discussed in a review that the different *RRR*-α-Toc remaining in liver (such as γ-Toc or *SRR*-α-Toc) will probably be excreted in the bile. Thus we can note the difference in the metabolism of *RRR*- and *SRR*-α-Toc *in vivo*.

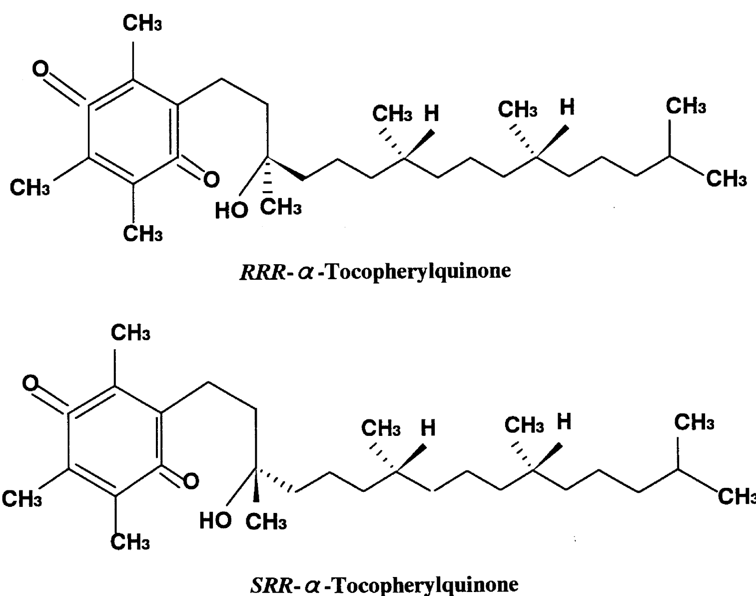
The first metabolite of α-Toc is assumed to be α-tocopherylquinone (α-TQ). Stereoisomerism of α-TQ arises from an asymmetric carbon at the 2-position on the chroman ring similar to α-Toc (Scheme 1). Regarding the determination of α-TQ, Hughes and Tove (14) reported that rat liver contained 124 nmol/g of α-TQ (the amount of α-TQ was determined spectrophotometrically). In contrast, Bieri and Tolliver (15) reported that 1–4 nmol/g of α-TQ was present in the liver of rats fed low and high levels of dietary α-Toc [the amount of α-TQ was determined by thin-layer chromatography (TLC) and HPLC]. Moreover, Koskas *et al.* (16) reported the determination of α-TQ by the reverse-phase HPLC method, and Vatassery and Smith (17) reported the determination of it in biological samples by using the reverse-phase HPLC method. Their results showed that the concentration of α-TQ in normal human serum was extremely small, only 0.02–0.05% of the α-Toc concentration. In this brief review we show that there have been many reports on the determination of the amounts of α-TQ in biological samples, but no reports on the determination of α-TQ stereoisomers, and that an HPLC method to separate *RRR*- and *SRR*-α-TQ has not yet been developed.

Therefore in this study we devised a method to determine *RRR*- and *SRR*-α-TQ in biological specimens by chiral phase HPLC so that we could estimate the amounts of *RRR*- and *SRR*-α-TQ present *in vivo*. Furthermore, we examined the distribution of *RRR*- and *SRR*-α-TQ in rat tissues after oral administration of 2-*ambo*-α-Toc.

EXPERIMENTAL PROCEDURES

Materials. *RRR*-α-Toc, 2-*ambo*-(2*RS*,4′*R*,8′*R*) α-Toc, and 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) as an internal standard were donated by Eisai Co. (Tokyo, Japan).

Preparation of 2-ambo-*α-TQ.* Mixed in the separation funnel were 200 mL of ethanolic 2-*ambo*-α-Toc solution (2-*ambo*-α-Toc, 6 mg/mL) and 100 mL of ethanolic $FeCl_3$ solution ($FeCl_3$ 50 mg/mL), and the funnel was shaken vigorously for 3 min. Next, the solution was shaken with 100 mL of *n*-hexane and 100 mL of water for 1 min. After shaking, the solution was



RRR-α-Tocopherylquinone

SRR-α-Tocopherylquinone

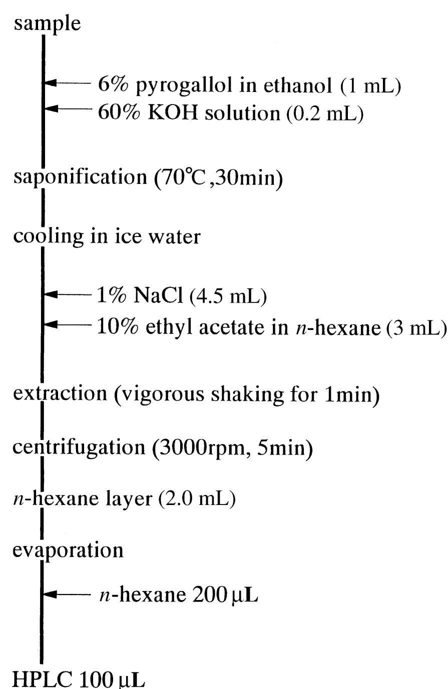
SCHEME 1

permitted to stand for a few minutes, and the water phase was removed. Water was again added to the *n*-hexane layer and shaken for 1 min. This process was repeated three times. The washed hexane layer was dehydrated by filtration through Na_2SO_4. The 2-*ambo*-α-TQ in hexane was purified by using silica gel column chromatography. The mobile phase used was 10% chloroform in hexane, and the concentration of chloroform was raised gradually to 50%. A few fractions of the purified 2-*ambo*-α-TQ solution were extracted, and 2-*ambo*-α-TQ in each fraction was confirmed by TLC. The mobile phase of TLC was benzene/chloroform (15:1, vol/vol). The standard used for TLC was α-tocopherol quinone (purchased from ICN Nutritional Biochemicals, Cleveland, OH). The purity of 2-*ambo*-α-TQ was checked by HPLC and was found to be greater than 97%. *RRR*-α-TQ was also prepared in a method similar to the one described above. The identification of 2-*ambo*-α-TQ was performed by liquid chromatography–mass spectrometry (LC–MS).

Vitamin E-deficient rats. Male Sprague-Dawley strain rats (3 wk of age) were purchased from Nippon Clea Co (Tokyo, Japan). They were initially fed a commercial diet (CE-2; Nippon Clea Co.) for a week to allow them to adapt to the new environment. These rats were then fed a diet deficient in vitamin E (AIN-76; Eisai Co.) for 8 wk. This diet was composed of 23.68% sucrose, 23.68% glucose, 18.95% vitamin-free casein, 14.21% cornstarch, 4.74% filter paper, 3.32% mineral mixture, 0.95% vitamin mixture except vitamin E, 0.28% DL-methionine, 0.19% choline bitartrate, and 10% stripped corn oil. Animals were housed individually in cages at 22°C and 55% humidity with a 12 h light/dark cycle. The feed and water were supplied *ad libitum.*

Determination of RRR- *and* SRR-*α-Toc and their quinones in rats.* Corn oil, stripped of vitamin E, was used as a vehicle. After 18 h fasting overnight, the vitamin E-deficient rats were divided into four groups; one was a control group and the others were experimental groups. The experimental groups of six rats each were given a dosage of 0.5 mL corn oil preparation containing 50 mg of 2-*ambo*-α-Toc per rat. One group was killed under anesthesia at 3 h after oral administration, and the other groups were killed after 6 and 24 h. The control group was given a dosage of 0.5 mL stripped corn oil (depleted of vitamin E) per rat, then killed at 24 h after oral administration. Plasma was separated from heparinized blood by centrifugation. Each of the tissue and the plasma were stored at −80°C until analysis of α-Toc and α-TQ stereoisomers.

Extraction of α-Toc and-TQ stereoisomers from the biological specimens. Plasma (0.2 mL) was pipetted into a 10-mL centrifuge tube. About 0.5 g of each tissue, cut into small pieces, was placed in a 10-mL centrifuge tube. Then 0.1 mL of 1% NaCl (wt/vol) solution was added to the tubes to accelerate the saponification by dispersion of the samples. Ethanolic pyrogallol solution (1 mL, 6%, wt/vol) was added to each tube during stirring. After 0.2 mL of 60% (wt/vol) KOH solution had been added to each tube, the contents were saponified with occasional shaking at 70°C for 30 min. After the saponified solution was cooled in ice water, 4.5 mL of 1%



SCHEME 2

NaCl solution was added to it and the medium was shaken vigorously with 3 mL of 10% ethyl acetate in hexane for 1 min. The mixture was centrifuged at 600 × *g* for 5 min, and 2 mL of the upper layer was collected and evaporated. The residue was dissolved in 200 μL of hexane for the determination by HPLC. Scheme 2 shows the simultaneous extraction procedure for α-Toc and α-TQ stereoisomers.

Chromatographic apparatus and conditions. The HPLC system consisted of a Water's LC Module 1 (Japan Millipore Ltd., Tokyo, Japan) and an SK-25 column oven (Nihon Seimitsu Kagaku, Co., Ltd., Tokyo, Japan). The analysis of α-Toc and α-TQ stereoisomers was performed at 30°C by using Chiralcel OD-H column (250 × 4.5 mm i.d., Daicel Chem. Ind., Tokyo, Japan) and Sumichiral OA4100 column (250 × 4.5 mm i.d., Sumika Chemical Analysis Service, Ltd., Tokyo, Japan) connected in series. The mobile phase was hexane/isopropyl alcohol (97.3:2.7, vol/vol) at a flow rate of 0.3 mL/min. The detection wavelength was 268 nm.

Statistical analysis. All results are expressed as mean ± SD. Student's *t*-test was used for a comparison of *RRR*- and *SRR*-isomer of α-Toc and for α-TQ in each tissue. Differences were considered significant at $P < 0.05$.

RESULTS AND DISCUSSION

Separation of standard samples. Figure 1 shows HPLC chromatograms of standard samples of 2-*ambo*-α-Toc, 2-*ambo*-α-TQ, and PMC as the internal standard. Both 2-*ambo*-α-Toc and 2-*ambo*-α-TQ were separated into two peaks each (peak area ratio, 1:1) by chiral HPLC. For α-Toc, the first peak was *RRR*-α-Toc and the second *SRR*-α-Toc; the first peak of α-TQ was the *SRR*-isomer and the second was *RRR*. The con-

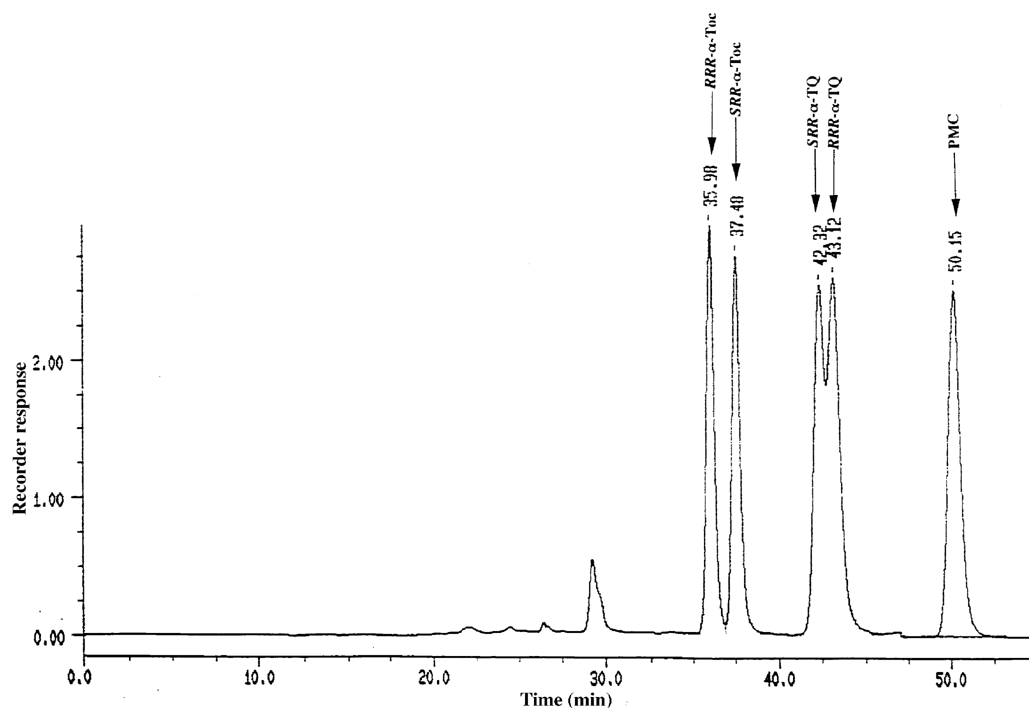


FIG. 1. A high-performance liquid chromatogram of 2-*ambo*-α-tocopherol (Toc), 2-*ambo*-α-tocopherylquinone (TQ), and 2,2,5,7,8-pentamethyl-6-hydroxychroman (PMC) as an internal standard.

firmation of each peak was performed by comparing the retention time with *RRR*-α-Toc and *RRR*-α-TQ.

The detection wavelength used in this method was determined to be 268 nm, the ultraviolet-absorption maximum wavelength for 2-*ambo*-α-TQ. Because α-TQ content compared with α-Toc might be a small amount *in vivo*, and because the ultraviolet absorption wavelength of 2-*ambo*-α-TQ may overlap those of 2-*ambo*-α-Toc and PMC, we used an analytical wavelength of 268 nm in this study (Fig. 2).

Calibration curves (Fig. 3). Calibration curves were prepared to evaluate the appropriate quantitative analysis of this methodology with authentic samples of 2-*ambo*-α-Toc, 2-*ambo*-α-TQ, and PMC as the internal standard.

One milliliter of 5.0, 10.0, 20.0, or 40.0 μg/mL ethanolic 2-*ambo*-α-Toc or 1 mL of 0.5, 1.0, 2.0, or 4.0 μg/mL ethanolic 2-*ambo*-α-TQ solutions was saponified with 5.0 μg/mL PMC. The extracts were analyzed by chiral HPLC. The correlation between the concentration ratio (x) and the peak area ratio (y) to the internal standard of each peak was determined. Concerning the results, the linear regression equation of *RRR*-α-Toc were $y = 1.43 \times 10^6x + 9.58 \times 10^4$ (correlation coefficient: $r = 0.999$, $P < 0.05$); that of *SRR*-α-Toc was $y = 1.45 \times 10^6x + 5.35 \times 10^4$ (correlation coefficient: $r = 1.000$, $P < 0.05$) (Fig. 3A); that of *RRR*-α-TQ was $y = 1.87 \times 10^6x + 5.13 \times 10^4$ (correlation coefficient: $r = 0.996$, $P < 0.05$); and that of *SRR*-α-TQ was $y = 1.52 \times 10^6x + 4.54 \times 10^4$ (correlation coefficient: $r = 0.996$, $P < 0.05$) (Fig. 3B).

Recoveries of 2-ambo-*α-Toc and -TQ added to rat plasma and tissues.* The standard samples of 2-*ambo*-α-Toc and -TQ

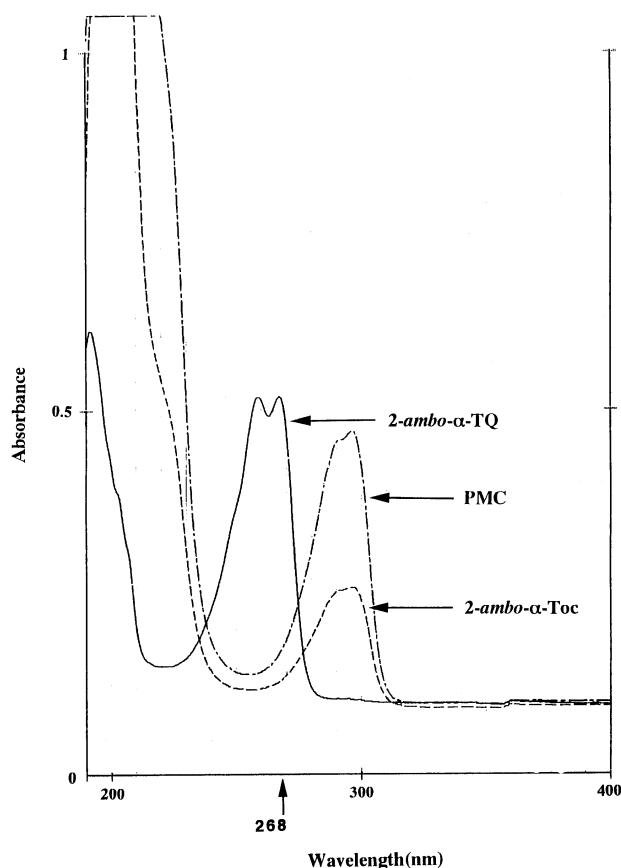


FIG. 2. Ultraviolet absorption spectra of 2-*ambo*-α-Toc, 2-*ambo*-α-TQ, and PMC in hexane. For abbreviations see Figure 1.

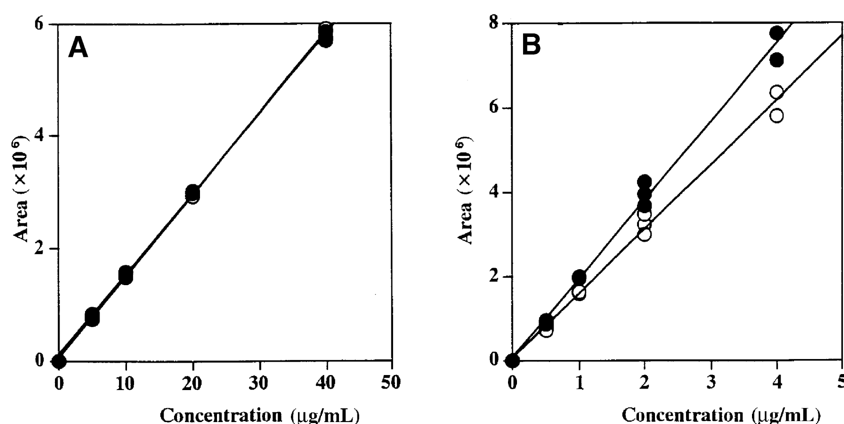


FIG. 3. Calibration curves of *RRR*- and *SRR*- α-Toc and -TQ. (A) Calibration curves of *RRR*- (●) and *SRR*- (○) α-Toc. One milliliter of 5.0, 10.0, 20.0, or 40.0 μg/mL ethanolic 2-*ambo*-α-Toc solution was saponified with PMC and each concentration of α-TQ. These extracted solutions were analyzed by chiral high-performance liquid chromatography (HPLC). (B) Calibration curves of *RRR*- (●) and *SRR*- (○) α-TQ. One milliliter of 0.5, 1.0, 2.0, or 4.0 μg/mL ethanolic 2-*ambo*-α-TQ solution was saponified with PMC and each concentration of α-Toc. These extracted solutions were analyzed by chiral HPLC. For abbreviations see Figure 1.

was added to plasma or tissues of rats, and the analytical recoveries of *RRR*- and *SRR*-α-Toc and their quinones were measured by this method. Pipetted into a 10-mL centrifuge tube was 0.2 mL of rat plasma. About 0.1 g of rat liver, cut into small pieces, was placed in a 10-mL centrifuge tube. Then 0.1 mL of 1% NaCl (wt/vol) solution was added to the tubes to accelerate the saponification by dispersion of the samples. One milliliter of ethanolic standard solution contained 2.0 μg of 2-*ambo*-α-TQ (1.0 μg each of *RRR*- and *SRR*-α-TQ) and 20 μg of 2-*ambo*-α-Toc (10 μg each of *RRR*- and *SRR*-α-Toc), and 20 μg/mL PMC solution and 1 mL of 6% (wt/vol) ethanolic pyrogallol solution were separately added to each tube during stirring. The samples added to each ethanolic 2-*ambo*-α-Toc and -TQ were saponified and extracted, similar to the method described above, and the analytical recoveries of *RRR*-, *SRR*-α-Toc and *RRR*-, *SRR*-α-TQ were determined by this method. According to this method of standard addition, the analytical recoveries of *RRR*-, *SRR*-α-Toc and *RRR*-, *SRR*-α-TQ exceeded 95% (Table 1). Moore and Ingold (18) demonstrated *in vivo* that orally administered α-TQ is converted to α-Toc in human plasma; from our results, this conversion *in vitro* was not observed.

In this examination, we used an antioxidant agent, pyrogallol, during saponification of the samples. Vatassery and Smith (17) reported the effect of antioxidant combinations by determining α-Toc and α-TQ in human red blood cells. According to their results, antioxidant combinations effectively inhibited the oxidation of α-Toc and the "artificial" formation of α-TQ (as a result of the saponification process). Therefore, we used only pyrogallol as an antioxidant in this study, and we found that by this method the simultaneous recoveries of α-Toc and α-TQ stereoisomers (*RRR*- and *SRR*-isomer) in each tissue to which a standard solution had been added exceeded 95%. Furthermore, artificial α-TQ resulting from the extraction procedure itself was scarcely formed in this method.

Distribution of α-Toc and α-TQ stereoisomers in rat tissues. We next investigated by this HPLC method changes of the distribution of *RRR*-, *SRR*-α-Toc and their quinones in rat plasma and tissues after oral administration of 2-*ambo*-α-Toc. The corn oil preparation (100 mg/mL of 2-*ambo*-α-Toc) was checked by HPLC, and 2-*ambo*-α-TQ was not detected.

The α-Toc and α-TQ stereoisomers in tissues were not detected 3 h after oral administration. Figure 4 shows that changes in the concentrations of α-Toc and α-TQ stereo-

TABLE 1
Analytical Recoveries of 2-*ambo*-α-Tocopherol and -Tocopherylquinone Added to Rat Plasma and Tissues[a]

	RRR-α-Toc (%)	*SRR*-α-Toc (%)	*RRR*-α-TQ (%)	*SRR*-α-TQ (%)
Plasma (*n* = 3)[c]	99.0 ± 1.0[b]	95.4 ± 2.9	98.5 ± 2.2	99.6 ± 2.8
Liver (*n* = 4)	100.2 ± 4.4	99.0 ± 2.2	99.0 ± 4.3	103.8 ± 3.9

[a]1.6 μg of 2-*ambo*-α-tocopherylquinone (TQ) and 20 μg of 2-*ambo*-α-tocopherol (Toc) were added to samples.
[b]Each value is expressed as mean ± SD.
[c]*n*, number of determinations.

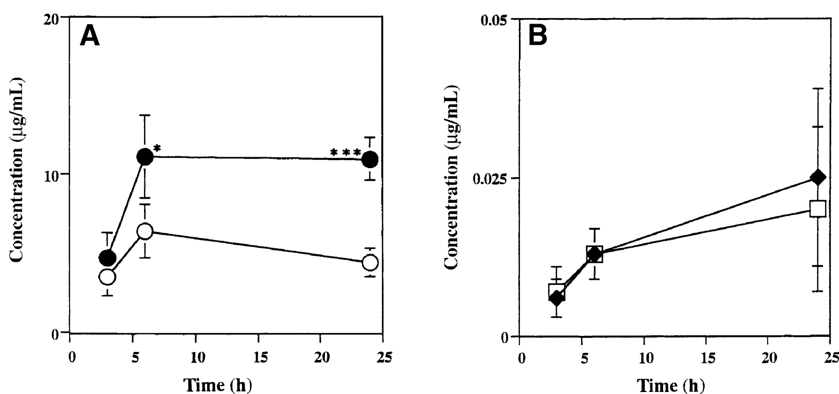


FIG. 4. Concentrations of α-Toc and α-TQ stereoisomers in rat plasma. Values are mean ± SD of 5–6 rats. (A) Change in concentration of *RRR*- (●) and *SRR*- (○) α-Toc in rat plasma after oral administration of 2-*ambo*-α-Toc. Values with asterisks mean a significant difference between *RRR*- and *SRR*-isomer; *** $P < 0.001$; * $P < 0.05$ (by *t*-test). (B) Change in concentration of *RRR*- (◆) and *SRR*- (□) α-TQ in rat plasma after oral administration of 2-*ambo*-α-Toc. For abbreviations see Figure 1.

isomers did occur in rat plasma after oral administration. The concentration of *RRR*-α-Toc was significantly higher than that of *SRR*-α-Toc up to 6 h, and remained at a similar level until 24 h. The total α-TQ content in plasma was approximately 0.2% of total α-Toc. The concentration of both *RRR*- and *SRR*-α-TQ gradually increased after oral administration, but no significant difference was found between *RRR*- and *SRR*-isomers. On the contrary, the concentration of *SRR*-α-Toc in liver was higher than that of *RRR*-α-Toc from 6 h to 24 h after oral administration. Concerning α-TQ, total α-TQ content in liver was <1% of total α-Toc in liver. As to *RRR*- and *SRR*-isomers, the concentration of *SRR*-α-TQ was higher than that of *RRR*-α-TQ up to 6 h after oral administration. The concentration of *SRR*-α-TQ at 24 h after oral administration had decreased in comparison with the level after 6 h, and *RRR*-α-TQ at 24 h had slightly increased in comparison. However, the concentration of α-TQ in liver was not significantly different between *RRR*- and *SRR*-isomer at all times (Fig. 5). In adrenal glands, the increases of *RRR*- and *SRR*-α-Toc contents continued until 24 h after oral administration; then the concentration of *RRR*-α-Toc became significantly higher than that of *SRR*-α-Toc. The concentration of α-TQ also gradually increased until 24 h after oral administration, but no significant difference was noted between *RRR*- and *SRR*-α-TQ (Fig. 6). The total α-TQ content in adrenal glands was approximately 0.2% of total α-Toc. In brain, only *RRR*-α-Toc was found in small amounts up to 24 h; no *SRR*-α-Toc or α-TQ stereoisomers were detected until 24 h.

We studied α-TQ, the first product of α-Toc oxidation, because the differentiation of the turnover for α-Toc stereoisomers *in vivo* has been investigated. Howell and Smith (19) and others (15,17) established the measurement of α-TQ in the biological specimens, but reports of separation and determination of *RRR*- and *SRR*-α-TQ had not yet been made. In

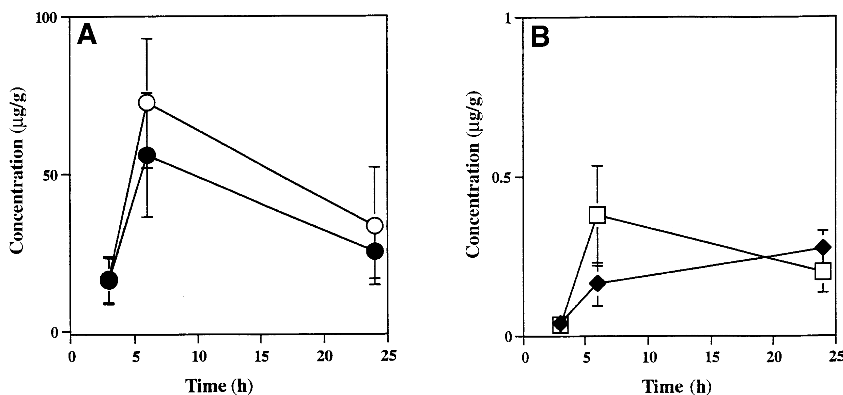


FIG. 5. Concentrations of α-Toc and α-TQ stereoisomers in rat liver. Values are means ± SD of 5–6 rats. (A) Change in concentration of *RRR*- (●) and *SRR*-(○) α-Toc in rat liver after oral administration of 2-*ambo*-α-Toc. (B) Change in concentration of *RRR*-(◆) and *SRR*- (□) α-TQ in rat liver after oral administration of 2-*ambo*-α-Toc. For abbreviations see Figure 1.

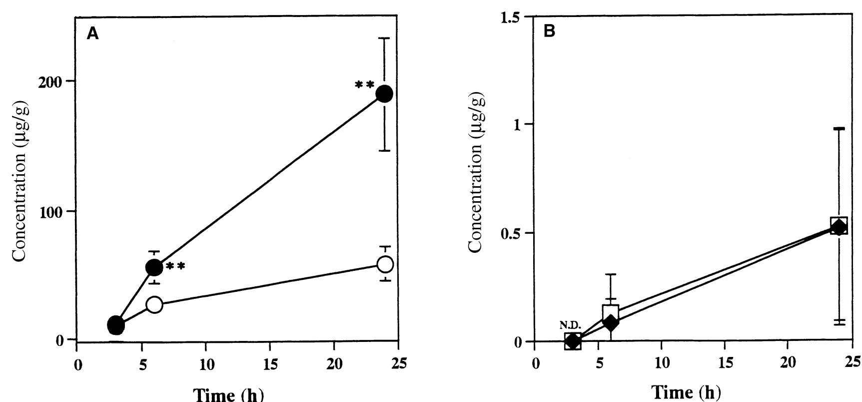


FIG. 6. Concentrations of α-Toc and α-TQ stereoisomers in rat adrenal glands. Values are mean ± SD of 5–6 rats. (A) Change in concentration of *RRR*-(●) and *SRR*- (○) α-Toc in rat adrenal glands after oral administration of 2-*ambo*-α-Toc. Values with asterisks mean a significant difference between *RRR*- and *SRR*-isomers; $^{**}P < 0.01$ (by *t*-test). (B) Change in concentration of *RRR*- (◆) and *SRR*- (□) α-TQ in rat adrenal glands after oral administration of 2-*ambo*-α-Toc. For abbreviations see Figure 1.

this experiment, we established the determination of α-Toc and α-TQ stereoisomers in biological specimens by chiral HPLC. In plasma and adrenal glands, the concentration of *RRR*-α-TQ was not significantly different from that of *SRR*-α-TQ, but the *SRR*-α-TQ content in liver was higher than that of *RRR*-α-TQ up to 6 h after oral administration (Fig. 4). Therefore, we suggested that the turnover of *SRR*-α-Toc was more rapid than that of *RRR*-α-Toc in rat liver.

The mechanism of α-Toc metabolism is suggested as follows: α-Toc is oxidized to α-TQ first; then it is reduced to α-TQH, which is followed by degradation of the side chain. Finally, these are produced as the Simon metabolites. The Simon metabolites were first found in the urine of rabbits (20), in which they are present mainly as glucuronide conjugates. Dutton *et al.* (21) suggested that Simon metabolites are oxidation products derived from α-TQ. In this study, we investigated the distribution only of α-TQ stereoisomers *in vivo*, not of α-TQH stereoisomers. However, because the total α-TQ content in liver was less than 1% of the total α-Toc content, we assumed that a part of α-Toc might be metabolized into other types of metabolite (e.g., the degradation of the side chain without oxidative splitting of the chroman ring). Schultz *et al.* (22) reported that 2,5,7,8-tetramethyl-2-(2′-carboxyethyl)-6-hydroxy-chroman (α-CEHC) was the major urinary metabolite of α-Toc. They suggested that α-Toc can undergo ω-oxidation without oxidation and opening of the chroman ring, and that this pathway will be responsible for generation of the major urinary α-Toc metabolite in healthy humans.

In conclusion, we established a procedure to simultaneously determine *RRR*- and *SRR*-α-Toc and their quinones in rat plasma and tissues by chiral HPLC and investigated the distributions of α-Toc and α-TQ stereoisomers *in vivo*. We found that *RRR*- and *SRR*-α-TQ were present in very low concentrations in tissues, compared with *RRR*- and *SRR*-α-Toc. However, the concentrations of *RRR*- and *SRR*- α-TQ in each tissue were not significantly different. Therefore, we suggest that the formation of α-TQ *in vivo* is not different from the *RRR*- and *SRR*-isomers of α-Toc and that the formation of α-TQ had no effect on the proportion of α-Toc stereoisomers present *in vivo*. Furthermore, we conjecture that the *SRR*-isomer is rapidly metabolized into other types of metabolites, except α-TQ.

Using *SRR*-α-[5-methyl-^{14}C]Toc, we are now studying the differences in metabolism of *RRR*- and *SRR*-α-Toc in the rat.

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COMMUNICATION

The Occurrence and Possible Significance of Diacylglyceryl Ether Lipids in Abalone

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ABSTRACT: Gonadal and foot tissues of the green abalone, *Haliotis fulgens,* raised on artificial diets, were analyzed for lipids using thin-layer chromatography/flame-ionization detection and gas chromatography–mass spectrometry (GC–MS). Diacylglyceryl ether (DAGE) was 0.7% of total lipids in the gonad. The major alkyl constituents of the glyceryl ether diols in the gonad (as % of total diols) were 16:0 (38%) and 18:1 (36%). While levels of DAGE in the abalone foot were below flame-ionization detection limits, glyceryl ether diols from them were detected using the more sensitive GC–MS procedure. The major diol components in the foot were 18:0 (39%) and 18:1 (32%). To our knowledge, this is the first report of DAGE in abalone tissues. Although the precise role of DAGE in abalone remains to be determined, a possible structural role may exist.

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Knowledge of lipid composition provides a basis for understanding trophodynamic interactions and thereby possible nutritional requirements, energy storage, and buoyancy strategies of marine organisms. The most common lipids and those most often studied have been fatty acids, sterols (ST), triacylglycerols (TAG), phospholipids, and wax esters (WE). Research and identification of ether-containing phospholipids in molluscs include studies performed by Koizumi *et al.* (1) on the oyster *Crassostrea gigas* and by Sugiura *et al.* (2) on a number of invertebrate species. Earlier pertinent studies on ether-containing phospholipids include those of the aquatic snail *Lymnaea stagnalis* (3) and two species of terrestrial slug (4). Diacylglyceryl ether (DAGE) is generally an uncommon neutral lipid in marine organisms, which is reflected in the paucity of literature concerning it. DAGE has recently been reported to occur in pteropod molluscs at 28% of the total lipid (5).

Lipid studies performed on abalone, a commercially important fishery and mariculture family (6), include those by Uki *et al.* (7–10), Dunstan *et al.* (11), and other authors (12, 13). Except for the suggestion that glyceryl ether phospholipids may be present in the abalone *Haliotis midae* (14) and discussion in a study by Floreto *et al.* (15), all previous studies have neither reported nor quantified the wider complex lipid class composition, including DAGE, in abalone. This work reports on lipid class distributions, including the occurrence of DAGE, in the gonad and foot of the green abalone, *H. fulgens*.

MATERIALS AND METHODS

Sample description. Female green abalone, *H. fulgens*, were supplied during December, 1996, by Marine Bioculture, Leucadia, California. Ovary tissue from two fully ovigerous individuals was carefully ablated from the underlying hepatopancreas. Foot columellar tissue (partly cartilagenous) was obtained from the same individuals. Samples were immediately frozen in a −70°C Kelvinator freezer and lyophilized in a Virtis Freezemobile II lyophilizer at the Scripps Institution of Oceanography, California, in the laboratory of Dr. V. Vacquier.

Lipid extraction. During March, 1997, lipid analyses were conducted on the lyophilized tissues. Samples were homogenized with a mortar and pestle and subsamples taken for analysis. Tissues were rehydrated with the addition of water and left standing for 1 h. Rehydration prior to extraction results in improved lipid yields in comparison to nonrehydrated lyophilized samples (16). Samples were quantitatively extracted overnight using a modified Bligh and Dyer one-phase methanol/chloroform/water extraction (17).

Lipid classes. Lipid classes were determined with an Iatroscan MK V TH-10 thin-layer chromatograph–flame-ionization detector (18), utilizing two solvent systems for lipid separation. The primary solvent system was hexane/diethyl ether/acetic acid (60:17:0.1 by vol), a mobile phase resolving nonpolar compounds such as WE, TAG, free fatty acids, and ST. A second nonpolar system of hexane/diethyl ether (96:4, vol/vol) resolved hydrocarbons, WE, TAG, and DAGE. The flame-ionization detector was calibrated for each lipid class with phosphatidylcholine, cholesterol, cholesteryl oleate, oleic acid, squalene, triolein, and DAGE that had been purified from shark liver oil; 0.1–10 µg range. Glyceryl ether diol composition (1-*O*-alkyl glycerols) was determined using a Fisons MD 800 gas chromatograph–mass spectrometer (GC–MS) (Manchester, United Kingdom). The GC–MS was fitted with an on-column injector and operated in scan mode

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Abbreviations: DAGE, diacylglyceryl ether; GE, 1-*O*-alkyl glycerols; ST, sterols; TAG, triacylglycerols; WE, wax esters.

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with an ionization voltage of 70 eV. Techniques used to isolate and analyze these components are described further by Bakes and Nichols (19).

RESULTS

Lipid classes. TAG was the major lipid class in the abalone gonadal tissue (62.9%, as percentage of total lipid, Table 1). The foot was dominated by polar lipids, essentially all phospholipids (74.8%), whereas TAG were low (<0.05%). ST were also abundant in the foot tissue (23.5%). All samples contained WE, although the gonad (4.3%) contained on average 43 times that in the foot (0.1%). Gonad contained small amounts of DAGE (0.7%, 0.9 mg/g, dry mass) and long-chain alcohol (0.2%). Free fatty acids were present (1–4%) in all samples (Table 1).

1-O-alkyl glycerols. In abalone gonad the major alkyl glyceryl ether diols (GE) were 16:0 (38.1%, as percentage of total GE, Table 2) and 18:1 (36.1%); minor GE included 16:1, 17:1, 18:0, 20:1, and 20:0. The abalone foot tissue also contained GE, with 18:0 (39.4%) and 18:1 (31.5%) most prominent, as well as 16:1, 16:0, 20:1, and 20:0 (Table 2). A representative mass spectrum of the 17:1 diol and a plot of the ion *m/z* 205 characteristic of the diols are shown in Figure 1. A [M − 147] ion (e.g., *m/z* 327, 17:1 GE) provided additional information on the alkyl chain length (20). Methoxyethers were not detected.

Sterols. In gonad tissue, the main ST was cholesterol (97.4%, as percentage of total ST, Table 3), with small amounts of cholestanol and desmosterol. The foot lipids were dominated by cholesterol (97.9%), and minor ST included dehydrocholesterol, cholestanol, desmosterol, 24-methylenecholesterol, and fucosterol (Table 3).

TABLE 1
Lipid Classes (% of total lipids) of *Haliotis fulgens*

	Percentage composition	
Lipid Class	Gonad	Foot
Wax ester	4.3 ± 0.4	0.1 ± 0.0
Long-chain alcohol	0.2 ± 0.0	—
Diacylglyceryl ether	0.7 ± 0.1	—
Triacylglycerol	62.9 ± 0.9	Trace[a]
Free fatty acid	3.2 ± 0.8	1.6 ± 0.2
Sterol	4.4 ± 0.1	23.5 ± 0.3
Polar lipid	24.4 ± 1.4	74.8 ± 0.4
Total	100.0	100.0

[a]*n* = 2; data presented as mean ± SD. Trace: less than 0.05%.

TABLE 2
Alkyl Glyceryl Ether Diol Composition (% of total diols) of *Haliotis fulgens*

	Percentage composition	
Ether alkyl chain	Gonad	Foot
16:1	8.6 ± 0.2	5.8 ± 3.9
16:0	38.1 ± 0.7	17.8 ± 1.2
17:1	1.9 ± 0.4	—
17:0	0.5 ± 0.1	Trace[a]
18:1	36.1 ± 0.3	31.5 ± 5.4
18:0	8.3 ± 0.1	39.4 ± 11.0
20:1	4.0 ± 0.1	2.0 ± 0.2
20:0	2.4 ± 0.0	3.5 ± 0.6
Total	100.0	100.0

[a]*n* = 2; data presented as mean ± SD. Trace: confirmed by gas chromatography–mass spectrometry.

TABLE 3
Sterol Composition (% of total sterols) of *Haliotis fulgens*[a]

	Percentage composition	
Sterol	Gonad	Foot
Dehydrocholesterol	—	0.5 ± 0.0
Cholesterol	97.4 ± 0.0	97.9 ± 0.7
Cholestanol	1.1 ± 0.0	0.3 ± 0.0
Desmosterol	1.4 ± 0.1	0.5 ± 0.1
24-Methylenecholesterol	—	0.5 ± 0.3
Fucosterol	—	0.4 ± 0.5
Total	100.0	100.0

[a]*n* = 2; data presented as mean ± SD.

DISCUSSION

The high proportions of polar lipid (essentially phospholipids) and of ST in the foot signify that it is composed mostly of membrane lipid, whereas the markedly high abundance of TAG in abalone gonad tissue suggests an energy storage function for transference to larvae. The main energy reserves for adult abalone (*H. cracherodii*) are stored in the foot tissue as glycogen, rather than lipid (21). Our results indicate the same energy storage pathway is also used by *H. fulgens*. In addition, the gonad is a temporally variable tissue, which at times is almost nonexistent. Therefore, TAG may function as an energy reserve in the yolk in lecithotrophic larvae of this mollusc. Observations on six California species of abalone (*H. corrugata*, *H. cracherodii*, *H. fulgens*, *H. kamtschatkana*, *H. rufescens*, and *H. sorenseni*) provided evidence that pigments in free swimming (veliger) larvae are derived from the parental lipids in the yolk (22). If fat-soluble carotenoids are passed to larvae, it follows that other lipids would be as well. The presence of WE in abalone and their macroalgal diet (Nelson, M., unpublished data) suggests a dietary source, and it appears that WE are selectively incorporated into the gonads. WE may also be transferred to larvae, although this lipid class has low metabolic activity and typically serves as a long-term energy reserve. The presence of small proportions of DAGE in ovary tissue is intriguing. DAGE has been suggested to have a buoyancy role in some molluscs (5) and in other selected marine organisms (23). However, in abalone the role of DAGE observed at such low levels is unclear, although a buoyancy role is not anticipated. Other molluscs reported to contain DAGE include sea mussels (24) and *Octopus dofleini* (25).

The detection of GE in abalone foot lipids confirms the presence of DAGE, which was present at only trace

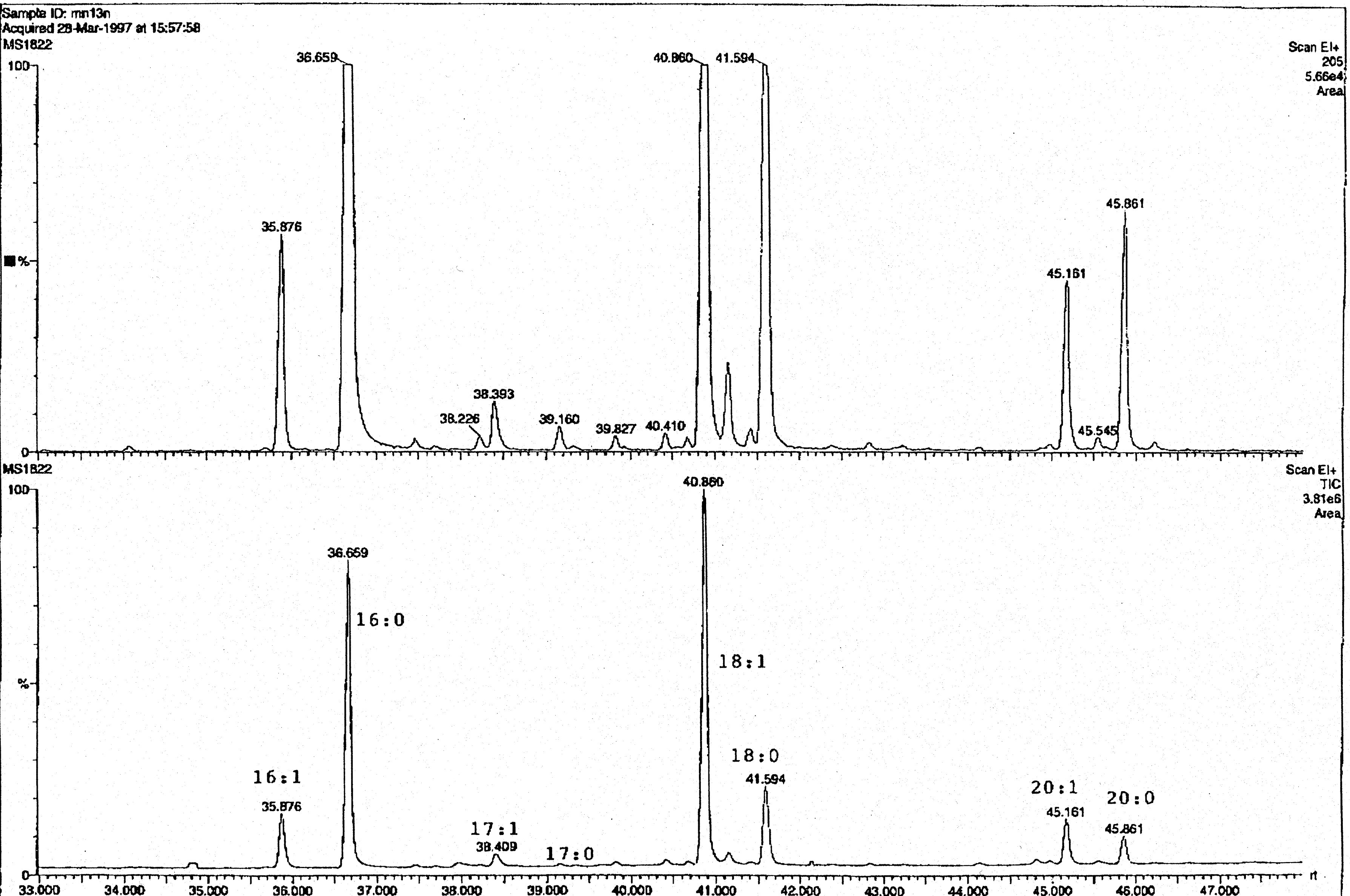

FIG. 1. (Below) Partial total recombinant ion chromatogram of glyceryl ether fraction from *Haliotus fulgens* as diol *O*-trimethylsilyl ethers. (Above) Partial recombinant ion chromatogram for *m/z* 205.

amounts(<0.05%), and was therefore not detected by thin-layer chromatograph–flame-ionization detector. A comprehensive taxonomic study by Isay *et al.* (26) revealed the occurrence of α-glyceryl ethers in 22 molluscs, including several species of limpets (*Collisella dorsuosa*, *Collisella* sp., and *Acmaea pallida*), which are archaeogastropods. This order also includes *Haliotis*. The major DAGE alkyl diol detected in abalone foot tissue in the present study was 18:0 (39.4%). In contrast to the foot, the dominant DAGE alkyl diol in abalone gonad was 1-hexadecylglyceryl ether (16:0; 38.1%), which was also found at high levels in three species of pteropods (5). Almost equally abundant in abalone gonad was the DAGE with 18:1 alkyl diol (36.1%). High proportions of 16:0 and octadec-9-enyglyceryl ether [18:1n-9c] were also found in the deep sea squid, *Moroteuthis robusta* (27). Furthermore, the ovaries of two asteroids were reported to contain 18:0, 20:1, and 16:0 as predominant DAGE diol components (28). The reasons for the difference between tissues in alkyl chain profiles are unknown, however these findings may provide insight to diet of and biosynthetic pathways in *H. fulgens*.

The major ST detected was cholesterol. The high proportions of phytol in algae (Nelson, M., unpublished data) is reflected in the very high proportions of cholesterol (Table 3) in sterols of abalone tissue, supporting the suggestion that abalone can convert phytosterols into cholesterol (29). The fucosterol common in algal diets was likely also converted to cholesterol or desmosterol, an intermediate.

Although only present in small quantities, the occurrence of DAGE in abalone tissue implies a possible structural requirement for it. These observations provide an impetus to clarify its function. DAGE may be synthesized by the abalone or it may be of dietary origin. However, it is noteworthy that neither DAGE nor GE were detected in macroalgae which are commonly part of the farmed abalone diet (Nelson, M., unpublished data). These data suggest that artificial diets for abalone culture may benefit from inclusion of small amounts of DAGE. Future research will examine the presence of DAGE in abalone larvae and juveniles, as well as in young adults undergoing gonad development. Their diet will also be examined, including macroalgae and epiphytic organisms associated with the macroalgae. These and other experiments will assist in elucidating the role of DAGE in abalone.

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ARTICLE

Fatty Acid Metabolism in Marine Fish: Low Activity of Fatty Acyl Δ5 Desaturation in Gilthead Sea Bream *(Sparus aurata)* Cells

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ABSTRACT: Marine fish have an absolute dietary requirement for C_{20} and C_{22} highly unsaturated fatty acids. Previous studies using cultured cell lines indicated that underlying this requirement in marine fish was either a deficiency in fatty acyl Δ5 desaturase or $C_{18–20}$ elongase activity. Recent research in turbot cells found low $C_{18–20}$ elongase but high Δ5 desaturase activity. In the present study, the fatty acid desaturase/elongase pathway was investigated in a cell line (SAF-1) from another carnivorous marine fish, sea bream. The metabolic conversions of a range of radiolabeled polyunsaturated fatty acids that comprised the direct substrates for Δ6 desaturase ([1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3), $C_{18–20}$ elongase ([U-^{14}C]18:4n-3), Δ5 desaturase ([1-^{14}C]20:3n-6 and [U-^{14}C]20:4n-3), and $C_{20–22}$ elongase ([1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3) were utilized. The results showed that fatty acyl Δ6 desaturase in SAF-1 cells was highly active and that $C_{18–20}$ elongase and $C_{20–22}$ elongase activities were substantial. A deficiency in the desaturation/elongation pathway was clearly identified at the level of the fatty acyl Δ5 desaturase, which was very low, particularly with 20:4n-3 as substrate. In comparison, the apparent activities of Δ6 desaturase, $C_{18–20}$ elongase, and $C_{20–22}$ elongase were approximately 94-, 27-, and 16-fold greater than that for Δ5 desaturase toward their respective n-3 polyunsaturated fatty acid substrates. The evidence obtained in the SAF-1 cell line is consistent with the dietary requirement for C_{20} and C_{22} highly unsaturated fatty acids in the marine fish the sea bream, being primarily due to a deficiency in fatty acid Δ5 desaturase activity.

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The essential fatty acid (EFA) requirements of freshwater and marine fish have long been known to differ qualitatively, for in freshwater fish 18:3n-3 and/or 18:2n-6 can satisfy the EFA requirement, whereas marine species require the longer-chain highly unsaturated fatty acids (HUFA) 20:5n-3 and 22:6n-3 to be supplied by the diet for optimal growth (1–3). This suggested that there was a difference between freshwater and marine fish in the fatty acid desaturation/elongation pathway (4,5). Dietary conversion studies performed on turbot *in vivo* indicated that marine fish were unable to produce 20:4n-6 and 20:5n-3 from dietary 18:2n-6 and 18:3n-3, respectively (6–8). However, the *in vivo* experiments were unable to locate precisely any potential deficiency in the fatty acid desaturation/elongation pathway.

The difference in fatty acid metabolism between freshwater and marine fish observed *in vivo* was also present in cultured cell lines (9). Studies involving supplementation of turbot fin (TF) cells in culture, compared to both rainbow trout cells (RTG-2) and Atlantic salmon cells (AS), with various n-3 and n-6 polyunsaturated fatty acids (PUFA) showed that a relative deficiency in the desaturase/elongase pathway in TF cells was located either at the C_{18} to C_{20} elongase ($C_{18–20}$ elongase) multienzyme complex or the fatty acyl Δ5 desaturase (9,10). To establish more precisely a deficient step in the desaturase/elongase pathway, the direct substrates for all the component enzymes in the pathway are required so that each activity can be assayed and determined in isolation from the influence of preceding activities in the pathway. However, isotopes for two crucial fatty acid substrates, 18:4n-3 (the direct substrate for $C_{18–20}$ elongase) and 20:4n-3 (the direct substrate for Δ5 desaturase) were not available commercially. Recently, Ghioni *et al.* (11) biosynthesized high-purity [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 and provided good evidence that the deficiency in TF cells was located at the $C_{18–20}$ elongase and not the Δ5 desaturase.

To date, a deficiency in marine fish fatty acid metabolism has been characterized in only one species, turbot. Results from various *in vivo* studies involving injection of radiolabeled fatty acid precursors such as [1-^{14}C]18:3n-3 and [1-^{14}C]20:5n-3 into other species such as gilthead sea bream (*Sparus aurata*) and golden grey mullet (*Liza aurata*) confirmed that an apparent deficiency in the desaturation/elongation pathway was present in those species and supported the view that it was common to all marine fish (12–14). However, as with the *in vivo* studies in turbot, Mourente and Tocher (12–14) were unable to identify the specific enzymic location responsible for a deficiency in the pathway. Recently, the

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Abbreviations: AS, Atlantic salmon cells; BHT, butylated hydroxytoluene; BSA, bovine serum albumin; EFA, essential fatty acid; FAF-BSA, fatty acid free-BSA; FAME, fatty acid methyl ester; FBS, fetal bovine serum; HBSS, Hank's balanced salt solution (without Ca^{2+} and Mg^{2+}); HPTLC, high-performance thin-layer chromatography; HUFA, highly unsaturated fatty acids (≥C_{20} with ≥3 double bonds); PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; RTG-2, rainbow trout cells; SAF-1, seam bream cell line, developed from fin tissue without immortalization; TF, turbot fin cells; TLC, thin-layer chromatography.

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availability of a cell line from sea bream, SAF-1, combined with the availability of [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 isotopes, has enabled the deficiency in the pathway to be characterized in another marine fish. We report here that, in contrast to TF cells, the primary deficiency in the desaturation/elongation pathway in SAF-1 cells from gilthead sea bream was clearly identified at the level of the fatty acyl Δ5 desaturase.

MATERIALS AND METHODS

Cell line and culture conditions. The gilthead seabream (*S. aurata* L.) cell line, SAF-1, developed from fin tissue without immortalization, was kindly provided by Dr. M.C. Alvarez (Department of Cell Biology and Genetics, University of Malaga, Spain) (15). The cell line displays fibroblast-like morphology and had been subcultured approximately 120 times over a 2-yr period. Karyotype and DNA content indicate a predominantly euploid and stable cell population. The cells were routinely maintained in Leibovitz L-15 medium supplemented with 2 mM glutamine, antibiotics (50 I.U. mL^{-1} penicillin and 50 mg·mL^{-1} streptomycin) and 10% fetal bovine serum (FBS). Cells were cultured at 22°C in 75 cm^2 sealed plastic tissue culture flasks (Corning Costar, High Wycombe, United Kingdom) and were routinely subcultured within 24 h of reaching confluence at a seeding density of 1×10^5 cells·cm^{-2}. Unless otherwise stated, for each experimental sample, 3×75 cm^2 flasks were seeded and this experiment was repeated three times to obtain the replicates. Therefore, all results are means ± SD of three such experiments.

Cell harvesting and lipid extraction. Medium was aspirated and the cultures washed twice with 20 mL of ice-cold Hanks' balanced salt solution without calcium and magnesium (HBSS). Cells were harvested by trypsinization with 0.05% trypsin/0.5 mM EDTA, washed with 5 mL ice-cold HBSS; and samples were taken for cell counting (16) and protein determination according to the method of Lowry *et al.* (17) after incubation with 0.25 mL of 0.25% (wt/vol) sodium dodecyl sulfate/1 M NaOH for 45 min at 60°C. The cells were washed with 5 mL HBSS containing 1% fatty acid-free bovine serum albumin (FAF-BSA), and total lipid was extracted from the cell pellets with 5 mL ice-cold chloroform/methanol (2:1, vol/vol) containing 0.01% butylated hydroxytoluene (BHT) as antioxidant, according to Folch *et al.* (18) as described in detail previously (19). Lipid content was determined gravimetrically after 1 h vacuum desiccation; lipid was resuspended in chloroform/methanol (2:1, vol/vol) containing BHT at a concentration of 10 mg·ml^{-1}, and samples were stored at −20°C prior to analyses.

Lipid class analysis. Lipid classes were separated by one-dimensional, double-development high-performance thin-layer chromatography (HPTLC) using methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) and hexane/diethyl ether/acetic acid (85:15;1.5, by vol) as developing solvents as described previously (20). Lipid classes were quantified by charring (21) followed by scanning densitometry using a Shimadzu CS-9000 dual wavelength flying spot scanner and DR-2 recorder (22).

Fatty acid analyses. Fatty acid methyl esters (FAME) were prepared from total lipid by acid-catalyzed transmethylation at 50°C for 16 h (23) and extracted and purified as described previously (20). FAME were analyzed in a Carlo Erba Vega GC6000 gas chromatograph (Carlo Erba Strumentazione, Milan, Italy) equipped with a CP Wax 51 fused-silica capillary column (50 m × 0.32 mm i.d., Chrompack U.K. Ltd., London), with on-column injection using hydrogen as carrier gas and a biphasic thermal gradient from 50 to 225°C. FAME were identified by comparison with known standards and a well-characterized fish oil and by reference to published data as described previously; they were quantified using a Carlo Erba DP800 data processor (20). All solvents contained 0.01% BHT as an antioxidant.

Incubation of cultures with ^{14}C-labeled PUFA and incorporation of radioactivity into total lipid. Cell cultures were routinely grown in 75 cm^2 flasks in medium containing 10% FBS and subcultured into medium containing 2% FBS 24 h prior to experimentation. All experiments were performed in triplicate with three flasks for [1-^{14}C]PUFA and one flask for [U-^{14}C]PUFA per sample per experiment. The medium was aspirated, cultures were washed with Dulbecco's phosphate-buffered saline (PBS), and fresh Leibovitz L-15 medium, as above, was added. The [1-^{14}C]PUFA (18:2n-6, 18:3n-3, 20:3n-6, 20:4n-6, and 20:5n-3) (0.25 µCi per flask; concentration, 0.35 µM) were added to the cell cultures bound to FAF-BSA in medium (24). The [U-^{14}C]PUFA (18:4n-3 and 20:4n-3) were both obtained as methyl esters and because of the low yield that characterized their production, with little mass available, these substrates were added directly as methyl esters in 50 µL of ethanol) (0.25 µCi per flask; concentration approximately 0.1 µM), to avoid the losses associated with saponification, purification of unesterified fatty acid, and sterile filtration (11). Preliminary experiments incubating cells with 25 µM unlabeled 18:4n-3 as fatty acid salt/BSA complex and as methyl ester in ethanol showed no difference in the metabolism to 20:4n-3, after 6 d of culture, and no accumulation of methyl ester in cellular lipids (11). Therefore, these two ways of presenting and delivering radiolabeled PUFA to the cells in culture can be considered equivalent under the conditions used in this study.

After addition of isotope, incubation continued for 6 d at 22°C. Cells were harvested, lipid extracted as above and the radioactive content of total lipid determined in three aliquots of 5 µL in mini-vials containing 2.5 mL scintillation fluid (Ecoscint A; National Diagnostics, Atlanta, GA) using a Tri-Carb 2000CA liquid scintillation spectrophotometer (United Technologies Packard). Results were corrected for counting efficiency and quenching using an appropriate calibration curve. All solvents contained 0.01% BHT as antioxidant.

Incorporation of radioactivity into glycerophospholipid classes. Samples of total lipid (100 µg) were applied as 1-cm streaks to HPTLC plates, and lipid classes separated by the one-dimensional, double-development method described

above (20). After desiccation, the lipid classes were visualized by brief exposure to iodine vapor, and areas corresponding to phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine, phosphatidylinositol, phosphatidic acid/cardiolipin, and total neutral lipid (the combined free fatty acid, triacylglycerol, and steryl ester areas) were scraped into scintillation vials containing 2.5 mL scintillation fluid and radioactivity determined as above.

Incorporation of radioactivity into PUFA. Total lipid extracts were transmethylated overnight at 50°C in methanolic sulfuric acid (23) and FAME extracted and purified as described above. TLC plates were impregnated by spraying with 2 g silver nitrate in 20 mL acetonitrile and activated at 110°C for 30 min. FAME were applied as 1-cm streaks and plates developed with toluene/acetonitrile (95:5, vol/vol) to separate PUFA (25). Autoradiography was performed using Kodak MR2 film for 7 d at room temperature. Silica corresponding to individual FAME was scraped into scintillation vials containing 2.5 mL scintillation fluid and radioactivity determined as described above.

Preparation of [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3. [U-^{14}C]18:4n-3 was prepared as described in detail by Ghioni *et al*. (11). Briefly, the unicellular marine alga *Isochrysis galbana* (Parke) (S.M.B.A. strain No. 58 C.C.A.P. strain 927/1) was incubated with sodium [^{14}C]bicarbonate and total lipid extracted. The 18:4-rich glycerolipids (mono- and digalactosyldiacylglycerols) were purified by TLC, transmethylated, and FAME extracted as described previously (11,20). FAME were separated by argentation TLC, individual FAME identified by autoradiography; and the [U-^{14}C]18:4n-3 methyl ester was eluted and quantified (11). [U-^{14}C]20:4n-3 was biosynthesized from [U-^{14}C]18:4n-3 as described by Ghioni *et al*. (11). Briefly, AS cells were incubated with [U-^{14}C]18:4n-3 methyl ester; total lipid was extracted and transmethylated, and FAME were separated by argentation TLC as above. Both [U-^{14}C] isotopes had specific activities of approximately 12 mCi/mmol and were >99% pure, determined by radio-gas chromatography as described by Buzzi *et al*. (26).

Materials. Sodium [^{14}C]bicarbonate (~50 mCi·mmol^{-1}) was purchased from ICN Biomedicals Ltd. (Thame, United Kingdom). [1-^{14}C] PUFA (all 50–55 mCi·mmol^{-1} and >98% pure) were obtained from NEN Life Science Products (Hounslow, United Kingdom). Leibovitz L-15 medium, HBSS, Dulbecco's modification phosphate-buffered saline (wtihout Ca^{2+} and Mg^{2+}), glutamine, penicillin, streptomycin, FBS, trypsin/EDTA, FAF-BSA, and BHT were obtained from Sigma Chemical Co. Ltd. (Poole, United Kingdom). TLC plates (20 cm × 20 cm × 0.25 mm) and HPTLC plates (10 cm × 10 cm × 0.15 mm), pre-coated with silica gel 60, were obtained from Merck, (Darmstadt, Germany). All solvents were of high-performance liquid chromatography grade and were obtained from Rathburn Chemicals (Walkerburn, Peebleshire, Scotland).

Statistical analysis. All results are means ± SD of three experiments. Where indicated, data were subjected to one-way analysis of variance, and where appropriate the significance of differences was determined by Tukey's test. When necessary, data were subjected to arc-sin transformation prior to statistical analysis. Differences are reported as significant when $P < 0.05$ (27).

RESULTS

The lipid content and class composition of SAF-1 cells cultured in 10% FBS are shown in Table 1. Nearly 75% of the total lipid was polar lipid with almost 30% PC, 19% PE, 7.3% phosphatidylinositol, and 6.3% phosphatidylserine, whereas the neutral lipid was predominantly cholesterol (20.3%). The fatty acid composition of total lipid of SAF-1 cells contained over 41% monounsaturated fatty acids, with 18:1n-9 constituting 25.2%, and approximately 25% saturated fatty acids, predominantly 16:0 (15%) and 18:0 (8.5%) (Table 2). The PUFA were characterized by 10% n-9 PUFA (90% of which was 18:2n-9, the single most abundant PUFA in the cells), 11.1% n-3 PUFA (mainly 22:6n-3 and 20:5n-3), and 9.5% n-6 PUFA (primarily 20:4n-6).

The recovery of radioactivity in total lipid from SAF-1 cells incubated with [1-^{14}C]20:5n-3 was significantly greater than any of the other PUFA investigated (Table 3). There were numerical differences between the other PUFA but, irrespective of the way that the recovery data were presented, few were statistically significant although there was a trend for the recovery of radioactivity from cells incubated with [1-^{14}C]18:2n-6, [1-^{14}C]18:3n-3, and [1-^{14}C]20:4n-6 to be higher than the recovery of radioactivity from incubations with [1-^{14}C]18:4n-3, [1-^{14}C]20:3n-6, and [1-^{14}C]20:4n-3. All the PUFA were predominantly esterified into the main glycerophospholipid classes with between 88 and 98% of radioactivity being recovered in these classes (Table 4). Higher in-

TABLE 1
Lipid Content and Lipid Class Composition (percentage of total lipid) of SAF-1 Cells Grown at 22°C in Medium Supplemented with 10% Fetal Bovine Serum

Lipid	Content[a]
Total lipid content	
μg lipid/10^6 cells	59.8 ± 4.7
μg lipid/mg protein	135 ± 7
Lipid class composition	
Phosphatidylcholine	29.8 ± 0.8
Phosphatidylethanolamine	19.0 ± 0.6
Phosphatidylserine	6.3 ± 0.2
Phosphatidylinositol	7.3 ± 0.2
Phosphatidic acid/cardiolipin	4.5 ± 0.2
Sphingomyelin	2.4 ± 0.3
Sulfatides/cerebrosides	4.9 ± 0.3
Total polar lipids	74.2 ± 1.7
Total neutral lipids	25.8 ± 1.7
Cholesterol	20.3 ± 0.7
Triacylglycerol	1.7 ± 0.4
Steryl ester	3.8 ± 2.0
Free fatty acids	Trace

[a]Results are means ± SD (n = 3); Trace, <0.05%. SAF-1, gilthead seabream (*Spartus aurata* L.) cell line.

TABLE 2
Fatty Acid Compositions (percentage of weight) of Total Lipid from SAF-1 Cells Grown at 22°C in Medium Supplemented with 10% Fetal Bovine Serum

Fatty acid	Percentage[a]
14:0	1.0 ± 0.1
15:0	0.2 ± 0.0
16:0	15.0 ± 0.8
17:0	0.5 ± 0.2
18:0	8.5 ± 0.2
Total saturated	25.2 ± 1.1
16:1n-9	3.9 ± 0.5
16:1n-7	4.6 ± 0.2
18:1n-9	25.2 ± 1.0
18:1n-7	7.0 ± 0.3
20:1n-9	Trace
20:1n-7	0.1 ± 0.1
24:1	0.6 ± 0.1
Total monounsaturated	41.4 ± 1.6
18:2n-9	9.0 ± 0.9
20:2n-9	0.4 ± 0.2
20:3n-9	0.1 ± 0.1
22n-9	0.5 ± 0.4
Total n-9 PUFA	10.0 ± 1.0
18:2n-6	1.2 ± 0.2
18:3n-6	1.2 ± 0.2
20:2n-6	0.1 ± 0.1
20:3n-6	1.6 ± 0.1
20:4n-6	5.4 ± 0.5
Total n-6 PUFA	9.5 ± 0.6
18:4n-3	0.1 ± 0.1
20:4n-3	0.2 ± 0.2
20:5n-3	1.4 ± 0.9
22:5n-3	3.5 ± 0.1
22:6n-3	5.5 ± 0.7
Total n-3 PUFA	10.7 ± 1.7
Total dimethylacetals	1.7 ± 1.5
Total PUFA	30.2 ± 1.5
Total unidentified	1.5 ± 0.4

[a]Results are means ± SD (n = 3). PUFA, polyunsaturated fatty acids. For other abbreviation see Table 1.

TABLE 3
Recovery of Radioactivity in Total Lipid from SAF-1 Cells Incubated with ^{14}C-PUFA

	Recovery[a]	
^{14}C-PUFA	(pmol/million cells)	(pmol/mg protein)
[1-^{14}C]18:2n-6	3.82 ± 0.94[b]	1.14 ± 0.32[b]
[1-^{14}C]18:3n-3	3.71 ± 0.99[b]	1.22 ± 0.12[b]
[U-^{14}C]18:4n-3	1.36 ± 0.31[c]	0.44 ± 0.10[c]
[1-^{14}C]20:3n-6	2.43 ± 0.60[b,c]	0.76 ± 0.10[b,c]
[1-^{14}C]20:4n-6	3.14 ± 0.64[b,c]	1.18 ± 0.02[b]
[U-^{14}C]20:4n-3	1.95 ± 0.03[b,c]	0.63 ± 0.01[b,c]
[1-^{14}C]20:5n-3	6.53 ± 1.10[a]	2.21 ± 0.46[a]

[a]Results are means ± SD (n = 3). Data were subjected to one-way analysis of variance followed, where appropriate, by Tukey's multiple comparison test. Means in the same column with different superscript letters are significantly different (P < 0.05). For other abbreviations see Tables 1 and 2.

TABLE 4
Recovery of Radioactivity in Lipid Classes from SAF-1 Cells Incubated with ^{14}C-PUFA

	^{14}C-PUFA added[a]		
Lipid class	18:2n-6	18:3n-3	18:4n-3
PC	51.1 ± 1.1[c]	51.1 ± 0.8[c]	70.5 ± 1.8[a]
PE	18.1 ± 0.5[e]	20.4 ± 0.9[d,e]	10.2 ± 1.0[f]
PS	5.3 ± 0.5[b]	4.1 ± 0.3[c]	1.9 ± 0.2[d]
PI	5.1 ± 0.1[d,e]	5.3 ± 0.5[d]	3.0 ± 0.2[e]
PA/CL	12.8 ± 0.3[a]	8.9 ± 0.5[c]	2.4 ± 0.2[d]
TP	92.4 ± 0.2[b,c]	89.8 ± 0.3[c,d]	87.9 ± 3.2[c,d]
TN	7.6 ± 0.2[a,b]	10.2 ± 0.3[a]	12.1 ± 3.2[a]

	^{14}C-PUFA added			
Lipid class	20:3n-6	20:4n-6	20:4n-3	20:5n-3
PC	35.5 ± 0.9[d]	11.9 ± 1.4[f]	61.4 ± 2.0[b]	20.8 ± 1.1[e]
PE	26.6 ± 0.5[c]	43.0 ± 1.6[b]	22.0 ± 0.9[d]	63.2 ± 1.6[a]
PS	6.7 ± 0.3[a]	2.2 ± 0.2[d]	1.9 ± 0.2[d]	2.6 ± 0.1[d]
PI	15.0 ± 1.4[b]	32.8 ± 1.3[a]	4.6 ± 0.2[d,e]	8.8 ± 0.3[c]
PA/CL	10.8 ± 0.1[b]	8.1 ± 0.8[c]	2.4 ± 0.3[d]	2.8 ± 0.1[d]
TP	94.7 ± 0.2[b]	98.1 ± 0.1[a,b]	92.2 ± 2.9[b,d]	98.1 ± 0.2[a,b]
TN	5.3 ± 0.2[b,c]	1.9 ± 0.1[c]	7.8 ± 2.9[a,b]	1.9 ± 0.2[c]

[a]Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3). Data were subjected to one-way analysis of variance followed, where appropriate, by Tukey's multiple comparison test. Means for each individual lipid class with different superscript letters are significantly different (P < 0.05). CL, cardiolipin; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine; TN, total neutral lipids; TP, total polar lipids. For other abbreviations see Tables 1 and 2.

corporation into the glycerophospholipids was generally observed with the C_{20} PUFA compared to the C_{18} PUFA. Both [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 were predominantly incorporated into PE whereas all the other PUFA were incorporated predominantly into PC, these differences being statistically significant (Table 4). Significantly more [U-^{14}C]18:4n-3 and [U-^{14}C]20:4n-3 were incorporated into PC compared to the other PUFA; and significantly greater proportions of [1-^{14}C]20:4n-6 and, to a lesser extent, [1-^{14}C]20:3n-6 were incorporated into phosphatidylinositol compared to the other PUFA.

The main metabolic product of [1-^{14}C]18:2n-6 recovered from SAF-1 cells was 18:3n-6 (over 35% of radioactivity recovered in this fraction) whereas the main metabolic products of [1-^{14}C]18:3n-3 were 18:4n-3 and 20:4n-3 (almost 41 and 21% of radioactivity recovered in these products, respectively) (Table 5). With all the other PUFA, over 80% of the radioactivity was recovered in the form of the fatty acid originally added to the cells. With [U-^{14}C]18:4n-3, the only major products were the elongation products 20:4n-3 (13.2%) and 22:4n-3 (5.1%), with only 0.7% being desaturated to 20:5n-3. Similarly, with [1-^{14}C]20:3n-6, elongation products constituted almost 85% of the total metabolized. The main products of the metabolism of [U-^{14}C]20:4n-3 in SAF-1 cells were 22:4n-3 and the chain-shortened product, 18:4n-3 (Table 5). Both [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 were mainly elon-

TABLE 5
Metabolism of ^{14}C-PUFA by Desaturation and Elongation in SAF-1 Cells

Fatty acid	^{14}C-PUFA added		
	18:2n-6	20:3n-6	20:4n-6
18:2n-6	56.0 ± 1.6	N.D.	N.D.
20:2n-6	0.8 ± 0.1	N.D.	N.D.
18:3n-6	35.1 ± 1.8	N.D.	N.D.
20:3n-6	3.8 ± 0.3	84.1 ± 0.7	N.D.
22:3n-6	Trace	13.4 ± 0.8	N.D.
20:4n-6	2.5 ± 0.5	2.0 ± 0.2	93.4 ± 1.9
22:4n-6	1.8 ± 0.3	0.5 ± 0.1	5.5 ± 1.9
24:4n-6	N.D.	N.D.	1.1 ± 0.2
22:5n-6	N.D.	Trace	Trace

Fatty acid	^{14}C-PUFA added			
	18:3n-3	18:4n-3	20:4n-3	20:5n-3
18:3n-3	32.1 ± 1.0	N.D.	N.D.	N.D.
20:3n-3	1.9 ± 0.3	N.D.	N.D.	N.D.
18:4n-3	40.9 ± 0.9	81.0 ± 2.0	8.3 ± 1.4	N.D.
20:4n-3	20.7 ± 0.9	13.2 ± 1.4	85.8 ± 1.4	N.D.
22:4n-3	4.5 ± 0.3	5.1 ± 0.2	5.2 ± 1.7	N.D.
18:5n-3	N.D.	N.D.	N.D.	N.D.
20:5n-3	Trace	0.7 ± 0.5	0.7 ± 0.4	89.0 ± 0.7
22:5n-3	N.D.	N.D.	N.D.	9.9 ± 0.8
24:5n-3	N.D.	N.D.	N.D.	0.6 ± 0.1
22:6n-3	N.D.	N.D.	N.D.	0.4 ± 0.0

[a]Results are expressed as percentages of total radioactivity recovered and are means ± SD (n = 3). N.D., not detected; trace, <0.05%.

gated with only a small fraction of radioactivity being recovered as 22:6n-3 in [1-^{14}C]20:5n-3-supplemented cells. Table 6 is a summary of the results obtained in the isotope experiments and is derived from the data contained in Table 5 but presented as sums of products of each individual step in the desaturation/elongation pathway taking into account all the fatty acids that are derived from that step, irrespective of subsequent conversions. The results obtained for each enzyme activity when incubating the cells with the fatty acid which is its direct substrate (i.e., 18:4n-3 for $C_{18–20}$ elongase and 20:4n-3 for Δ5 desaturase) are of particular interest as they show the potential of each activity independent of the previous enzymes. Table 6 shows that considerable amounts of radioactivity are recovered as Δ6 desaturase products in SAF-1 cells incubated with the main fatty acid substates for Δ6 desaturase, [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6. Although the majority of the [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 metabolized to Δ6 desaturase products was not further metabolized, about 27 and 9% of the total radioactivity recovered in the case of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6, respectively, were elongated by $C_{18–20}$ elongase. Very little radioactivity from [1-^{14}C]18:2n-6 and, especially, [1-^{14}C]18:3n-3, was recovered in Δ5 desaturated products. When SAF-1 cells were incubated with the direct n-3 substrate of $C_{18–20}$ elongase, [U-^{14}C]18:4n-3, 19% was elongated but only 0.7% was recovered in Δ5 desaturated products. The very low activity of fatty acyl Δ5 desaturase in SAF-1 cells was confirmed using direct substrates for Δ5 desaturase, [1-^{14}C]20:3n-6 and

TABLE 6
Products of Desaturase and Elongase Activities in SAF-1 Cells Incubated with Various ^{14}C-Labeled PUFA[a]

Fatty acid added	Δ6 desaturase products	$C_{18–20}$ elongase products	Δ5 desaturase products	$C_{20–22}$ elongase products
[1-^{14}C]18:2n-6	43.2 ± 1.5	8.9 ± 0.5	4.3 ± 0.8	1.8 ± 0.3
[1-^{14}C]18:3n-3	66.1 ± 1.1	27.1 ± 0.6	Trace	4.5 ± 0.3
[U-^{14}C]18:4n-3	—	19.0 ± 2.1	0.7 ± 0.5	5.1 ± 0.2
[1-^{14}C]20:3n-6	—	—	2.5 ± 0.2	13.9 ± 0.7
[U-^{14}C]20:4n-3	—	—	0.7 ± 0.4	5.2 ± 1.7
[1-^{14}C]20:4n-6	—	—	—	6.6 ± 1.9
[1-^{14}C]20:5n-3	—	—	—	10.9 ± 0.8

[a]Results are presented as percentages of total radioactivity recovered and are means ± SD (n = 3). All fatty acid metabolites for a given step in the pathway were summed, irrespective of subsequent metabolism by another activity (e.g., for [1-^{14}C]18:2n-6, "Δ6 products" represents the sum of radioactivity recovered as 18:3n-6, 20:3n-6, 20:4n-6, and 22:4n-6). Trace, <0.05%.

[U-^{14}C]20:4n-3, where little radioactivity was recovered in Δ5 desaturated products (Table 6). $C_{20–22}$ elongase products were found in the cells with all the fatty acids used and in higher percentages compared to Δ5 desaturation products.

DISCUSSION

The results of the present study indicate that the sea bream cell line has a deficiency in the desaturation/elongation pathway similar in effect to that observed in turbot and sea bream *in vivo* and TF cells *in vitro*. Specifically, the results show, first, that the lipid and fatty acid compositions of cell lines derived from marine fish have several features that distinguish them from cell lines from freshwater fish; second, that one of the main factors underpinning these differences is a relative deficiency in the pathway for the desaturation and elongation of EFA in marine fish cells; and third, that although the effect of the deficiency is similar in different marine fish, its location in the pathway is different.

The first feature distinguishing cells from a marine fish was the lipid class composition. The SAF-1 cells cultured in 2% serum displayed a lipid content (59.8 ± 4.7 μg/10^6 cells) comparable to levels previously reported in other fish cell lines cultured in 2% serum, being similar to that observed in AS cells (50.4 ± 10.0 μg/10^6 cells) but slightly higher than that found in another marine fish cell line, TF, (41.4 ± 3.3) and lower than that found in RTG-2 (87.2 ± 6.0) (9,28). However, the proportion of neutral lipids in SAF-1 cells (25.8%) was very similar to that found in TF cells (27.3%), both of which were much lower than the levels found in the freshwater fish cell lines, 37.2 and 39.2% for AS (cell line prepared from freshwater fry stage) and RTG-2 cells, respectively, which were characterized by higher percentages of triacylglycerols (9,28).

Importantly however, the fatty acid composition of SAF-1 cells was characterized by the presence of substantial amounts of n-9 PUFA, 90% of which was 18:2n-9, the most abundant PUFA in SAF-1 cells. This is an identical feature to

that observed in TF cells, in which 18:2n-9 was also the most abundant PUFA and its level was increased to over 22% by reducing the serum supplement to 2% (9). In contrast, 18:2n-9 is a relatively minor PUFA in AS and RTG-2 cells, amounting to only 1.2 and 1.8%, respectively, in cells cultured in 2% serum (9,28). The presence of large amounts of this unusual PUFA (18:2n-9) in marine fish cells, compared to freshwater fish cells and mammalian cell lines, on its own strongly suggests a deficiency in fatty acid desaturation/elongation as a principal factor responsible for the differences in EFA requirements between marine and freshwater fish.

The higher level of total n-9 PUFA in TF cells compared to AS and RTG-2 cells was hypothesized to be due to the marine fish cells having a higher requirement for n-3 HUFA (4,29) and thus experiencing a greater EFA deficiency when grown in relatively n-3 HUFA-deficient, n-6 PUFA-rich mammalian serum (9). This, combined with the specific location of the apparent deficiency in the EFA desaturation/elongation pathway in marine fish, resulted in the specific accumulation of 18:2n-9 in TF cells. Although the level of 18:2n-9 is lower than in TF cells, the present data in SAF-1 cells support this hypothesis and indicate that high levels of 18:2n-9 may be a characteristic fatty acid marker for all marine fish cells in culture. Consistent with the hypothesis that marine fish cells require higher levels of n-3 HUFA, the two marine fish cell lines retained the highest proportions of n-3 PUFA in their total lipid, with n-3 PUFA accounting for 54 and 45% of total PUFA (not counting n-9 PUFA) in SAF-1 and TF cells, respectively, but only 38 and 34% in AS and RTG-2 cells, respectively (9,28). These observations in cell lines derived from marine fish are supported by the dietary traits of the marine species with both sea bream and turbot being carnivorous, specifically piscivorous, species with diets consisting almost entirely of smaller fish. As such their natural diets are rich in the long-chain n-3 HUFA, 20:5n-3 and 22:6n-3 (5,29,30). In contrast, freshwater salmonids, such as rainbow trout, have diets richer in C_{18} PUFA and much lower long-chain HUFA (4,29,31).

In contrast to the lipid and fatty acid composition data, the fatty acid incorporation data show surprisingly little difference between marine and freshwater cell lines. The data in Table 3 suggest (i) that the SAF-1 cells retained [1-^{14}C]20:5n-3 in their lipids to a greater extent than any other PUFA studied and (ii) that the PUFA, which are present normally in only very small amounts in fish lipids and are generally regarded as more transient intermediates in the PUFA pathway, specifically 18:4n-3, 20:3n-6 and 20:4n-3, were retained the least. This is consistent with the observed fatty acid composition of fish in general, with 20:5n-3 and 22:6n-3 being the major PUFA. However, these data represent the net recovery of radioactivity in the cells after incubation with the fatty acid isotopes. The radioactivity recovered will depend on the amount of fatty acid initially taken up by the cells, less the amount of fatty acid lost due to other metabolic processes such as oxidation or conversion to eicosanoids. Owing to the limited amount of pure U-^{14}C-labeled isotopes available, they were not used at exactly the same concentration as the 1-^{14}C-labeled isotopes, thereby possibly affecting the amount initially taken up by the cells. In addition, the 1-^{14}C-labeled isotopes lose the radiolabel after one round of β-oxidation whereas the U-^{14}C-labelled isotopes will not, also affecting the relative recoveries of radioactivity from the differently labeled isotopes. Therefore, some caution is required in directly comparing the data in Table 3. Previously, we have shown in several fish cell lines that there was little difference between the recovery of radioactivity when incubated with the 1-^{14}C-labeled isotopes of 18:2n-6, 18:3n-3, 20:4n-6, and 20:5n-3 (10).

The pattern of incorporation of the PUFA into lipid classes was very similar to that reported previously in AS, RTG-2, and TF cell lines (10). In that earlier study, 86–94%, and 96–98% of radioactivity from C_{18} PUFA and C_{20} PUFA, respectively, were recovered in polar lipids compared with figures of 88–92% for C_{18} PUFA and 92–98% for C_{20} PUFA obtained with SAF-1 cells in the present study (10). The preferential incorporation of C_{18} PUFA into PC was also observed in the earlier study as was the preferential incorporation of 20:4n-6 into PI and 20:5n-3 into PE (10). The present study has shown that the pattern obtained with 20:3n-6 was basically as expected for a C_{20} PUFA in general, but particularly similar to 20:4n-6, whereas the incorporation of 20:4n-3 was more similar to that for C_{18} PUFA. However, there was no major difference between the freshwater fish cell lines, AS and RTG-2, and the marine fish cell lines, TF and SAF-1, in the incorporation of PUFA into lipid classes. These results appear to indicate that the ability of the cells to metabolize the fatty acids *via* desaturation and elongation does not greatly influence their uptake, retention or distribution in cell lipids, at least at the low concentrations used in the present study.

The primary aim of the present study was to determine if the deficiency in the desaturase/elongase pathway, established in C_{18-20} elongase in TF cells (11), was identical in another marine fish cell line. The results obtained using the direct substrates for each individual enzymic activity in the pathway enabled the deficient step in SAF-1 cells to be identified. The fact that 43.2 and 66.1% of added [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3, respectively, were desaturated showed that SAF-1 cells expressed a highly active Δ6 desaturase. In addition, 19% of the direct substrate for C_{18-20} elongase, [U-^{14}C]18:4n-3, was elongated. In contrast, only 2.5 and 0.7% of added [1-^{14}C]20:3n-6 and [U-^{14}C]20:4n-3 were recovered as Δ5 desaturation products. Almost 7 and 11% of [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3, respectively, were C_{20-22} elongated. In summary, the apparent activities of Δ6 desaturase, C_{18-20} elongase, and C_{20-22} elongase were approximately 94-, 27-, and 16-fold greater than that for Δ5 desaturase toward their respective n-3 PUFA substrates. These results showed that, in contrast to TF cells, fatty acyl Δ5 desaturation was the step with the lowest activity in the pathway biosynthesizing 20:5n-3 (or 20:4n-6) in the SAF-1 cells.

The Δ5 desaturase activity in SAF-1 cells was particularly ineffective toward the n-3 substrate. This was unexpected, as

desaturases in general are normally more active toward n-3 fatty acids in mammals (32,33) and fish cells (10,11,34). Furthermore, the other enzymes in the pathway, Δ6 desaturase and the C_{18-20} and C_{20-22} elongases, showed greater activity toward n-3PUFA in SAF-1 cells.

Deficiencies in fatty acyl desaturase activities have precedents in terrestrial carnivores such as cats in which Δ6 and Δ5 desaturase activities may both be very low (35–38). It has been hypothesized that this situation may be an evolutionary adaptation to carnivorous diets rich in preformed C_{20} and C_{22} HUFA. This may be paralleled in fish because, as described above, the diets of the generally more herbivorous/omnivorous freshwater fish are rich in C_{18} PUFA and do not contain much C_{20} or C_{22} HUFA, whereas marine fish that are predominantly carnivorous consume diets rich in 20:5n-3 and 22:6n-3 (30,31). Therefore, the differences in the fatty acid desaturase/elongase pathways between fish species may be an evolutionary response to dietary differences. However, this cannot explain the why turbot should have low C_{18-20} elongase and sea bream low Δ5 desaturase.

In both cases of marine fish studied, Δ5 desaturase in SAF-1 cells and C_{18-20} elongase in TF cells, there is not a complete lack of the enzyme activity responsible for the deficiency in the pathway. This is now also known to be the case in cats. In early studies, a complete lack of Δ6 desaturation enzymatic activity in felines was originally reported (35–38), although recent and more sensitive studies conducted with deuterated fatty acids showed that a low level of Δ6 desaturase is present in the cat (39).

The present study provides good evidence that SAF-1 cells have a deficiency in fatty acyl Δ5 desaturase activity in contrast to TF cells in which C_{18-20} elongase is deficient. Both of these relative deficiencies in the fatty acid desaturation/elongation pathway are entirely consistent with the known EFA requirements of marine fish. However, it is important to determine if this is a reflection of the situation *in vivo* and if the cell lines are a model for the whole animal. Studies with mammalian cell lines have shown that three out of six expressed little Δ6 desaturase activity, Δ5 desaturase activity appeared to be absent in four cell lines, and only one cell line expressed any desaturation activity beyond Δ5 (40). An apparent loss of stearoyl-CoA Δ9 desaturase has been reported in cultured murine T lymphocytes (41). Therefore, experiments with stable isotopes, similar to those performed by other authors in felines (39) and humans (42,43) are required to confirm the deficiencies in fatty acid desaturation and elongation in marine fish *in vivo*.

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Screening of Lipase Inhibitors from Marine Algae

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ABSTRACT: The possible presence of an inhibitor of pancreatic lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) was screened in 54 marine algae. An active inhibitor, caulerpenyne, was purified from an extract of *Caulerpa taxifolia,* using ethyl acetate extraction, followed by successive chromatographies on ODS and silica gel columns. The purified inhibitor was identified by thin-layer chromatography, infrared and nuclear magnetic resonance spectroscopy. Caulerpenyne competitively inhibited lipase activities using emulsified triolein and dispersed 4-methylumbelliferyl oleate (4-MU oleate) as substrates. The concentrations producing 50% inhibition against triolein and 4-MU oleate hydrolysis were 2 mM and 13 μM, respectively. *In vivo,* oral administration of corn oil with or without caulerpenyne to rats demonstrated a reduced and delayed peak plasma triacylglycerol concentration with caulerpenyne.

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Pancreatic lipase is a key enzyme of dietary triacylglycerol absorption, hydrolyzing triacylglycerols to 2-monoacylglycerols and fatty acids. Typical substrates for these enzymes are long-chain triacylglycerols which are separated from the aqueous medium by the surface phase. Thus, lipase must be adsorbed on the lipid surface, and the nature of the surface of the substrate is an important factor for lipase activity. Therefore, amphoteric substances would be expected to influence the lipase reaction rate. There have been many reports of lipase inhibitors derived from natural materials, for example, proteins (1,2), phytic acid (3), and tannin (4). Most of these inhibitors are amphoteric substances. It is well known that bile salts and synthetic detergents (5) behave as inhibitors of lipolysis. Hydrophobic proteins, such as bovine serum albumin and β-lactoglobulin (6), inhibit lipase activity toward triacylglycerol substrates by competing for the substrate surface.

Only a few substances directly interact with lipases themselves, one example being lipstatin (7), from *Streptomyces toxytricini,* which strongly inhibits lipases. In this study, we report the isolation from marine algae of caulerpenyne, an inhibitor which interacts directly with pancreatic lipase.

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Abbreviations : 4-MU oleate, 4-methylumbelliferyl oleate.

MATERIALS AND METHODS

Materials. The enzyme substrates and reagents used were obtained as follows. Triolein, cholesterol oleate, taurocholic acid, pancreatic lipase (Type VI-S, from porcine pancreas), and 4-methylumbelliferyl oleate (4-MU oleate) were purchased from Sigma Co. (St. Louis, MO). Silica gel (Kiesel gel 60, Merck) was purchased from Kanto Chemicals (Tokyo, Japan). Dimethyl sulfoxide and octadecyl silica gel columns (Cosmosil 75C18-PREP) were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). A normal-phase high-performance liquid chromatographic column (YMC-pack SIL-06) was obtained from YMC Co. (Kyoto, Japan).

Measurement of lipase activity and inhibitory activity. Lipase activity was determined by measuring the rate of release of oleic acid from triolein (8). A suspension of 90 μmol triolein, 45 mg gum arabic, and 9.45 μmol taurocholic acid in 9 mL 0.1 M *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (pH 7.0, containing 0.1 M NaCl) was sonicated for 5 min. The assay system comprised the following components in a total volume of 0.2 mL: 0.05 mL enzyme solution, 0.05 mL inhibitor solution, 1 μmol triolein, 0.106 μmol taurocholic acid, 0.5 mg gum arabic, 20 μmol *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid, and 20 μmol NaCl. Incubation was carried out at pH 7.0 and 37°C for 30 min, and the amount of oleic acid produced was determined by the method of Zapf *et al*. (9) with a slight modification (10). The incubation mixtures were added to 3 mL of a 1:1 (vol/vol) mixture of chloroform/heptane containing 2% (vol/vol) methanol and extracted by shaking the tubes horizontally for 10 min in a shaker. The mixture was centrifuged (2000 × *g*, 10 min), the upper aqueous phase was removed, and 1 mL of copper reagent (10) was added to the lower organic phase. The tubes were then shaken for 10 min, the mixture was centrifuged (2000 × *g*, 10 min), and 1 mL of the upper organic phase, which contained the copper salts of the extracted oleic acid, was treated with 1 mL 0.1% (wt/vol) bathocuproine containing 0.05% (wt/vol) 3-*tert*-butyl-4-hydroxyanisole and the absorbance determined at 480 nm.

Lipase activity was also measured using 4-MU oleate as a substrate (11). The reaction mixture was prepared with 0.1 mL 0.1 mM 4-MU oleate and 0.05 mL inhibitor solution. The reaction was started by adding 0.05 mL lipase, all in a final volume of 0.2 mL. After incubation at 37°C for 15 min, 0.5

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mL of 0.1 M HCl and 1 mL of 0.1 M sodium citrate were added. The amount of 4-methylumbelliferone released by the lipase was measured fluorometrically at an excitation wavelength of 320 nm and an emission wavelength of 450 nm.

The concentration of the tested compounds giving 50% inhibition of the enzyme activity was estimated from the least-squares regression line of the plots of the logarithm of the concentration vs. the inhibitory activity.

Extraction from marine algae. Fresh marine algae were collected around Shikoku Island, Japan, in 1993–1994, freeze-dried, powdered, and kept at −20°C. Ten grams of the marine algal powder was extracted with 100 mL methanol (or ethyl acetate) with shaking at room temperature for 24 h. After filtration, the extract was evaporated *in vacuo*, dissolved in 2 mL dimethyl sulfoxide, and used to measure the lipase inhibitory activity.

Purification of inhibitor. Caulerpa taxifolia, obtained from a tropical fish store, was cultured in a tank. The cultured alga was freeze-dried and ground to give 100 g of powder and then extracted with 1000 mL of ethyl acetate. After filtration, the extract was evaporated *in vacuo*. The evaporated sample was dissolved in a small amount of acetonitrile, passed through an octadecyl silica gel column (Cosmosil 75C18-PREP, 40 g) to remove pigments such as chlorophyll, and eluted with acetonitrile. The eluate was concentrated *in vacuo*, applied to a silica gel column (Kiesel gel 60, 200 g), and eluted with a stepwise solvent system of *n*-hexane/ethyl acetate. The eluate from hexane/ethyl acetate (4:1, vol/vol) that exhibited lipase inhibitory activity was applied to a normal-phase high-performance liquid chromatographic column (YMC-pack SIL-06) and eluted with hexane/ethyl acetate (2:1, vol/vol). The fractions containing lipase inhibitory activity were pooled and concentrated to dryness *in vacuo*.

Oral administration of caulerpenyne. A suspension of 6 mL corn oil, 80 mg cholic acid, and 2 mg cholesteryl oleate in 6 mL water was sonicated for 5 min. Male Wistar King rats, weighing 150–190 g, were starved overnight, then divided into two groups, and 1 mL corn oil suspension was administered to each rat *via* a stomach tube (8). One group received this suspension containing 1 mL caulerpenyne (15 mg) solution and the control group received the suspension containing 1 mL water. After caulerpenyne administration, blood samples were collected from the tail vein or artery into heparinized microcapillary tubes, at regular intervals, and centrifuged immediately at 10,000 rpm for 5 min. Plasma triacylglycerol concentrations were determined using the Triglyceride E-Test (Wako Pure Chemical Industries, Osaka, Japan).

Statistics. The data were analyzed for statistical significance using Student's *t*-test.

RESULTS

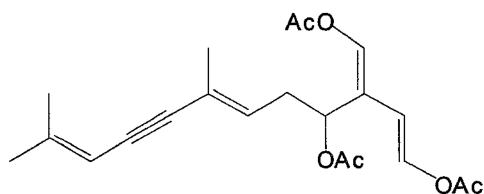
Screening of lipase inhibitors. Marine algae (54 species of natural materials) were extracted using methanol or ethyl acetate, and the inhibitory activities of these extracts were screened against triolein-hydrolyzing activity of porcine pancreatic lipase. The results from 27 species are shown in Table 1. Inhibitory activity was not observed in the extracts from *Gracilaria bursa-pastoris*, *G. chorda*, *G. textorii*, *Gymnogongrus flabelliformis*, *Eckloniopsis radicosa*, *Chaetomorpha crassa*, and algae of the genus *Laurencia* (data not shown). Higher activity was found in methanol extracts from *C. taxifolia* and *Asparagopsis tociformis.*

Purification of inhibitor. Since both the methanol and ethyl acetate extracts from *C. taxifolia* had relatively high lipase inhibitory activity, isolation of the lipase inhibitor from its ethyl acetate extract was attempted. Although the methanol extract had a higher lipase inhibitory activity than that of the ethyl acetate extract, it also contained a high amount of polyphenols or their oxidized compounds such as tannins. Since tannin-like compounds bind strongly to enzyme proteins and may inhibit their enzyme activity (12), we isolated the lipase inhibitor from the ethyl acetate extract. Purification was achieved by sequential chromatography on octadecyl silica gel, silica gel and normal-phase high-performance liquid chromatographic columns. The purified inhibitor (0.2 g, slightly yellowish oil) was obtained from 100 g of dried *C. taxifolia*. The purified inhibitor was identified as caulerpenyne by comparing its thin-

TABLE 1
Inhibition of Lipase Activity by Algal Extracts

Species	Inhibition activity (%)	
	Methanol extract	Ethyl acetate extract
Phaeophyta		
Cutleria cylindrica	88	14
Dictyopteris latiuscula	90	—[a]
D. prolifera	97	—
Hizikia fusiformis	71	21
Ishige okamurai	77	41
I. sinicola	76	22
Myelophycus simplex	62	49
Padina arborescens	52	—
P. crassa	—	98
Sargassum muticum	94	—
S. ringgoldianum	85	—
S. thunbergii	63	—
Chlorophyta		
Caulerpa okamurae	94	—
C. toxifolia	100	52
Codium latum	95	3
C. pugniformis	94	5
Rhodophyta		
Asparagopsis taxiformis	100	10
Chondrus giganteus	51	—
Eucheuma amakusaensis	70	39
E. serra	44	31
Gloiopeltis tenax	94	—
Gracilaria verrucosa	—	41
Gracilariopsis chorda	58	5
Hypnea charoides	100	1
H. japonica	88	—
Lomentaria catenata	43	13
Porphyra tenera	63	—

[a]Not detected.



SCHEME 1

layer chromatographic, infrared and nuclear magnetic resonance spectroscopic, and electron impact-mass spectrometric data with those reported previously (13) (Scheme 1). ^{1}H and ^{13}C nuclear magnetic resonance spectra were measured using EX-270 (270 MHz) (JEOL Ltd., Tokyo, Japan), and electron impact-mass spectrometric data were obtained using JMS-SX102A (JEOL Ltd.).

Inhibitory action of caulerpenyne. The hydrolytic activity of porcine pancreatic lipase toward triolein emulsified with gum arabic and 4-MU oleate was determined in the presence of increasing concentrations of caulerpenyne (Figs. 1 and 2). In a concentration dependent manner caulerpenyne inhibited the triolein- and 4-MU oleate-hydrolyzing activities of pancreatic lipase, showing 50% inhibition at 2 mM and 13μM, respectively. Caulerpenyne also inhibited the tributyrin-hydrolyzing activity in a concentration-dependent manner (data not shown). The inhibitory action of caulerpenyne was examined using different concentrations of pancreatic lipase and substrates. In using triolein as a substrate, the inhibitory activity of caulerpenyne was not affected by an increase in the triolein concentration (Fig. 3A), but it decreased with increasing enzyme concentration (Fig. 3B). Similar results were observed using 4-MU oleate as a substrate; the inhibition was not affected by an increase of 4-MU oleate, but it decreased with an increase in enzyme concentration (data not shown). To clarify the enzyme-inhibiting mechanism, the kinetics of the inhibitory effect of caulerpenyne on lipase activity were studied. The mode of inhibition was analyzed by changing the concentration of substrate in the presence of two concentrations of caulerpenyne. A double-reciprocal plot of the reaction rate vs. triolein concentration was linear in the presence and absence of caulerpenyne, suggesting that the observed inhibition of lipase activity by caulerpenyne was due to competitive inhibition of the enzyme (data not shown). A similar result was obtained using 4-MU oleate as a substrate (data not shown).

Oral administration of caulerpenyne. Figure 4 shows the time course of the plasma triacylglycerol concentration when corn oil suspension with or without caulerpenyne was administered orally to rats. Two, 3 and 6 h after caulerpenyne administration, the plasma triacylglycerol concentration decreased significantly in comparison with the controls. The peak plasma triacylglycerol concentration was reduced and delayed by caulerpenyne administration.

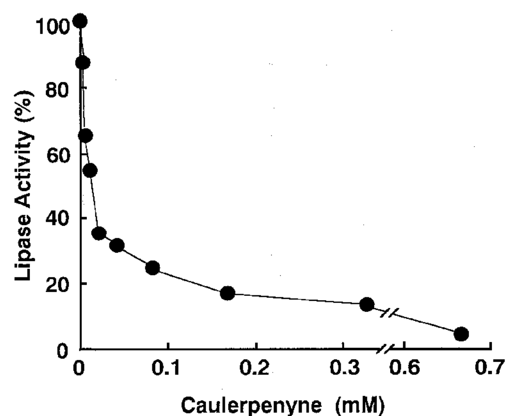


FIG. 2. Effect of increasing concentrations of caulerpenyne on the rate of hydrolysis of 4-methylumbelliferyl (4-MU) oleate by pancreatic lipase (18.75 μg/mL). The specific activity of 4-MU oleate-hydrolyzing activity was 17.8 μmol 4-methylumbelliferone released/mg protein of pancreatic lipase/min.

DISCUSSION

Caulerpenyne competitively inhibited the lipase activity. The inhibitory activity of caulerpenyne was dependent on the li-

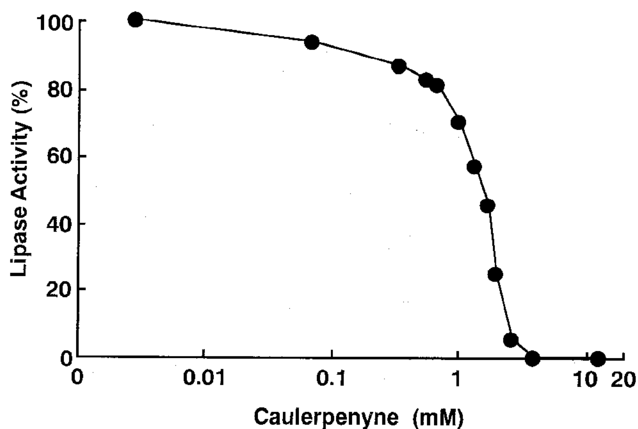


FIG. 1. Effect of increasing concentrations of caulerpenyne on the activity in hydrolysis of triolein emulsified with gum arabic by pancreatic lipase (1.0 μg/mL).

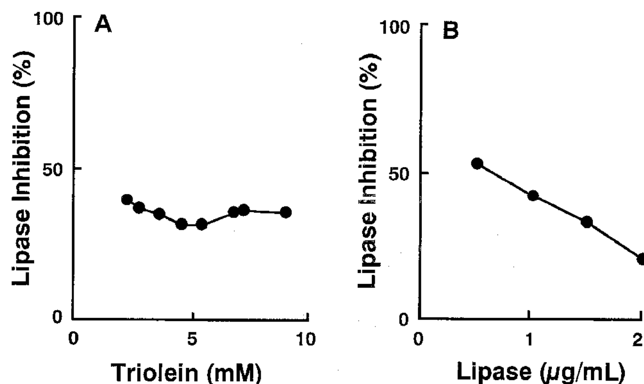


FIG. 3. Effect of substrate and enzyme concentrations on the inhibition by caulerpenyne. Lipase activity was determined using triolein as substrate with 330 μM caulerpenyne. (A) Effect of substrate concentrations (lipase, 1.0 μg/mL); (B) effect of enzyme concentrations (triolein, 5 mM).

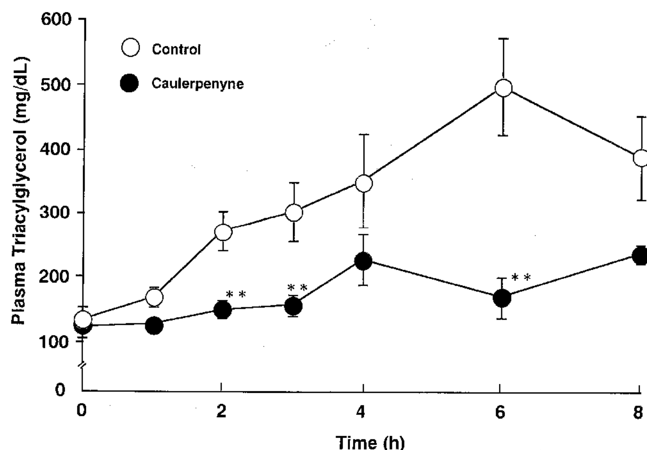


FIG. 4. Effect of caulerpenyne on rat plasma triacylglycerol levels after oral administration of lipid emulsion (○) or lipid emulsion containing caulerpenyne (●). The results are expressed as means ± SE of 10 experiments. $^{**}P < 0.01$ (compared with the corresponding control, Newman-Keuls' range test).

pase concentration but independent of substrate concentration (Fig. 3). Caulerpenyne also inhibited lipase activity for monomeric substrate such as 4-MU oleate or tributyrin, as well as when using emulsified triolein. These results suggest that caulerpenyne interacts directly with the lipase protein, rather than interacting with the substrate.

Caulerpenyne was the major metabolite in *C. taxifolia*, which is widely distributed in the tropics and is presently invading part of the Mediterranean (14). Fischel *et al.* (15) reported that caulerpenyne inhibited cell growth. However, as a result of evaluations conducted using human hematopoietic progenitors, melanocytes, and keratinocytes in culture, the toxicological risk of caulerpenyne to humans has been reported as low (16). In the Philippines, *C. taxifolia* is actually available as a food for medicinal purposes. It acts as an antifungal agent and reportedly decreases blood pressure (17). These results suggest that caulerpenyne (or *C. taxifolia*) may have potential as an agent that inhibits lipid absorption.

For lipase catalysis, the surface characteristics of substrate lipid micelles are the most important factors. Lipases are sometimes activated or denaturated by adsorption (or penetration) on to the substrate surface (18). Most lipase inhibitors reported thus far are adsorbed onto (or penetrate) the lipid surface, thus blocking the surface and affecting lipase activity. Several proteins, including mellitin, β-lactoglobulin A, serum albumin, ovalbumin and myoglobin, inhibit pancreatic lipase activity, and their inhibitory effects might be the result of lipase desorption from the substrate due to a change in interfacial quality (19,20). Hydrocarbons, fatty alcohols, and fatty acids also adsorb on the substrate interface and inhibit lipase activity (21–23). In this study, we purified caulerpenyne, a substance which may interact directly with lipase. A few substances acting as direct inhibitors of lipase have been reported. Lipstatin, isolated from *S. toxytricini,* is a stable covalent inhibitor of lipase (7). It interacts with lipase, probably with 1:1 stoichiometry, and inactivates the enzyme through the formation of a stable covalent intermediate. Fat absorption from the intestine of rats is also inhibited by lipstatin: the absorption of emulsified triolein was significantly decreased by 10^{-4} M lipstatin using the constant infusion method (24). We showed that caulerpenyne, administered orally in a single dose, reduced the concentration of plasma triacylglycerol in an *in vivo* experiment using rats (Fig. 4).

Marine algae (or seaweeds) have long been a food source for maritime people, as sea vegetables. In Japan, they have been eaten as a healthy food since antiquity. They contain proteins, fats, carbohydrates, cellulose, ash, minerals, and nucleic acids and are especially rich in iodine and certain vitamins. In this study, we demonstrated that many marine algae inhibited the activity of pancreatic lipase (Table 1), the extracts from Phaeophyta, Chlorophyta, and Rhodophyta having particularly high inhibitory activities. Phaeophyta generally contain large amounts of polyphenols such as tannin, which has lipase-inhibiting activity. It has been reported that most compounds with a porphyrin structure inhibit lipase activity (11). The weak inhibitory activities recognized in many algae might be caused by chlorophyll in the algal extracts. Therefore, algae are one of the most useful sources of lipase inhibitors.

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3-Thia Fatty Acid Treatment, in Contrast to Eicosapentaenoic Acid and Starvation, Induces Gene Expression of Carnitine Palmitoyltransferase-II in Rat Liver

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ABSTRACT: The aim of the present study was to investigate the hepatic regulation and β-oxidation of long-chain fatty acids in peroxisomes and mitochondria, after 3-thia- tetradecylthioacetic acid (C_{14}-S-acetic acid) treatment. When palmitoyl-CoA and palmitoyl-L-carnitine were used as substrates, hepatic formation of acid-soluble products was significantly increased in C_{14}-S-acetic acid treated rats. Administration of C_{14}-S-acetic acid resulted in increased enzyme activity and mRNA levels of hepatic mitochondrial carnitine palmitoyltransferase (CPT)-II. CPT-II activity correlated with both palmitoyl-CoA and palmitoyl-L-carnitine oxidation in rats treated with different chain-length 3-thia fatty acids. CPT-I activity and mRNA levels were, however, marginally affected. The hepatic CPT-II activity was mainly localized in the mitochondrial fraction, whereas the CPT-I activity was enriched in the mitochondrial, peroxisomal, and microsomal fractions. In C_{14}-S-acetic acid-treated rats, the specific activity of peroxisomal and microsomal CPT-I increased, whereas the mitochondrial activity tended to decrease. C_{14}-S-Acetyl-CoA inhibited CPT-I activity *in vitro*. The sensitivity of CPT-I to malonyl-CoA was unchanged, and the hepatic malonyl-CoA concentration increased after C_{14}-S-acetic acid treatment. The mRNA levels of acetyl-CoA carboxylase increased. In hepatocytes cultured from palmitic acid- and C_{14}-S-acetic acid-treated rats, the CPT-I inhibitor etomoxir inhibited the formation of acid-soluble products 91 and 21%, respectively. In contrast to 3-thia fatty acid treatment, eicosapentaenoic acid treatment and starvation increased the mitochondrial CPT-I activity and reduced its malonyl-CoA sensitivity. Palmitoyl-L-carnitine oxidation and CPT-II activity were, however, unchanged after either EPA treatment or starvation. The results from this study open the possibility that the rate control of mitochondrial β-oxidation under mitochondrion and peroxisome proliferation is distributed between an enzyme or enzymes of the pathway beyond the CPT-I site after 3-thia fatty acid treatment. It is suggested that fatty acids are partly oxidized in the peroxisomes before entering the mitochondria as acylcarnitines for further oxidation.

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Abbreviations: C_{14}-S-Acetic acid, tetradecylthioacetic acid; BSA, bovine serum albumin; CMC, carboxymethyl cellulose; CPT, carnitine palmitoyltransferase; EPA, eicosapentaenoic acid; PPAR, peroxisome proliferator activated receptor, Enzymes: carnitine palmitoyltransferase (EC 2.3.1.21); acetyl-CoA carboxylase (EC 6.4.1.2).

Not only are the 3-thia fatty acids peroxisome proliferators, they also increase the hepatic mitochondrial mass (1). The results from many years of studies strongly suggest that increased mitochondrial fatty acid oxidation is important for the hypotriglyceridemic effect of 3-thia fatty acids, such as tetradecylthioacetic acid (C_{14}-S-acetic acid) (1–4), and fibrates (5). Eicosapentaenoic acid (EPA), the hypotriglyceridemic component of fish oil (6–8), probably mediates its triglyceride-lowering effect by the same mechanism, as EPA also is reported to be a mitochondrion proliferator (5). Because of the potential use of EPA and 3-thia fatty acids as treatment for hypolipidemia in humans, this similarity is of especial interest.

Transport of long-chain fatty acyl groups into the mitochondrial matrix to undergo β-oxidation is controlled by the carnitine palmitoyltransferase (CPT) enzyme system. It is generally accepted that this process is regulated by carnitine palmitoyltransferase (CPT)-I, through different mechanisms: changes in activity and the transcription rate, changes in the concentration of its physiological inhibitor malonyl-CoA, and changes in its sensitivity to malonyl-CoA [reviewed by McGarry and Brown (9)]. CPT-II, on the other hand, is usually not considered rate-controlling for fatty acid oxidation, and less is known about the regulation of this activity. The effects of 3-thia fatty acids on the CPT system were investigated in order to study by which mechanisms the fatty acid oxidation rate in liver is increased.

The molecular mechanism involved in gene regulation by fatty acids is not fully elucidated. However, dietary polyunsaturated fatty acids directly modulate gene transcription as a primary event, rather than exert their influence by modifying membrane lipid fatty acid composition, which is a later event (10). Whether fatty acids and peroxisome proliferators act through the same or distinct mechanisms remains unclear. Chatelain *et al.* (11) suggested that the mechanism of action of long-chain polyunsaturated fatty acids differs from that of the classical peroxisome proliferators, such as clofibrate, which presumably works *via* the peroxisome-proliferator activated receptor (PPAR) α. Besides being fatty acids, the 3-thia fatty acids are both mitochondrion- and peroxisome-proliferators (1). The 3-thia fatty acids are activated to their respective CoA esters, accumulate in the phospholipid fraction

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of the liver (12,13) and are metabolized as normal long-chain fatty acids, except that they are not β-oxidized (1), and, like a variety of other lipid-like compounds, they transcriptionally activate PPAR α (14–16).

The mechanism by which 3-thia fatty acids increase hepatic fatty acid oxidation is not known. In this study we report that in contrast to EPA treatment and starvation, 3-thia fatty acids marginally affect mitochondrial CPT-I activity and mRNA levels, but increase CPT-II activity and mRNA levels.

EXPERIMENTAL PROCEDURES

Chemicals and drugs. [^{32}Pα]dCTP, L-[methyl-^{14}C]carnitine hydrochloride, [1-^{14}C]palmitoyl-L-carnitine, and [1-^{14}C] palmitoyl-CoA were purchased from the Radiochemical Centre (Amersham, England). [1-^{14}C]Palmitic acid (50 mCi/mmol) was obtained from New England Nuclear (Boston, MA). Nylon membranes and slot-blot equipment were obtained from Schleicher & Schuell (Kassel, Germany). The sulfur-substituted fatty acids were prepared at Department of Chemistry, University of Bergen (Bergen, Norway), as earlier described (17). EPA as ethyl ester (94% pure) was obtained from Norsk Hydro AS, Research Centre (Porsgrunn, Norway). The CoA ester of C_{14}-S-acetic acid was synthesized as described by Kawaguchi *et al.* (18). Etomoxir was from Research Biochemicals International (Natick, MA). All other chemicals and solvents were of reagent grade from common commercial sources.

Treatment of animals. Male Wistar rats from Møllegard Breeding Laboratory (Ejby, Denmark), weighing about 250 g each, were housed in metal wire cages in pairs and maintained at a 12 h cycle of light and dark at 20 ± 3°C. The animals were acclimatized under these conditions for at least 1 wk before the experiments. The fatty acids were suspended in 0.5% sodium carboxymethyl cellulose (CMC) and administered by orogastric intubation once a day for 1 wk at different doses. The control animals received CMC only. One group was starved for 3 d with free access to water. Control and fatty acid-treated animals had free access to water and standard rat pellet food during the experiment. Each test and control group consisted of four animals. After 12 h fasting, the rats were anesthetized with 0.2 mL Hypnorm-Dormicum® (fluanisone-fentylmidazolam)/100 g body weight. Cardiac puncture was performed and blood was collected in vacutainers. The livers and hearts were removed, samples immediately freeze-clamped, weighed, and stored at −80°C. Other pieces were chilled on ice and weighed. The use of the animals was approved by the Norwegian State Board of Biological Experiments with Living Animals.

Preparation of subcellular fractions and protein measurements. The livers from individual rats were homogenized in ice-cold sucrose medium [0.25 M sucrose, 10 mM HEPES (pH 7.4), and 2 mM EDTA]. The hearts were homogenized in 0.25 M sucrose, 5 mM HEPES (pH 7.4), and 0.5 mM EGTA. Subcellular fractions were prepared according to DeDuve *et al.* (19) using preparative differential centrifugation. Modifications, purity, and yield were described earlier (20). Bio-Rad protein kit (Bio-Rad, Richmond, CA) was used for protein measurement. Bovine serum albumin (BSA) dissolved in distilled water was used as a standard.

Measurement of enzyme activities. Acid-soluble products were measured in the mitochondrial-enriched fractions, using palmitoyl-CoA and palmitoyl-L-carnitine as substrates (21). CPT-I activity was measured essentially as described by Bremer (22) in all subcellular fractions. The assay for CPT-I contained 20 mM HEPES, pH 7.5, 70 mM KCl, 5 mM KCN, 100 μM palmitoyl-CoA, and 10 mg BSA/mL, plus 0.6 mg tissue protein/mL. The reaction was started with 200 μM [methyl-^{14}C]-L-carnitine. When included, malonyl-CoA was added prior to start of the reaction. Assay conditions for CPT-II were identical except that BSA was omitted and 0.01% Triton-X included. Tissue protein concentration was 0.1 mg/mL.

Measurement of malonyl-CoA and CoASH. Malonyl-CoA and CoASH were measured by reversed-phase high-performance liquid chromatography according to Demoz and Netteland (23), with the following modifications of tissue preparation: (i) 100 mg liver was homogenized in 900 μL ice-cold 2.33 M $HClO_4$ and 2 mM DL-dithiothreitol. (ii) Aliquots of the homogenate were centrifuged at 10,000 × *g* for 2 min. (iii) 180 μL ice-cold 3 M K_2CO_3 with 0.5 M triethanolamine was added to 500 μL of the supernatant. (iv) After 10 min on ice, the solution was centrifuged at 10,000 × *g* for 2 min. (v) 20 μL of the homogenate was injected onto the high-performance liquid chromatography column. Owing to rapid degradation of malonyl-CoA, separate animals were used for this purpose, where parts of the liver were immediately freeze-clamped.

Preparation of cell suspensions. Cells were isolated from rats treated with C_{14}-S-acetic acid or palmitic acid at a dose of 300 mg/d/kg body weight for 12 wk, by *in vitro* perfusion of the liver using a two-step procedure as earlier described (24). After collagenase perfusion small tears were made in the capsule and the cells were released by gentle shaking in a 145-mM NaCl solution containing 5 mM KCl, 0.4 mM KH_2PO_4, 0.3 mM Na_2HPO_4, 0.8 mM $MgSO_4$, 1 mM $CaCl_2$, 20 mM HEPES, and 1% BSA. The cell suspension was subsequently preincubated at 37°C for 25–30 min in a shaking waterbath. The parenchymal cells were separated from the nonparenchymal cells by centrifugation (700 rpm/1 min). The precipitate containing the heavier parenchymal cells was discarded and the supernatant was recentrifuged at 1500 rpm for 5 min to remove the nonparenchymal cells from the solution. These cells were washed in 40–50 mL of incubation solution, recentrifuged, and assessed for viability.

The cells were subsequently resuspended in a preincubation medium (Dulbecco's modified Eagle's medium) containing 20 mM HEPES and 0.5 mM L-carnitine, at a concentration of 1×10^6 cells/mL medium. The cells were preincubated at 37°C for 30 min, with or without 50 μM etomoxir. The reaction was started with [1-^{14}C]palmitic acid bound to BSA at a ratio 2.5:1. The reaction tubes were immediately sealed. After 1 h the tubes were put on ice, and 250 μL 0.75 M $HClO_4$ was added. $^{14}CO_2$ was trapped from sealed culture flasks es-

sentially as described by Christiansen and Davies (25). Free fatty acids and lipids were then precipitated with addition of 1.0 mL ice-cold 0.75 M $HClO_4$ and 0.45% BSA. The extract was centrifuged at 1800 × *g* for 10 min, and 0.5 mL of the supernatant was assayed for radioactivity by liquid scintillation counting.

Purification of RNA and hybridization analysis. Total RNA was isolated using the guanidinium thiocyanate–phenol method (26), and the RNA concentrations were determined by measuring the absorbance at 260 nm. Northern- and slot-blotting were performed as earlier described (24). Three different RNA concentrations were applied. Hybridization reactions were performed as described by Sambrook *et. al.* (27), and the membranes stringently washed three times; Kodak XAR-5 X-ray films were exposed to the membranes, and autoradiograms were obtained as described earlier (24). The relative levels of mRNA expression were estimated as the amount of radioactive probe hybridized to each sample of RNA relative to the levels of 28S rRNA in each sample.

Preparation of hybridization probes. The appropriate DNA fragments were cut from plastids by restriction enzymes. Purified fragments were then ^{32}P-labeled using the oligolabeling technique (28), resulting in specific activities ranging from 0.8–5 × 10^9 cpm/mg. The probes were purified fragments of: CPT-I, 2600 bp *Eco*RI fragment of pBK2-CPT-I ; partial CPT-II, 1600 bp *Xho* I/ *Xba*I fragment of PbKs-CPT-II.4; and partial acetyl-CoA carboxylase cDNA, 485 bp *Eco*RI fragment of pUC 19.

Statistical analysis and presentation of data. The data are presented as mean ± standard deviation (SD) from 4 animals and were evaluated by a two-sample variance Student's *t*-test (two-tailed distribution) where relevant. The level of statistical significance was set at $P < 0.05$.

RESULTS

EPA feeding and especially starvation increased the oxidation palmitoyl-CoA both in the presence and absence of malonyl-CoA in isolated liver mitochondria, compared to palmitic acid treatment (Fig. 1A). In the absence of malonyl-CoA, the mitochondrial palmitoyl-CoA oxidation was increased 1.5- and 3.1-fold after EPA feeding and starvation, respectively. Moreover, EPA feeding and especially starvation lowered the sensitivity of palmitoyl-CoA oxidation to malonyl-CoA (Fig. 1A), i.e., in the presence of 12 μM malonyl-CoA the palmitoyl-CoA oxidation was inhibited 45 and 22% in EPA-fed and -starved rats, respectively, compared to 55% in palmitic acid-treated rats.

It is generally assumed that the rate-limiting enzyme of palmitoyl-CoA oxidation is mitochondrial CPT-I. Figure 1B shows that EPA feeding and starvation increased the mitochondrial CPT-I activity in both the absence and presence of malonyl-CoA, compared to palmitic acid treatment. Addition of 12 μM malonyl-CoA inhibited mitochondrial CPT-I activity by 35 and 47% in EPA-fed and -starved animals, respectively, compared to 59% in palmitic acid-treated rats (Fig. 1B). The mRNA levels of CPT-I reflected the activities measured in absence of malonyl-CoA after EPA feeding and starvation (Table 1).

In sharp contrast, after C_{14}-S-acetic acid treatment, the

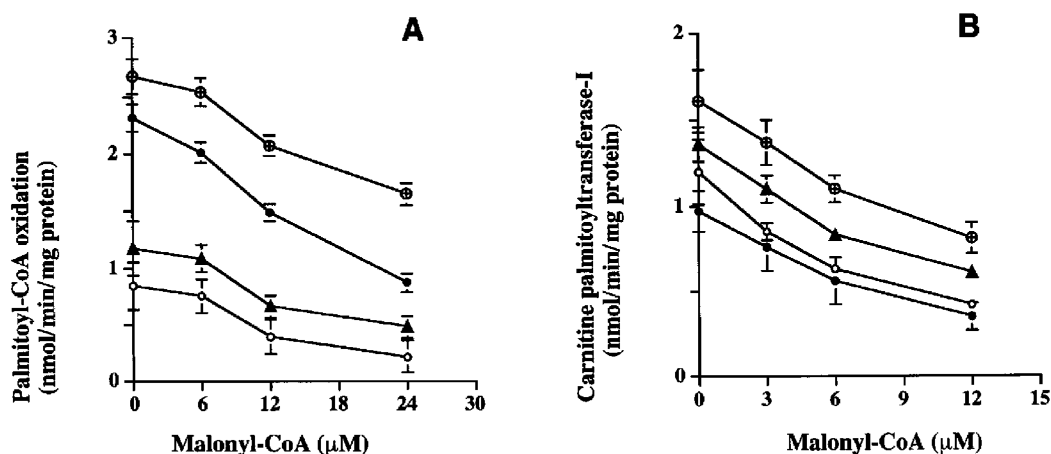


FIG. 1. Effect of tetradecylthioacetic acid (C_{14}-S-acetic acid) treatment (—●—), eicosapentaenoic acid (EPA) feeding (—▲—), and starvation (—⊕—) on mitochondrial palmitoyl-CoA oxidation (A) and mitochondrial carnitinepalmitoyltransferase (CPT)-I activity (B) in the presence and absence of malonyl-CoA. Rats were fed EPA at a dose of 1000 mg/d/kg body weight or C_{14}-S-acetic acid suspended in 0.5% carboxymethylcellulose (CMC) at a dose of 300 mg/d/kg body weight or for 7 d. Control animals received palmitic acid (—○—) suspended in 0.5% CMC at a dose of 300 mg/d/kg body weight or for 7 d. One group of rats was starved for 3 d. Palmitoyl-CoA oxidation and CPT-I activity were measured in isolated mitochondria as described in the Experimental Procedures section in four individual rat livers in the absence and in the presence of malonyl-CoA. The values are presented as mean ± SD.

TABLE 1
Effect of Starvation, EPA, Palmitic and Thia Fatty Acid Treatment on the mRNA Levels of CPT-I and CPT-II[a]

Treatment	Dose (mg/d/kg BW)	mRNA (fold increase) CPT-I	CPT-II
Control		1.00 ± 0.08	1.00 ± 0.04
Palmitic acid	150	1.05 ± 0.11	0.97 ± 0.07
	300	1.21 ± 0.15	1.06 ± 0.09
C_{14}-S-Acetic acid	150	1.17 ± 0.23	2.23 ± 0.31*
	300	1.32 ± 0.25*	3.79 ± 0.63*
EPA	1500	1.49 ± 0.18*	1.26 ± 0.32
Starvation		2.13 ± 0.21*	0.95 ± 0.09

[a]Rats were fed fatty acids at different doses for 7 d or starved for 3 d. The mRNA levels of CPT-I and CPT-II were measured as described in the Experimental Procedures section in four individual rats in each experimental group. The relative mRNA levels were normalized to the corresponding 28S rRNA levels. Control values were set to 1.0 and experimental values are given as fold increase ± SD. Values are means ± SD (n = 4). *Significantly different from control, P < 0.05. Abbreviations: BW, body weight; EPA, eicosapentaenoic acid; CPT, carnitine palmitoyltransferase; C_{14}-S-acetic acid, tetradecylthioacetic acid.

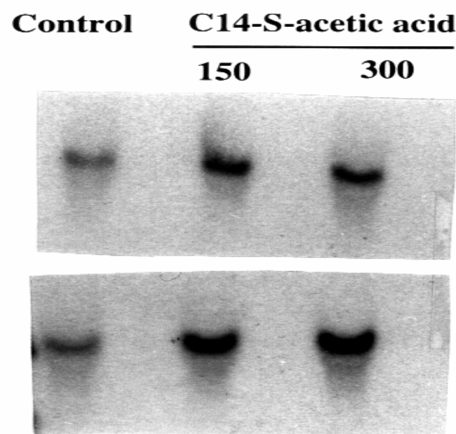


FIG. 2. Effects of C_{14}-S-acetic acid on CPT-I and CPT-II mRNA levels in liver. Rats were fed C_{14}-S-acetic acid suspended 0.5% in CMC at a dose of 150 or 300 mg/d/kg body weight for 7 d. Control animals received CMC only. Total mRNA were purified from each individual liver. Northern blotting and hybridization to immobilized RNA were performed as described in the Experimental Procedures section. Figure shows representative mRNA levels of CPT-I and CPT-II. For abbreviations see Figure 1.

palmitoyl-CoA oxidation increased 2.6-fold (Fig. 1A), whereas the specific mitochondrial CPT-I activity tended to decrease (Fig. 1B) in the absence of malonyl-CoA compared to palmitic acid treatment. Addition of 12 μM malonyl-CoA inhibited the CPT-I activity 55%, suggesting the sensitivity of CPT-I to malonyl-CoA was unchanged after C_{14}-S-acetic acid treatment (Fig. 1B). C_{14}-S-Acetic acid treatment increased the mRNA levels of CPT-I compared to control, but not compared to palmitic acid treatment (Fig. 2 and Table 1).

In contrast to EPA feeding and starvation, C_{14}-S-acetic acid treatment increased the oxidation of palmitoyl-L-carnitine 1.7-fold in isolated mitochondria (Fig. 3A), suggesting that C_{14}-S-acetic acid mediates its action on step(s) subsequent to CPT-I. Indeed, a 2.8-fold increased mitochondrial CPT-II activity was observed after C_{14}-S-acetic acid treatment (Fig. 3B). Moreover, the mRNA levels of CPT-II increased after different doses of C_{14}-S-acetic acid (Table 1), whereas both the mRNA levels and activities of CPT-II were unchanged after either EPA feeding or starvation (Fig. 3B, Table 1).

Figure 4A shows CPT-II activity in the liver of rats treated with different chain-length monocarboxylic 3-thia fatty acids, which are reported to increase β-oxidation in different ways (24). Significantly increased β-oxidation (24) and CPT-II activity (Fig. 4A) were observed in rats treated with C_{11}-S- to

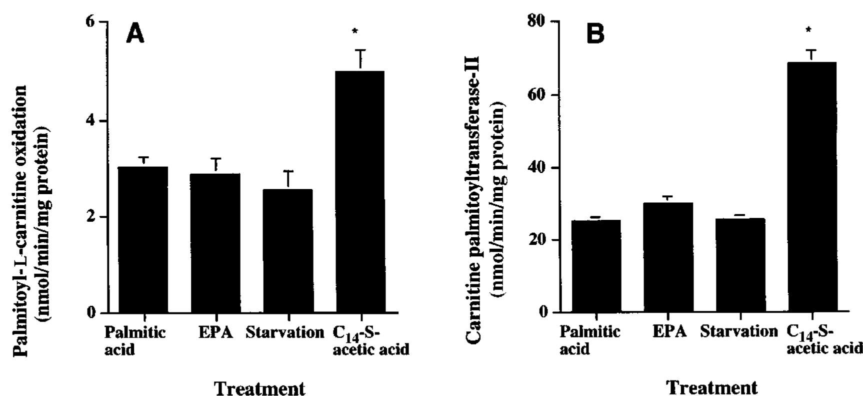


FIG. 3. Effect of C_{14}-S-acetic acid treatment, EPA feeding, and fasting on mitochondrial palmitoyl-L-carnitine oxidation (A) and mitochondrial CPT-II activity (B). Rats were fed EPA at a dose of 1000 mg/d/kg body weight or C_{14}-S-acetic acid suspended in 0.5% CMC at a dose of 300 mg/d/kg body weight for 7 d. Control animals received palmitic acid suspended in 0.5% CMC at a dose of 300 mg/d/kg body weight for 7 d. One group of rats was starved for 3 d. Palmitoyl-L-carnitine oxidation and CPT- II activity were measured in isolated mitochondria as described in the Experimental Procedures section in four individual rat livers in the absence, and in the presence of malonyl-CoA. The values are presented as mean ± SD. * P < 0.05. For abbreviations see Figure 1.

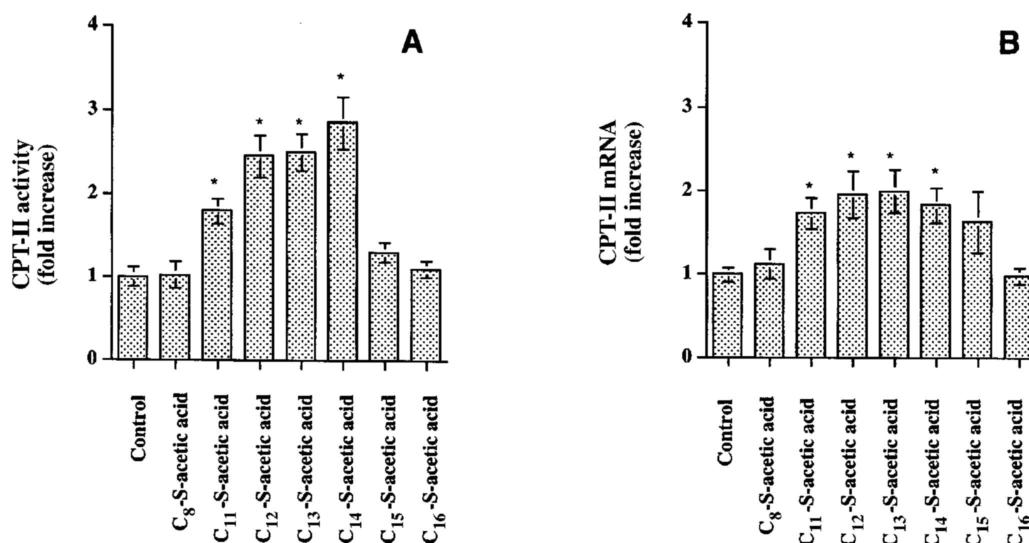


FIG. 4. Effects of different chain-length 3-thia fatty acids on CPT-II activity and mRNA levels in liver. Rats were fed palmitic or 3-thia fatty acids with different chain lengths at a dose of 150 mg/d/kg body weight for 7 d. CPT-II activities were measured in postnuclear homogenates, and the mRNA levels were measured as described in the Experimental Procedures section in four individual rats in each experimental group. The relative mRNA levels were normalized to the corresponding 28S rRNA levels. Control values were set to 1.0 and experimental values are given as fold increase ± SD. The activities in palmitic acid-treated animals were 5.18 ± 1.03 nmol/min/mg protein in liver postnuclear homogenate. *Indicates statistical difference between thia fatty acid- and palmitic acid-treated rat, fed a dose of 150 mg/d/kg body weight, $P < 0.05$

C_{14}-S-acetic acid. Concomitant increases in CPT-II mRNA levels were observed (Fig. 4B). There was a positive correlation between CPT-II activity and oxidation rate of both palmitoyl-CoA and palmitoyl-L-carnitine (Fig. 5), suggesting a regulatory function of CPT-II rather than CPT-I in oxidation of palmitic acid after 3-thia fatty acid treatment.

The rate of hepatic mitochondrial fatty acid oxidation might also be regulated through changes of the malonyl-CoA

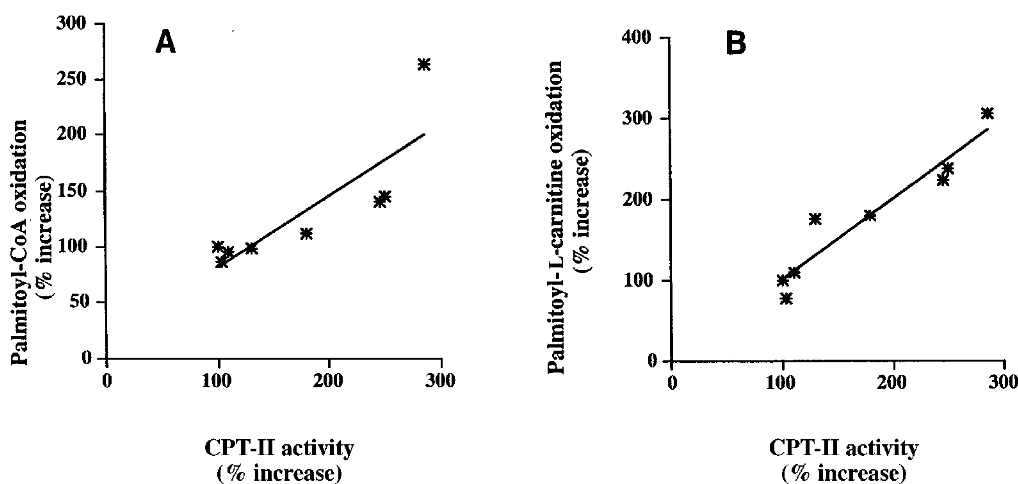


FIG. 5. Correlation between mitochondrial β-oxidation and CPT-II activity in 3-thia fatty acid-treated rats. Rats were fed palmitic or 3-thia fatty acids with different chain length at a dose of 150 mg/d/kg body weight for 7 d. CPT-II activity was measured in postnuclear liver homogenate as described in the Experimental Procedures section and presented in Figure 4. The mitochondrial β-oxidation was measured in liver homogenate as described in the Experimental Procedures section (24). The blots represent the mean of four animals in each group. The CPT-II activity was correlated to mitochondrial β-oxidation using palmitoyl-CoA (A) ($y = 0.641\ x + 17.624$, $r^2 = 0.703$) and palmitoyl-L-carnitine (B) ($y = 0.985x + 2.837$, $r^2 = 0.913$) as substrates, respectively. For abbreviations see Figure 1.

TABLE 2
Effect of Fatty Acid Treatment on the Hepatic Activities and mRNA Levels of Acetyl-CoA Carboxylase Activities and the Levels of Malonyl-CoA and CoASH[a]

Treatment	Acetyl-CoA carboxylase mRNA (fold increase)	Malonyl-CoA (nmol/mg liver)	CoASH (nmol/mg liver)
CMC	1.0 ± 0.1	8 ± 2	98 ± 9
Palmitic acid	1.0 ± 0.2	N.D.	N.D.
C_{14}-S-acetic acid	1.7 ± 0.2*	27 ± 5*	403 ± 41*

[a]Rats were fed palmitic or C_{14}-S-acetic acid at a dose of 300 mg/d/kg body weight suspended in 0.5% CMC for 7 d. Control animals received CMC only. The mRNA levels and activities of fatty acyl-CoA carboxylase were measured as described in the Experimental Procedures section. The relative mRNA levels were normalized to the corresponding 28S rRNA levels. Control values were set to 1.0 and experimental values are given as fold increase ± SD. The malonyl-CoA and CoASH levels were measured in separate animals as described in the Experimental Procedures section. Values are means ± SD (n = 4).
*Significant different from control $P < 0.05$. Abbreviations: ND, not determined; CMC, carboxymethylcellulose; for other abbreviation see Table 1.

in the liver. After treatment with C_{14}-S-acetic acid, the hepatic malonyl-CoA levels increased more than threefold (Table 2). Also the CoASH levels and the mRNA levels of the malonyl-CoA-producing enzyme, acetyl-CoA carboxylase, were increased (4.1- and 1.7-fold, respectively), suggesting that the production of malonyl-CoA is stimulated (Table 2). We earlier demonstrated that C_{14}-S-acetic acid is converted to its CoA ester in rat liver and C_{14}-S-acetic acid accumulates especially in the phospholipid fraction after treatment (12,13), suggesting that C_{14}-S-acetyl-CoA is formed *in vivo* (29). The C_{14}-S-acetyl-CoA is a poor substrate for mitochondrial CPT-I (30) and, when it was present in the assay mixture, the CoA ester of C_{14}-S-acetic acid consequently inhibited the conversion of palmitoyl-CoA to palmitoyl-L-carnitine (Fig. 6A). Figure 6A shows that the inhibition of CPT-I activity by C_{14}-S-acetyl-CoA was more pronounced in mitochondria isolated from C_{14}-S-acetic acid-treated rats than animals treated with palmitic acid. On the other hand, the CPT-II activity was not affected by the CoA ester of C_{14}-S-acetic acid (Fig. 6B).

Peroxisomal β-oxidation is independent of carnitine (31). However, an isoform of CPT-I is present in the peroxisomes (32), and acyl-carnitines are formed in this organelle (33). Significantly increased CPT-I activities measured in the peroxisomal- and microsomal-fractions after C_{14}-S-acetic acid treatment (Table 3) resulted in a shift of the subcellular distribution of CPT-I toward these organelles (Fig. 7). CPT-II activity was increased in all subcellular fractions (Table 3). However, more than 90% of the total CPT-II activity was localized in the mitochondria, and C_{14}-S-acetic acid treatment did not change the subcellular distribution (Fig. 8).

The formation of acid-soluble products + CO_2 expresses total fatty acid oxidation. As it appears that C_{14}-S-acetic acid increases the fatty acid oxidation independent of CPT-I, we measured the oxidation of [1-^{14}C]palmitic acid in cell suspensions prepared from rats treated with palmitic- or C_{14}-S-acetic acid. The formation of [1-^{14}C]-labeled acid-soluble products from [1-^{14}C]palmitic acid was 30-fold higher in cells cultured from C_{14}-S-acetic than from palmitic acid-treated rats (Table 4). Whereas addition of the CPT-I inhibitor etomoxir inhibited the formation of [1-^{14}C]-labeled acid-soluble products 91% in cells from control rats, the inhibition was only 21% in the cells from C_{14}-S-acetic acid-treated rats (Table 4). Addi-

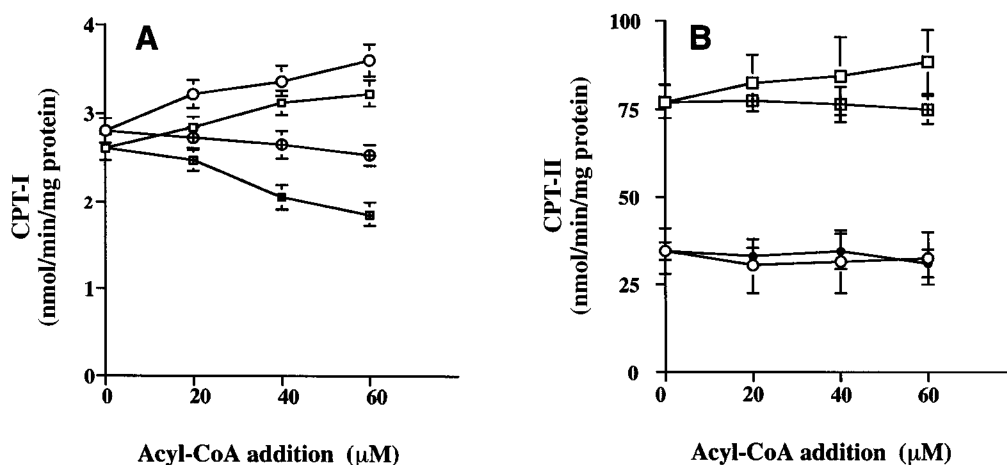


FIG. 6. Effect of C_{14}-S-acetyl-CoA on the mitochondrial CPT-I (A) and CPT-II (B) activity. Rats were fed C_{14}-S-acetic acid at a dose of 300 mg/d/kg body weight suspended in 0.5% CMC for 7 d. Control animals received CMC only. The CPT activities were measured in isolated mitochondria from four different control rat livers as described in the Experimental Procedures section using 100 μM palmitoyl-CoA as a substrate. The CPT-I activity in control rats was measured with additional 20, 40, and 60 μM palmitoyl-CoA (—○—) and C_{14}-S-acetyl-CoA (—⊕—) in the assay mix. The CPT-I activity in C_{14}-S-acetic acid-treated rats was also measured with additional palmitoyl-CoA (—□—) and C_{14}-S-acetyl-CoA (—⊞—) in the assay mix. The values are presented as mean ± SD. For abbreviations see Figure 1.

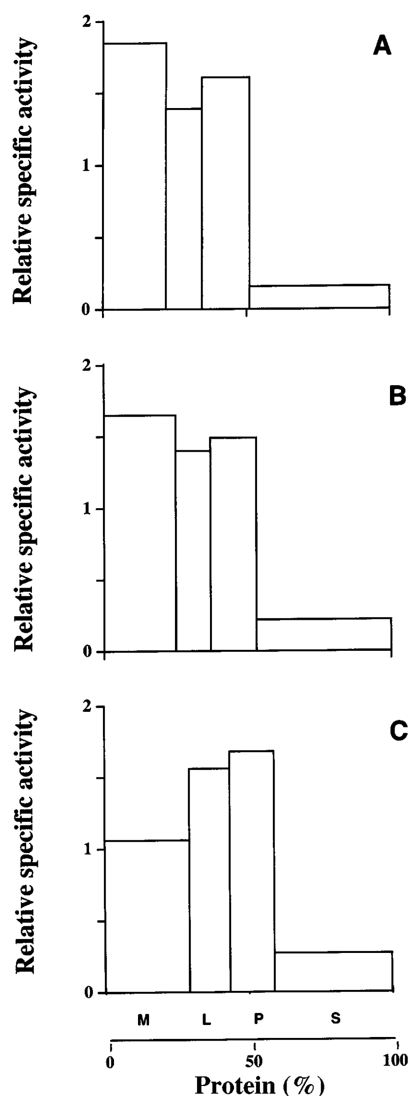


FIG. 7. The effects of C_{14}-S-acetic acid and palmitic acid on the subcellular distribution of CPT-I. Rats were fed palmitic (B) or C_{14}-S-acetic (C) acid at a dose of 300 mg/d/kg body weight suspended in 0.5% CMC for 7 d. Control animals received CMC only (A). The postnuclear homogenates from the livers were fractionated into mitochondrial (M), peroxisomal (L), microsomal (P), and cytosolic (S) fractions, and the CPT-I activity was measured as described in the Experimental Procedures section. The abscissa represents the cumulative protein content for each fraction as a percentage of the total postnuclear protein. The ordinate represents relative specific activity, i.e., the percentage of the total enzyme activity in the fraction over the percentage of total protein in the fraction. For abbreviations see Figure 1.

tionally, as the $^{14}CO_2$ formation was not statistically different, it is presumably that the first cycle of β-oxidation mainly occurred in the peroxisomes in C_{14}-S-acetic acid-treated rats (Table 4). Acyl-carnitines formed in the peroxisomes might be delivered to the mitochondria for further oxidation. (Scheme 1, where FAO is fatty acyl-CoA oxidase and CTL is carnitine translocase).

DISCUSSION

The present paper presents evidence that the effect of 3-thia fatty acids on CPT-I and CPT-II is different from those of EPA and starvation. Mitochondrial CPT-I are considered to be rate-controlling for mitochondrial β-oxidation through different mechanisms, such as changes in activity and the transcription rate, changes in the concentration of its physiological inhibitor malonyl-CoA, and changes in its sensitivity to malonyl-CoA (9). However, in contrast to starved and EPA-fed rats, there was unexpectedly no correlation between mitochondrial β-oxidation and mitochondrial CPT-I activity in C_{14}-S-acetic acid-treated rats (Fig. 1). Nor did C_{14}-S-acetic acid treatment increase the mRNA levels of CPT-I, compared to palmitic acid treatment (Table 1). In addition, the hepatic levels of malonyl-CoA increased and the sensitivity of malonyl-CoA to CPT-I activity appeared unchanged after C_{14}-S-acetic acid treatment (Fig. 1).

The increased hepatic malonyl-CoA levels after C_{14}-S-acetic acid treatment (Table 2) might inhibit CPT-I activity. Inhibition of CPT-I by increased hepatic levels of CoASH (Table 2) and C_{14}-S-acetyl-CoA (Fig. 6A) might also occur. By which mechanism the hepatic malonyl-CoA increases needs further investigation. However, increased mRNA levels of acetyl-CoA carboxylase and hepatic levels of CoASH (Table 2) suggest increased formation rather than reduced degradation by malonyl-CoA hydrolase.

In contrast to starvation and EPA feeding, C_{14}-S-acetic acid treatment also stimulated mitochondrial formation of acid-soluble products when palmitoyl-L-carnitine was used as substrate (Fig. 3), suggesting that enzyme(s) subsequent to CPT-I are involved in the regulation of fatty acid oxidation. CPT-II is not usually regarded as a regulatory enzyme for fatty acid oxidation, but our results are consistent with earlier findings of McGarry and co-workers (34,35), who showed a direct proportionality between ketogenesis from oleate and L-octanoylcarnitine in perfused livers from rats treated in different ways. Indeed, C_{14}-S-acetic acid treatment increased both CPT-II activity and gene-expression (Figs. 2 and 3 and Table 1). Increased CPT-II activities and mRNA levels were also observed in the liver of rats treated with different chain-length monocarboxylic 3-thia fatty acids (Fig. 4), which correlated to oxidation of both palmitoyl-CoA and palmitoyl-L-carnitine (Fig. 5).

It was recently reported that fibrate action on CPT-II gene-expression was PPARα-dependent (36). The 3-thia fatty acids might increase the CPT-II mRNA levels by PPARα-activation, as the trancriptional activity of C_{14}-S-acetic acid correlates with its ability to bind PPARα (15). The CPT-I mRNA levels were, on the other hand, marginally although significantly increased after C_{14}-S-acetic acid treatment compared to controls (Table 1 and Fig. 2). The increased mRNA levels were, however, not statistically significant when palmitic acid treatment was used as control (Table 1). Conversely, starvation

TABLE 3
Effect of Palmitic- and C_{14}-S-Acetic Acid Treatment on CPT-I and CPT-II Activities in Different Subcellular Fractions[a]

Treatment	E	M	L	P	S
CPT-I activity					
CMC	246 ± 23	102 ± 5	46 ± 5	64 ± 13	28 ± 4
Palmitic acid	248 ± 35	108 ± 12	45 ± 6	73 ± 24	21 ± 2
C_{14}-S-Acetic acid	296 ± 25*	109 ± 9	70 ± 7*	87 ± 22*	37 ± 8*
CPT-II activity					
CMC	1121 ± 190	981 ± 270	23 ± 13	28 ± 13	91 ± 27
Palmitic acid	1061 ± 226	878 ± 59	36 ± 17	30 ± 14	116 ± 31
C_{14}-S-Acetic acid	5211 ± 616*	4550 ± 529*	162 ± 11*	127 ± 62*	371 ± 75*

[a]Rats were fed palmitic- or C_{14}-S-acetic acid at a dose of 300 mg/d/kg body weight suspended in 0.5% CMC for 7 d. Control animals received CMC only. The postnuclear homogenates (E) from the livers were fractionated into mitochondrial (M), peroxisomal (L), microsomal (P), and cytosolic (S) fractions, and the CPT-I and CPT-II activities (recorded as nmol/min/g liver) were measured as described in the Experimental Procedures section. Values are means ± SD ($n = 4$).
*Significantly different from control $P < 0.05$. For other abbreviations see Tables 1 and 2.

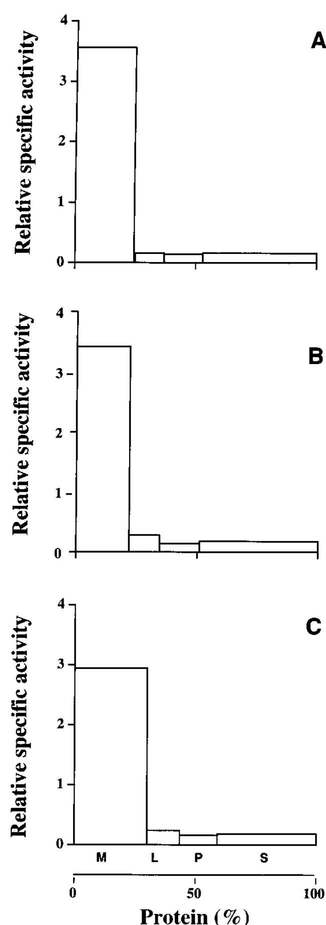


FIG. 8. The effects of C_{14}-S-acetic acid and palmitic acid on the subcellular distribution of CPT-II. Rats were fed palmitic (B) or C_{14}-S-acetic acid (C) at a dose of 300 mg/d/kg body weight suspended in 0.5% CMC for 7 d. Control animals received CMC only (A). The postnuclear homogenates from the livers were fractionated into mitochondrial (M), peroxisomal (L), microsomal (P), and cytosolic (S) fractions, and the CPT-II activity was measured as described in the Experimental Procedures section. The abscissa represents the cumulative protein content for each fraction as a percentage of the total postnuclear protein. The ordinate represents relative specific activity, i.e., the percentage of the total enzyme activity in the fraction over the percentage of total protein in the fraction. For abbreviations see Figure 1.

and EPA feeding significantly increased the mRNA levels of CPT-I, but not CPT-II (Table 1). It thus appears that C_{14}-S-acetic acid influences the CPT system by a different mechanism from that of EPA feeding and starvation.

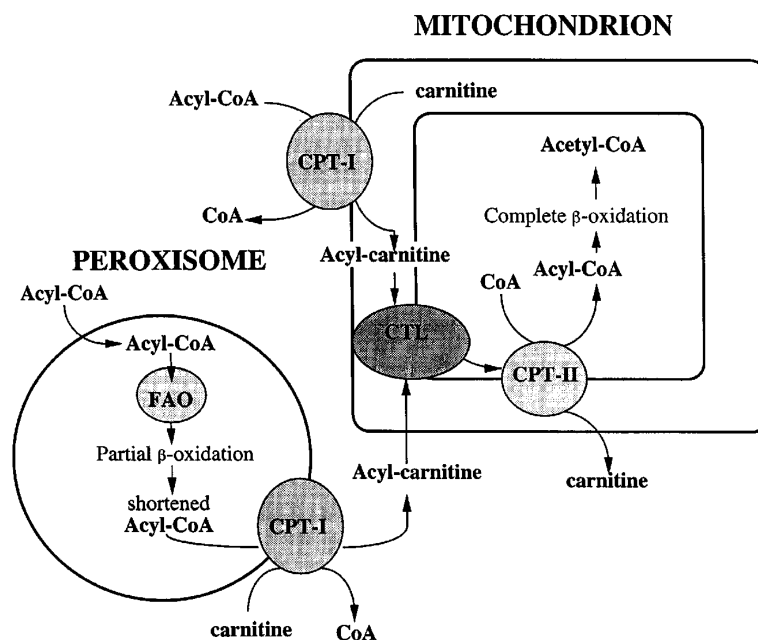
It is difficult to explain how the oxidation of palmitic acid can increase without any concomitant increased CPT-I activity, as it is believed that palmitate enters the mitochondia *via* this enzyme. However, the formation of [^{14}C]acid-soluble products, but not $^{14}CO_2$, from [1-^{14}C]palmitic acid, was increased in cell suspensions prepared from C_{14}-S-acetic acid-compared to palmitic acid-treated rats (Table 4). This suggest that the first cycle of β-oxidation in the liver of C_{14}-S-acetic acid treated rats primarily occurs in the peroxisome. The activity and gene-expression of the rate-limiting enzyme of peroxisomal β-oxidation, fatty acyl-CoA oxidase, are indeed upregulated by C_{14}-S-acetic acid treatment (24). Moreover, the formation of [^{14}C]acid-soluble products from [1-^{14}C]palmitic acid was marginally affected by the CPT-I inhibitor etomoxir in cell suspensions prepared from C_{14}-S-acetic acid-, as compared to palmitic acid-treated rats (Table 4).

Although hepatic CPT-I activity is shifted toward peroxisomes (and microsomes) (Fig. 7), the role of peroxisomal CPT-I is not elucidated. As acyl-CoA is transported into peroxisomes and can undergo peroxisomal oxidation independent of carnitine (31), presumably peroxisomal CPT-I are not involved in the transport of fatty acids into the peroxisomes.

TABLE 4
Oxidation of [1-^{14}C]Palmitic Acid in Hepatocytes Cultured from Palmitic Acid- and C_{14}-S-Acetic Acid-treated Rats[a]

		Acid-soluble products	
Fatty acid	CO_2	– Etomoxir	+ Etomoxir
Palmitic acid	1.8 ± 0.6	90 ± 11	8 ± 3
C_{14}-S-Acetic acid	2.2 ± 0.7	316 ± 48	249 ± 39

[a]Hepatocytes from rats treated with palmitic acid and C_{14}-S acetic acid-treated rats were cultured. Cells (2 × 10^6) were preincubated for 30 min in the presence of L-carnitine (0.5 mM) with and without the addition of 50 μM Etomoxir. The suspension was then incubated for 1 h, with labeled 200 μM palmitic acid as described in the Experimental Procedures section. The values represent nmol/h and are presented as means ± SD from at least three independent experiments. For abbreviations see Table 1.



SCHEME 1

Acyl-carnitines are also formed in the peroxisomes (33). Thus, it is possible that palmitate is partially oxidized in the peroxisomes and enters the mitochondria *via* peroxisomal CPT-I and mitochondrial CPT-II (Fig. 9), like phytanic and pristanic acids (37). Oxidation of palmitic acid by such a mechanism would be dependent on peroxisomal CPT-I and mitochondrial CPT-II, which indeed are upregulated after C_{14}-S-acetic acid (Figs. 2, 4, and 7 and Table 3).

In conclusion, the present study demonstrates that the effects of 3-thia fatty acids on CPT-I and CPT-II are different from those of EPA and starvation. Our results suggest that the crucial role of mitochondrial CPT-I in the control of entry- and β-oxidation of fatty acids in the hepatic mitochondria after EPA feeding and starvation does not seem evident after treatment with mitochondrion and peroxisome-proliferating 3-thia fatty acids. More work needs to be done to fully understand the mechanism by which CPT-II is being regulated.

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Cross-Influence of Membrane Polyunsaturated Fatty Acids and Hypoxia-Reoxygenation on α- and β-Adrenergic Function of Rat Cardiomyocytes

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ABSTRACT: The purpose of the present investigation was to determine whether the beneficial effects of polyunsaturated fatty acids (PUFA) may influence ischemia–reperfusion-induced alterations of myocardial α- and β-adrenoceptor (α-AR, β-AR) responsiveness. This study was carried out using monolayer cultures of neonatal rat ventricular myocytes in a substrate-free, hypoxia-reoxygenation model of ischemia. The cardiomyocytes (CM) were incubated during 4 days in media enriched either with n-6 PUFA (arachidonic acid, AA) or with n-3 PUFA (eicosapentaenoic acid, EPA, and docosahexaenoic acid, DHA). The n-6/n-3 ratio in n-3 CM was close to 1.2, compared to 20.1 in n-6 CM. The contractile parameters of n-6 CM and n-3 CM were similar in basal conditions as well as during hypoxia and reoxygenation. In basal conditions, the phospholipid (PL) enrichment with long chain n-3 PUFA resulted in an increased chronotropic response to isoproterenol (ISO) and to phenylephrine (PHE). After posthypoxic reoxygenation, the chronotropic response to β-AR activation in n-6 CM was significantly enhanced as compared with the control response in normoxia. In opposition, the ISO-induced rise in frequency in n-3 CM in control normoxia and after reoxygenation was similar. In these n-3 CM, the changes in contractile parameters, which accompanied the chronotropic response, were also similar in reoxygenation and in normoxic periods, although the rise in shortening velocity was slightly increased after reoxygenation. In response to PHE addition, only the chronotropic effect of n-6 CM appeared significantly enhanced after hypoxic treatment. These results suggested that increasing n-3 PUFA in PL reduced the increase in α- and β-AR functional responses observed after hypoxia-reoxygenation. This effect may partly account for the assumed cardiac protective effect of n-3 PUFA, through the attenuation of the functional response to catecholamines in the ischemic myocardium.

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Abbreviations: AA, arachidonic acid; ANOVA, analysis of variance; AR, adrenoceptor; CD20, duration at 20% contraction amplitude from peak contraction; CD80, the duration at 80% contraction amplitude from peak contraction; CM, cardiomyocytes; +Cmax, time for 20–80% shortening; −Cmax, time for 20–80% relaxation; CR, contraction rate; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; ISO, isoproterenol; PHE, phenylephrine; PL, phospholipids; PUFA, polyunsaturated fatty acids.

The effects of dietary polyunsaturated fatty acids (PUFA) on serum lipids and membrane phospholipid (PL) fatty acid composition and their pathophysiological and biochemical consequences have been extensively studied in the cardiovascular system. Nevertheless, the mechanisms underlying the beneficial effects of PUFA, of either the n-6 or the n-3 series, are still unclear. In particular, previous results (1) challenged the view of the main influence of n-3 PUFA on vascular parameters and suggested that their protective effects may be mediated through other mechanisms, such as direct action on the myocardium.

The lipid composition of cardiac membranes can be modified by the diet according to the fatty acid unsaturation degree, with a specificity for the PL classes (2,3). Such changes were reported to influence the contraction rate and the systolic ejection volume of the rat heart and improve the metabolic recovery and oxygen consumption after ischemia and reperfusion (4,5). In addition, dietary PUFA appeared to influence the cardiac noradrenaline sensitivity through alteration of the receptor binding characteristics and the signalling pathway (6–8), although differences in methodological approaches led to discordant data (9,10). Less attention has been devoted to the specific effect of PUFA on sarcolemmal protein function in pathological conditions. It was shown that dietary fish oil may prevent the development of arrhythmias in the isolated working heart in response to ischemia (11,12). Also, we had shown from earlier studies that the enrichment of PL in C_{18} PUFA was able to modulate the cardiac myocyte resistance to substrate-free hypoxia and its recovery after reoxygenation (13). However, apart from the suggestion that the *in vitro* induction of tachyarrhythmias by isoproterenol (ISO) did not appeared to be influenced by PUFA (14), there are no available experimental data on the cross-influence of ischemia and PL fatty acids on the adrenergic function.

Therefore, the purpose of the present work was to assess the respective influence of the composition in long chain PUFA of PL on the adrenergic functional responsiveness of the postischemic myocardial cell. Monolayer cultures of ventricular myocytes from the neonatal rat were incubated in media enriched either with arachidonic acid (AA) or with a

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mixture of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The effects of these changes in PL composition of the cardiomyocytes (CM) were evaluated on their basal contractile properties and on their responses to α- and β-adrenergic stimulation in physiological conditions and in a substrate-free, hypoxia-reoxygenation cellular model of myocardial ischemia.

MATERIALS AND METHODS

Cell culture. Primary cultures of rat ventricular myocytes were prepared with the ventricles of neonatal Wistar rat hearts as previously described (15). The proportion of myocytes in the cell suspension was increased by a two-step selective adhesion procedure. The final suspension was plated in 6-cm diameter culture dishes (Falcon Primaria 3802, Becton Dickinson, Oxnard, CA) at a density of 2×10^6 cells per dish. The cells were grown at 37°C in a standard culture medium (Ham's F10 basal medium, Seromed, Berlin, Germany) containing 10% fetal calf serum (Seromed, Berlin, Germany), 10% human serum and antibiotics (200 international units (IU)/mL penicillin and 150 IU/mL streptomycin, Seromed) in a humidified atmosphere with 5% CO_2. After 24 hours, the cells were incubated for 4 additional days in the same medium supplemented with serum albumin-bound fatty acids: either AA (20:4n-6) or EPA (20:5n-3) plus DHA (22:6n-3), leading to two CM populations (n-6 CM and n-3 CM, respectively). Reference cells (Ham C CM) were obtained by 5-d incubation in the standard culture medium. Using this method, the cell attachment was achieved within hours after seeding and then the cell growth gave rise to a continuous layer of confluent cells beating synchronously. The myocyte-rich cultures were composed of at least 95% cardiomyocytes, as assessed with microscopic observation and immunofluorescence staining with an antibody against rat cardiac myosin.

Fatty acid analysis. Fatty acids were analyzed according to previously detailed procedures (13). The media were freeze-dried and the fatty acids were transmethylated without previous extraction. In the cell, the lipids were extracted and the phospholipids were separated from nonphosphorous lipids using silica gel cartridges (Setpack, Waters, Bedford, Massachusetts). Then, the lipids were transmethylated using BF_3-methanol and the fatty acid methyl esters were analyzed by gas chromatography on a Carbowax 20M capillary column. The composition of the media in PUFA of relevance is presented in Table 1, with content in the other fatty acids—i.e., 16:0, 18:0, 18:1, and 18:2n-6—being similar in the standard and in the PUFA-enriched experimental media.

Experimental set-up. The experiments were done in static bath conditions in the glucose-free, Puck's F balanced salt solution under a paraffin oil layer. Cultured neonatal CM display spontaneous and regular beatings in basal salt media, showing that the presence of serum factors or hormonal supplement, such as catecholamines, are not required for the initiation and/or the maintenance of automaticity and contractile function, even during long term experiments. Furthermore, it has been previously determined that cultured rat cardiomyocytes are able to display stable normal function for hours in the presence as well as in the absence of glucose in the bath (16). The culture dish was placed in a heated water-circulation, gas-controlled chamber (17), fitted on the moving stage of an inverted phase contrast microscope. The temperature was maintained at 37°C throughout the experiments. In normoxic conditions, the dish was continuously flushed with air (600 mL/min) and the average values of pH, p_{O_2} and p_{CO_2} were 7.4 ± 0.3, 118.2 ± 3.5 mm Hg and 10.8 ± 1.9 mm Hg, respectively. Hypoxia was obtained by a flow of 100% N_2 (600 mL/min) during 2.5 h. In these conditions, p_{O_2} was reduced to 20 ± 4.3 mm Hg, whereas pH remained constant at 7.31 ± 0.2 (mean ± standard error of the mean; n = 17). Thereafter, the initial normoxic p_{O_2} value was restored by flushing air during 1 h at the same rate.

TABLE 1
Long Chain (C_{20}) Polyunsaturated Fatty Acid (PUFA) Composition of the Standard Culture Medium (Ham C Med) and the n-3 and n-6 PUFA-Enriched Media (n-3 Med and n-6 Med, respectively)[a]

Fatty acids	Ham C Med	n-6 Med	n-3 Med
20:4n-6	6.7	12.5	6.1
20:5n-3	0.6	0.6	4.6
22:6n-3	2.0	1.7	6.5

[a]n-6 Med was supplemented in arachidonic acid (20:4n-6) and n-3 Med in both eicosapentaenoic acid (20:5n-3) and docosahexaenoic acid (22:6n-3). Values expressed as percentage of total fatty acids ($n = 3 \times 3$ three sets of each medium); minor fatty acids were omitted.

Recording of cell contractions. The contractile function of the cultured CM was assessed by video, single-edge detection (10), which is adapted to newborn heart cells in culture, i.e., to cells fully attached and spread out onto the bottom of the dish, as in the case of the present study (18). This technique relies on the measurement of light density changes on the screen of a video monitor using a high-precision, narrow-angle light-sensitive silicon photodiode (SD-1420 Honeywell, Minneapolis), selected for its good linearity and its maximal sensitivity (gray-green) adapted to the video image. The phototransducer was fixed in the middle of a black rubber disc, in order to attenuate external light sources. The edge of a cell was positioned on the video field covered by the photodiode by moving the stage of the microscope. The edge of the cell moves during contractions, which caused light intensity changes, which were converted into an electrical signal by the transducer. This contraction signal was displayed by a storage oscilloscope (Gould DSO 1604, Ilford, Essex, UK) and transcribed onto paper chart using an ink-jet recorder (Siemens-Elema EM 81, Solna, Sweden). The analyses of acquired data and calculations were performed on a personal computer using an ASYST-based processing program (Keithley Instrument, Taunton). This procedure gives access to the time and rate parameters of the rhythmic cell contractions, allowing the quantification of the chronotropic effects. Moreover, although direct measurement of the developed force was not feasible, inotropic status can be evaluated from the calculation of the

myocyte shortening velocity ($+C_{max}$, time for 20% to 80% contraction) and relaxation velocity ($-C_{max}$, time for 20% to 80% relengthening) (18, 19). In some hypoxia-reoxygenation protocols on reference cells, action potential recording was carried out, together with contraction monitoring, using conventional glass microelectrode as previously described (16).

Pharmacology. Stock solutions of ISO and phenylephrine (PHE) (Sigma Chemical CO, St. Louis, MO) were prepared in Puck's F salt solution and kept at 0–4°C in the dark until use. The cells were treated by addition of 10 µL of these solutions (ISO or PHE) with a Hamilton syringe (Bonaduz, Switzerland) using a micromanipulator (Leitz, Wetzlar, Germany). The final concentrations of ISO and PHE in the culture dishes were 10^{-7} M and 10^{-6} M, respectively. Single drug addition in each dish was done to avoid fast desensitization of adrenoceptors (AR). These concentrations and conditions have been selected according previous studies (10,20), taking into account functional and biochemical responses. The duration of exposure to adrenoceptor agonists was too short to induce myocyte hypertrophy (21), which could have interfered with the functional responses. In addition, we have controlled that the culture medium contain no measurable traces of catecholamines, which could have influenced the adrenoceptor properties prior to experiments (22).

Statistics. The experimental data were collected in the physiological conditions for each dish as the mean of 5 measurements made in 5 defined areas before addition of the drug, and 5 other measurements made 10 min after addition of the drug. Basal contractility data were subjected to a two-way analysis of variance (ANOVA) with a fixed factor (culture medium) and a random factor (dish). The analysis of the effects of ISO and PHE in n-3 and n-6 cells was made by a three-way ANOVA on data expressed as percentage of change induced by the drugs. In the investigations involving hypoxia and reoxygenation, 6 dishes were used in each group. The measurements were made at 30 min intervals during 2.5 h (H1 to H5) hypoxia and 1 h reoxygenation (R1 and R2). The results obtained for the contractile parameters were analyzed by a two-way ANOVA (medium, treatment). In the studies of the stimulation by ISO and PHE after hypoxia-reoxygenation, the data were expressed as percentage change from nonstimulated cells, and were analyzed by a three-way ANOVA. The differences were considered significant when $P < 0.05$. Reference data were obtained from cells maintained for 5 days in the standard culture medium and submitted to the same protocols. However, the statistical analysis was done on the PUFA-enriched cells only, because the large difference in total fatty acid supply between the experimental medium and control medium might have introduced cell differences which would not be interpretable in terms of qualitative phospholipid PUFA differences.

TABLE 2
PUFA Composition of the Phospholipids (PL) of Rat Cardiomyocytes Incubated 4 d in the n-3 and n-6 PUFA-Enriched Media (n-3 CM and n-6 CM, respectively), in Comparison of the Composition of the Phospholipids of CM Incubated in the Standard Ham C Medium (Ham C CM)[a]

Fatty acids	Ham C CM	n-6 CM	n-3 CM	ANOVA
18:2n-6	11.6 ± 0.25	4.4 ± 0.08	7.7 ± 0.14	c
20:2n-6	0.6 ± 0.06	0.4 ± 0.02	0.3 ± 0.03	a
20:3n-6	1.7 ± 0.05	0.7 ± 0.01	1.0 ± 0.02	c
20:4n-6	18.7 ± 0.79	28.1 ± 0.30	12.5 ± 0.56	c
22:4n-6	3.0 ± 0.12	8.2 ± 0.14	0.8 ± 0.04	c
22:5n-6	0.4 ± 0.02	0.4 ± 0.02	0.1 ± 0.02	b
20:5n-3	0.3 ± 0.07	0.1 ± 0.01	5.5 ± 0.24	c
22:5n-3	1.3 ± 0.06	0.6 ± 0.04	4.3 ± 0.15	c
22:6n-3	2.8 ± 0.13	1.5 ± 0.11	8.3 ± 0.26	b
SFA	36.2	36.5	37.4	NS
MUFA	23.4	19.2	22.1	NS
PUFA	40.4	44.3	40.5	NS
n-6 PUFA	36.0	42.2	22.4	c
n-3 PUFA	4.4	2.1	18.1	c
n-6/n-3	8.2	20.1	1.2	c

[a]Data are expressed as percentage of total fatty acids ($n = 9$ in each group of CM: three dishes per culture preparation and 3 culture preparations). SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; NS, not significant. Statistical analysis compared n-3 CM vs. n-6 CM, $^{a}P < 0.05$, $^{b}P < 0.01$, and $^{c}P < 0.001$.

RESULTS

PL PUFA. The fatty acid composition of cell PL is presented in Table 2. The content in saturated fatty acids, monounsaturated fatty acids, and PUFA were similar in the three groups of CM, and represented 35, 20, and 45 % of the total fatty acids, respectively. In contrast, altering the long chain PUFA content of the culture medium resulted in significant qualitative modifications of the PUFA fraction of PL, since the PL n-6/n-3 ratio was 20.1 in n-6 CM and 1.2 in n-3 CM, in comparison to 8.2 in the reference CM. The PUFA composition of PL of the n-3 cells was characterized by a high content in EPA (5.5%), docosapentaenoic acid (22:5n-3) (4.3%), and DHA (8.3%). The n-6 cells were enriched in AA (28.1% vs. 12.5% for n-3 cells) and docosatetraenoic acid, (22:4n-6) (8.2%).

Contractile properties. Myocardial cells exhibited sustained, regular rhythmic contractions, in synchrony in the confluent monolayer (see Fig. 3A, CTRL). This spontaneous activity was determined by one or a very few myocardial cells regaining automaticity after isolation (23). These pacemaker cells exhibited characteristic diastolic depolarization and the voltage dependence of the rate of activity (24). The propagation of this spontaneous activity throughout the cell monolayer was achieved *via* permeable, low-resistance gap junctions, as in the native, *in vivo* myocardial tissue. The bulk of the monolayer was thus composed of driven cells, characterized by the absence of the pacemaker diastolic depolarization.

The contractile parameters of the reference CM and of the PUFA-enriched cells are presented in Table 3. The contraction rate was in the range of 181.7 to 313.6 cycles/min, with a mean of 230.1 cycles/min. The mean values of duration of the contraction peak (CD20, duration at 20% contraction amplitude from peak contraction) and of the overall contraction duration (CD80, the duration at 80% contraction amplitude from

TABLE 3
Basal Contractile Parameters of Cardiomyocytes Grown in Standard Ham C medium (Ham C CM) and of Cardiomyocytes Incubated for 4 d in n-3 and n-6 PUFA-Enriched Media (n-3 CM and n-6 CM, respectively)[a]

	CR (cycles/min)	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Ham C CM	230 ± 14.8	78 ± 7.8	188 ± 11.7	49 ± 2.2	63 ± 3.7
n-6 CM	250 ± 23.8	77 ± 9.3	185 ± 17.3	49 ± 3.2	61 ± 4.9
n-3 CM	225 ± 20.3	82 ± 9.3	191 ± 16.2	51 ± 3.0	62 ± 4.7
P	NS	NS	NS	NS	NS

[a]Data are the means ± standard error of mean (SEM) (n = 13). CR, contraction rate; CD20, the duration at 20% contraction amplitude from peak contraction; CD80, duration at 80% contraction amplitude from peak contraction; $+C_{max}$, time for 20 to 80% shortening; $-C_{max}$, time for 20 to 80% relaxation; for other abbreviations see Table 2. As stated in the Materials and Methods section, the ANOVA comparison was between n-6 and n-3 CM.

peak contraction) were 77.7 and 188.4 ms, respectively. The time for 20 to 80% contraction ($+C_{max}$) varied from 38 to 66 ms. The time for 20 to 80% relaxation ($-C_{max}$) ranged between 46 and 78 ms.

The functional characteristics of the n-3 and n-6 CM appeared close to those expressed by the CM grown in the standard culture medium and defined as the cardiac muscle cells of reference. Therefore, important changes in the n-6/n-3 ratio did not significantly impede the development of normal contractile function of isolated CM. In basal conditions, moreover, the fatty acid composition did not influence the contractile properties of cardiomyocytes, as the apparent increase in contraction rate in n-6 CM was not significant. The modification of the n-6/n-3 PUFA ratio had no incidence on the contraction peak duration (CD20) or on the overall contraction duration (CD80) (76.8 and 185 ms, respectively). Similarly, the modifications in PL PUFA did not influence the shortening time ($+C_{max}$) or the relaxation time ($-C_{max}$) (49.5 and 61.6 ms, respectively).

Effect of ISO. In response to the addition of ISO (10^{-7}M), an increase in beating rate appeared within 2 min in the reference CM as well as in the PUFA-enriched cells (Fig. 1, inset). The effects of ISO on the measured values of contraction parameters measured from reference CM are presented in Table 4. In n-3 CM and n-6 CM, the β-agonist caused an increase in spontaneous beating rate and, as in the case of the basal contractile function, the ISO-induced functional responses in these two experimental cell groups were comparable to that observed in the reference CM (Fig. 1). Therefore, the PUFA enrichment did not affect the development of normal myocardial β-adrenergic responsiveness. The positive chronotropic effect was accompanied by a shortening of the contraction duration parameters (CD20 and CD80) (Table 4). Additionally, a decrease in the shortening velocity ($+C_{max}$) and in the relaxation velocity ($-C_{max}$) was observed, indicating a direct ISO-induced inotropic response (*cf.* Materials and Methods section).

The comparison of the chronotropic responses in the n-6 and n-3 CM (Fig. 1) showed that the ISO-induced increase in beating rate was significantly greater in n-3 CM than in the n-6 CM, since the addition of the drug increased the beating rate by 25% in the n-3 CM and by only 15% in the n-6 CM. The modifications of the other contraction parameters caused by ISO were not significantly influenced by the n-6/n-3 ratio of cell PL, although the ISO-induced decrease in the contraction duration indexes and in shortening and relengthening times seemed greater in n-3 CM (Fig.1).

Effect of PHE. The effects of PHE addition (10^{-6} M) on the myocardial cell contractions are shown in Figure 2. As in the case of the exposure to ISO, whatever the incubating medium, the addition of the α-agonist induced a gradual increase in the spontaneous beating rate. The peak response appeared within 2 min and was followed by a slight decline to a steady level, during which measurements were made (Fig. 2, inset). The corresponding changes in contraction parameters of reference CM are presented in Table 5. The functional response to PHE of n-3 CM and n-6 CM to PHE (Fig. 2) was close to that of Ham C CM, and therefore, the expression of a normal α-adrenergic response was not substantially influenced by the changes in n-6/n-3 ratio. This positive

TABLE 4
Effect of Isoproterenol on Contractile Parameters of Cardiomyocytes Grown in Standard Ham C Medium (Ham C CM)[a]

	CR (cycles/min)	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Control	240 ± 11.5	73 ± 7.1	162 ± 9.8	44 ± 2.1	68 ± 3.6
ISO	280 ± 13.5	62 ± 6.8	131 ± 10	37 ± 3.1	55 ± 4.0
P	<0.05	NS	<0.05	<0.05	<0.05

[a]Data are the mean ± SEM (n = 7). ISO, isoproterenol; for other abbreviations see Table 3.

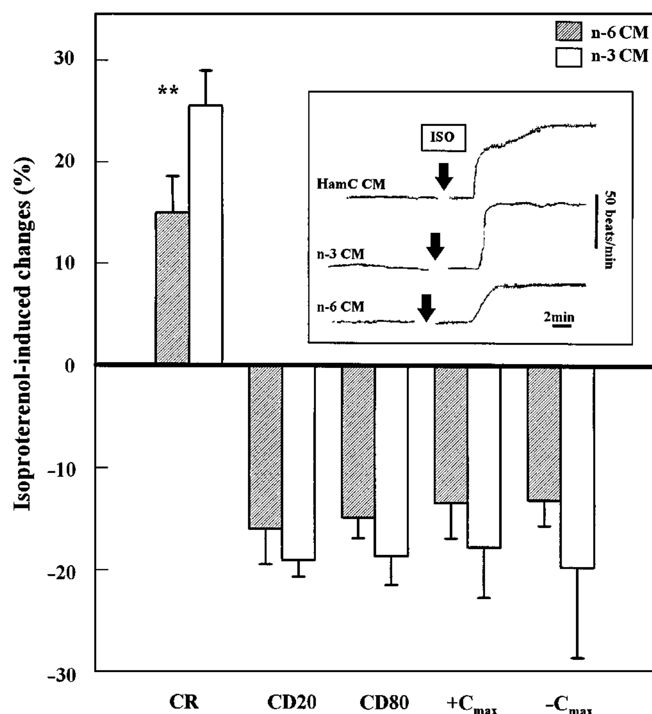

FIG. 1. Relation between the polyunsaturated fatty acid (PUFA) phospholipid (PL) composition (n-6 or n-3 profile) and isoproterenol (ISO) effects on the contractile parameters. Data are expressed as percentage of change induced by ISO stimulation (10^{-7} M). Values are means ± standard error of mean (SEM) (*n* = 7); **, $P < 0.05$. CR, contraction rate; CD20, duration at 20% contraction amplitude from peak contraction; CD80, the duration at 80% contraction amplitude from peak contraction; $+C_{max}$, time for 20–80% shortening; $-C_{max}$, time for 20–80% relaxation.

Inset. Continuous record of the spontaneous beating rate of ventricular myocytes before and after ISO (10^{-7} M) addition (arrow). Experiments were done after 4-d incubation in Ham C, n-3, and n-6 media. CM, cardiomyocytes.

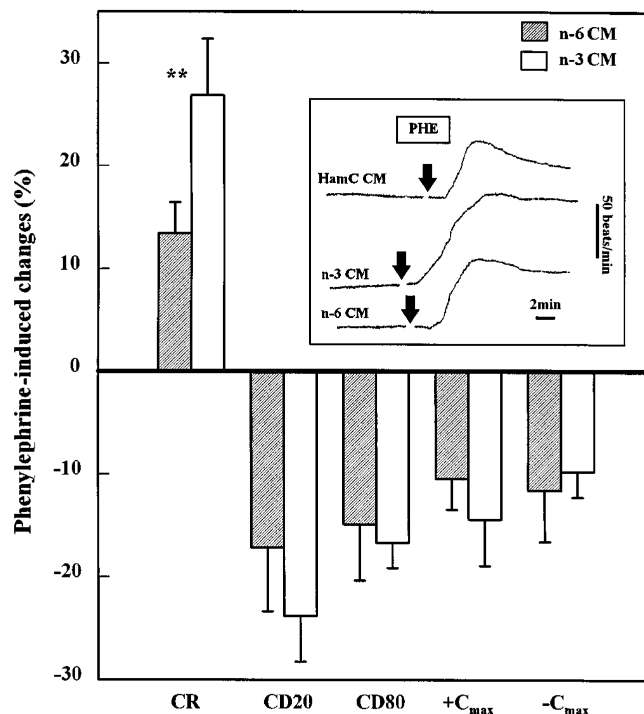

FIG. 2. Relation between PUFA PL composition (n-6 or n-3 profile) and phenylephrine (PHE) effects on the contractile parameters. Data are expressed as percentage of change induced by PHE stimulation (10^{-6} M). Values are means ± SEM (*n* = 6); ** $P < 0.05$. For abbreviations see Figure 1.

Inset. Continuous record of the spontaneous contraction rate of rat ventricular myocytes before and after PHE (10^{-6} M) addition (arrow). Experiments were done after 4-d incubation in Ham C, n-3, and n-6 media.

chronotropic response was accompanied by a decrease in the contraction duration parameters (CD20 and CD80). The addition of PHE decreased the shortening time ($+C_{max}$) significantly but similarly in the two experimental groups of CM.

The diagrammatic comparison of the chronotropic responses to PHE between the two experimental groups of CM (Fig. 2) shows that these responses differed with respect to the PUFA composition of the PL, since the increase in rate was significantly greater in n-3 CM than in n-6 CM (+28% and +13%, respectively). In contrast, there was no significant difference between n-6 CM and n-3 CM in the contraction duration parameters (CD20 and CD80) and in the relaxation time ($-C_{max}$), in spite of an apparent larger decrease in n-3 CM.

Substrate-free hypoxia and reoxygenation. Figure 3A depicts the typical alterations and recovery of the spontaneous electrical and contractile activity of reference Ham C CM during control normoxia and during 150 min substrate-free hypoxia followed by reoxygenation. As detailed previously, hypoxia caused a progressive decrease in action potential plateau and duration, accompanied by rhythm disturbances (Fig. 3A, H1, H2). Thereafter, a drastic fall in rate, action potential amplitude and contraction were observed (Fig. 3A, H3, H4). Hypoxia led finally to the complete arrest of the electrical activity (Fig. 3A, H5). After reoxygenation (Fig. 3A, R2), the spon-

TABLE 5
Effect of Phenylephrine on Contractile Parameters of Cardiomyocytes Grown in Standard Ham C Medium (Ham C CM)[a]

	CR (cycles/min)	CD20 (ms)	CD80 (ms)	$+C_{max}$ (ms)	$-C_{max}$ (ms)
Control	261 ± 12.9	70 ± 8.2	181 ± 10.2	47 ± 2.8	68 ± 4.0
PHE	289 ± 14.7	60 ± 6.7	150 ± 9.7	39 ± 4.8	58 ± 5.2
P	<0.05	NS	<0.01	<0.05	<0.05

[a]Data are the means ± SEM (*n* = 6). PHE, Phenylephrine; for other abbreviations see Table 3.

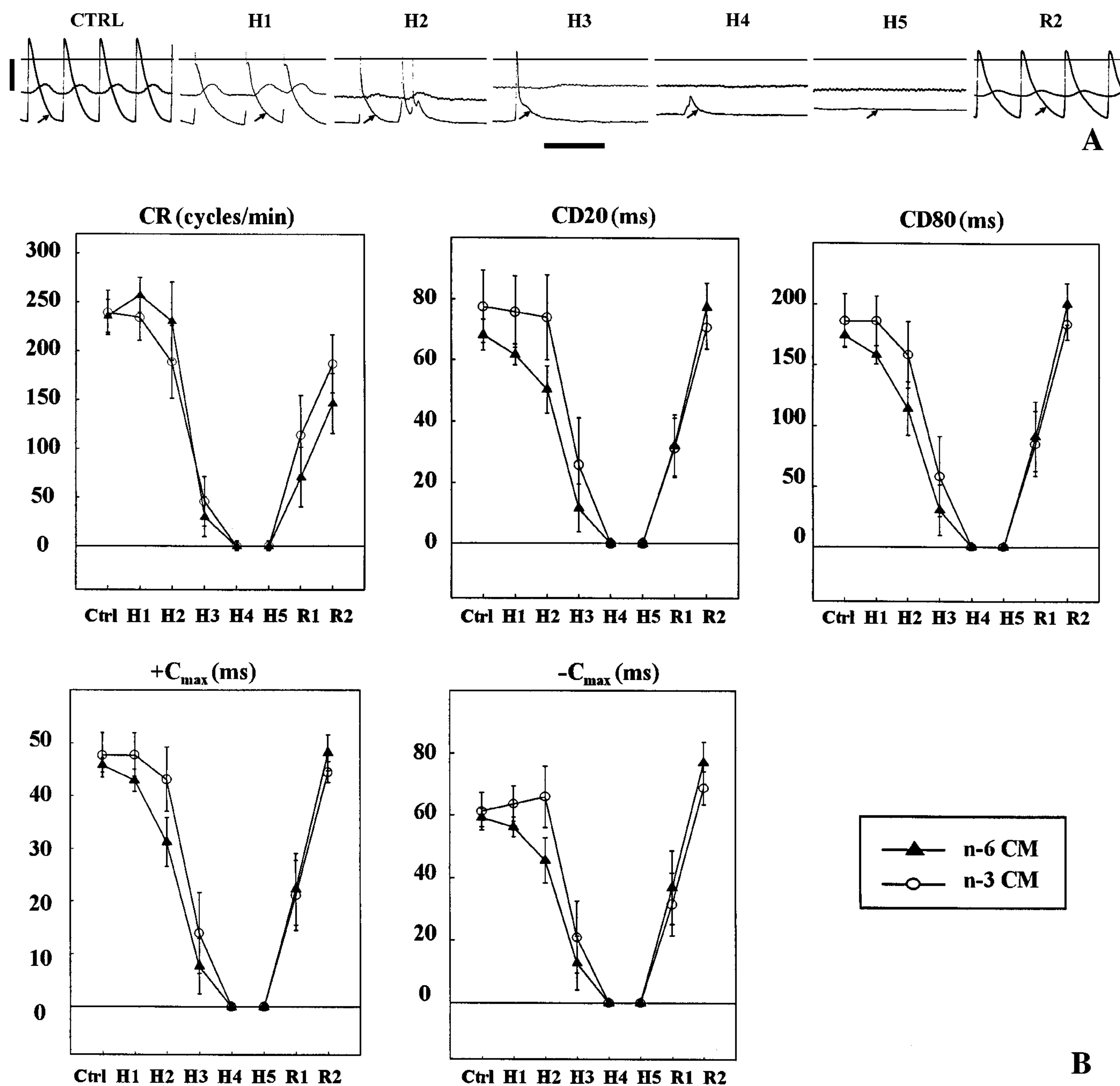

FIG. 3. (A) Typical changes in action potentials (arrows) and contractions (middle traces) simultaneously recorded from cultured rat ventricular myocytes before (CTRL, control) and during hypoxia (H1, H2, H3, H4, and H5; 0.5 h, 1 h, 1.5 h, 2 h, and 2.5 h N_2-induced, substrate-free hypoxia, respectively) followed by 1.0 h reoxygenation (R1 and R2; 0.5 h and 1 h air readmission). All records were obtained from the same cell culture dish. In each panel, the horizontal trace represents the zero potential level. Vertical bar: 40 mV; horizontal bar: 400 ms. Scale for contraction is arbitrary. Note the early rhythm irregularity (H1) followed by triggered afterpotentials (H2). (B) Time evolution of changes in the different contractile parameters before (Ctrl, control) and during hypoxia (H1, H2, H3, H4, and H5; 0.5 h, 1 h, 1.5 h, 2 h, and 2.5 h N_2-induced, substrate-free hypoxia, respectively) followed by 1.0 h reoxygenation (R1 and R2: 0.5 h and 1 h air readmission, respectively). n-3 CM, n-6 CM, cardiomyocytes incubated 4 days in n-3 and n-6 media, respectively. The data represent the mean ± SEM for 6 experiments per cell group. For abbreviations see Figure 1.

taneous electromechanical activity resumed, so that the action potential and contractions reverted close to their prehypoxic configurations. Dysrhythmias were never observed during reoxygenation nor under basal, normoxic conditions.

The time-evolution of the various contractile parameters of the n-3 and n-6 CM during substrate-free hypoxia is presented in Figure 3B. The rhythmic contractions were depressed after 1 h and ceased after 2 h hypoxia to a similar extent in the two PUFA groups of CM. The substrate-free hypoxia also altered the contraction duration and the shortening and relaxation times, which decreased significantly after 1 h hypoxia (H2). At the end of the 2.5 h period of hypoxia, the

CM were reoxygenated by resupplying the chamber with air for 1 h. During this reoxygenation period, the cardiomyocytes recovered spontaneous and regular automaticity and beatings. This recovery of contractile properties during reoxygenation was not influenced by the n-6/n-3 PUFA ratio of the CM. The changes in contractile parameters provoked by hypoxia-reoxygenation in n-3 and n-6 CM was also similar to those observed in the myocytes maintained in standard culture medium (Ham C CM) (16).

Posthypoxic adrenergic responses. Posthypoxic AR responsiveness was assessed through the addition of the receptor agonists 1 h after the reoxygenation following the hypoxic treatment. In these conditions, the response to the addition of ISO was a gradual increase of the contraction rate within 2 min. The corresponding changes in contractile parameters in the two experimental groups of CM are presented in Figure 4. In the n-6 CM, the ISO-induced rise in spontaneous frequency was significantly enhanced in comparison to the control response in normoxia (63.6% vs. 16.6%; Fig. 4A). In the n-3 CM conversely, the chronotropic effect measured after hypoxia-reoxygenation did not significantly differ from that obtained in normoxia. The ISO-induced changes in the other contractile parameters during normoxia and after hypoxia-reoxygenation were similar, although the rise in shortening velocity ($+C_{max}$) in n-3 CM was increased after reoxygenation (Fig. 4B).

The stimulation of the CM by PHE after hypoxia-reoxygenation induced a typical α-adrenergic positive chronotropic response followed by a partial progressive decline to a steady level. In comparison to normoxia, the chronotropic effect in n-6 CM was significantly increased after hypoxia-reoxygenation (55% vs. 17.4% in normoxia; Fig. 5). Conversely, the increases in contraction rate induced by PHE in n-3 CM were similar in normoxia and after hypoxia-reoxygenation. The PHE-induced modifications in the other contractile time parameters were not affected by hypoxia-reoxygenation treatment in n-3 CM and in n-6 CM (data not shown).

DISCUSSION

The objective of this work was to assess the cellular consequences of different fatty acid diets on the AR function in the normal and postischemic myocardium by the use of monolayer cell cultures from neonatal rat ventricles. In this cellular model, CM displayed regular and sustained spontaneous rhythm, without arrhythmias, in the basal conditions. Moreover, these cultivated CM retained differentiated electrical properties in monolayer cultures, including tetrodotoxin-sensitive rapidly rising action potentials (24), which are comparable with those of the tissue of origin (25).

Two groups of samples were prepared by incubating the cells in two different PUFA-enriched media. The PL of CM displayed similar global contents in saturated fatty acids, monounsaturated fatty acids, and PUFA. Conversely, their composition in individual PUFA was influenced by the fatty acids supplied by the media, leading to n-6 cells displaying an enhanced incorporation of AA in PL, and n-3 cells characterized by their high amount of docosapentaenoic acid and DHA. The n-6/n-3 PUFA ratio was hence close to 1 in n-3 cells and reached 20 in n-6 cells. These observations were in agreement with our preliminary results (26) and are comparable with those reported in the hearts of rats fed 8 weeks of fish oil or sunflower oil-enriched diets (4).

We found that the basal automaticity and contractile parameters of CM were not influenced by these large changes in

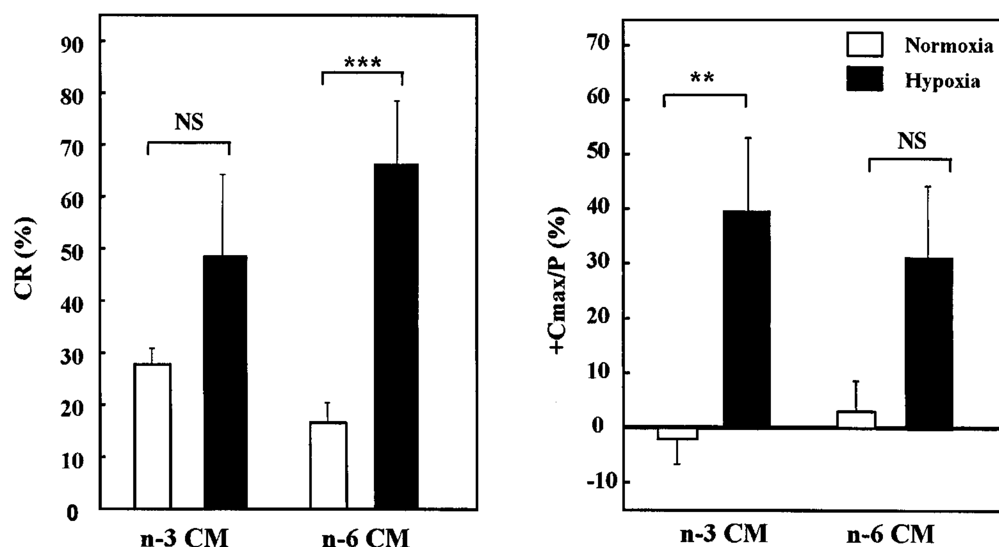

FIG. 4. Functional responses to isoproterenol addition (10^{-7} M) during normoxia (white bars) and during posthypoxic reoxygenation (black bars). n-3 CM, n-6 CM, cardiomyocytes incubated 4 days in n-3 and n-6 media, respectively; CR: contraction rate; $+C_{max}/P$: normalized shortening velocity, where P = 1/CR. Values are given as means ± SEM (n = 6 for each experimental group); *** $P < 0.001$; ** $P < 0.05$; NS, not significant.

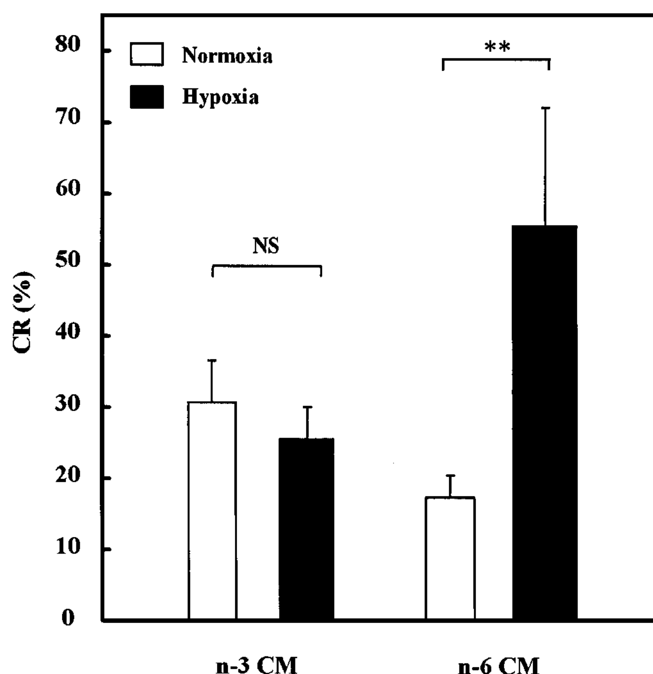

FIG. 5. Chronotropic responses to phenylephrine addition (10^{-6} M) during normoxia (white bars) and during posthypoxic reoxygenation (black bars). CR, contraction rate; n-3 CM, n-6 CM, cardiomyocytes incubated 4 days in n-3 and n-6 media, respectively. Values are given as means ± SEM (*n* = 6 for each group); ** $P < 0.05$; NS, not significant.

the n-6/n-3 PUFA ratio. These results are consistent with our previous study using C_{18} PUFA precursors (10,13) and with other reports (27). Similarly, *in vivo* studies have shown that modifications of cardiac PUFA profile do not alter myocardial performances (28–30), although in these conventional experimental models the difference between the n-6 and n-3 PUFA series has not been clearly explored.

The α- and β-AR functions were assessed by addition of PHE and ISO, respectively, on the basis of the subsequent changes in automaticity and cell contractility. It must be emphasized that there was no interference with possible AR-dependent hypertrophy (21), because of the short duration of exposure.The modifications of the PL n-6/n-3 PUFA ratio had no effect on the inotropic response to α- and β-adrenergic stimulation, because ISO and PHE induced a similar reduction of the shortening and relaxation velocities ($+C_{max}$ and $-C_{max}$, respectively). In contrast, the positive chronotropic response induced by both agonists were more pronounced in n-3 CM, indicating that the rise in the contraction rate after α- and β-stimulation was influenced by the modifications of the membrane PUFA composition. These results confirmed and extended our previous study indicating that CM enriched with C_{18} n-3 PUFA precursors exhibit an enhanced β-adrenergic positive chronotropy (10).

As previously detailed, substrate-free hypoxia induced a gradual decline in rhythmic contractions of CM followed by the cessation of beatings (16). These changes in automaticity and contractility have been correlated (31) to electrophysiological (Fig. 3A) and biochemical alterations. In the present work, the hypoxia-induced alterations in contractile characteristics were not influenced by the n-6/n-3 PUFA ratio. Consistently, Karmazyn *et al.* (32) showed in isolated CM that neither the developed force nor the resting tension following hypoxia were affected by the PL fatty acid content. The recovery of the mechanical properties during reoxygenation was also similar in the two groups of CM. However, the influence of PUFA seems to depend on the function studied, since we previously showed that the posthypoxic electrophysiological recovery was faster in n-3 CM (33). In addition, dietary fish oils were also assumed to be beneficial against ischemic myocardial dysfunction (5) and on mitochondrial oxidative capacity (4) in conventional intact heart models of cardiac
ischemia.

Despite the absence of influence of the n-6/n-3 ratio of PUFA on the CM contraction and automaticity throughout the course of hypoxia-reoxygenation, the chronotropic response caused by ISO and PHE in reoxygenation following hypoxia was significantly enhanced in n-6 CM as compared with the control response in normoxia. In contrast, the n-3 CM exhibited statistically identical ISO- and PHE-induced increases in rate in normoxia and in reoxygenation. In parallel to the functional investigation described herein, a companion study has been carried out to evaluate the influence of the PUFA composition on adrenergic messenger production by the posthypoxic cardiac muscle cells (34). This study showed that hypoxia affects the β-adrenergic messenger production and that a n-3 PUFA profile accentuates this change, because the decrease in the cAMP (cyclic adenosine 3′,5′-monophosphate) production in posthypoxic reoxygenation was more pronounced in DHA-enriched cells than in AA-enriched cells. This may account at least partly for the relatively attenuated β-adrenergic response in the posthypoxic n-3 CM. Conversely, the changes in phospholipid PUFA composition had no effect on the α-agonist-induced inositide phosphate production in the posthypoxic CM, suggesting that the decrease in α-adrenergic functional response in n-3 CM noted in the present study could not be linked to an alteration of the corresponding messenger production.

To our knowledge, the cross-influence of dietary PUFA and ischemia-reperfusion on the myocardial AR function have not been studied hitherto. However, Anderson *et al.* (35) suggested an enhanced role for the α_1-adrenergic system in initiating reperfusion-induced pathological events. It was also suggested that the rat heart contractile failure after ischemia was caused by an impaired second messenger system downstream from cyclic adenosine 3′,5′-monophosphate formation (36). In contrast, Strasser *et al.* (37) proposed that a sensitization of the β-adrenergic system may contribute to the increased sensitivity of the infarcted heart to catecholamines. This upregulation would be accompanied by an increased β-adrenergic receptor (β-AR) kinase activity (38), which involves a translocation of the enzyme to the membrane. Since the translocation of the G protein-coupled receptor kinases

(including β-AR kinase) seems to be modulated by membrane lipids (39), it could be hypothesized that the current enhancement of the posthypoxic response to α-stimulation in n-6 CM may involve an interplay between the G protein-coupled receptor kinase activity and membrane PL.

Nevertheless, although the data obtained suggested a modulation by PUFA of the posthypoxic responsiveness of the myocardial adrenoceptors, their direct extrapolation to the *in vivo* situation must be done cautiously. The isolated cardiac muscle cell, which is considered as a valuable model of the *in situ* cardiomyocyte, cannot be representative of the much more complex situation *in vivo* that involves interference with the complex, extrinsic autonomic mechanisms regulating the cardiovascular functions.

Within these limitations, it can be concluded that the PUFA-induced modulation of the α- and β-adrenergic responsiveness could be considered as functional correlates of the assumed dependence of the membrane functional proteins on membrane dynamic properties (40). Other mechanisms could be involved, however, such as alterations in the intracellular steps of the transduction pathways, as suggested earlier (36–38). Moreover, this study demonstrates that PL enrichment with n-3 PUFA is able to attenuate the increase in α- and β-AR functional responses after posthypoxic reoxygenation. This effect may contribute to the beneficial effect of the n-3 PUFA, which may involve a depressed response to endogenously released catecholamines in the ischemic myocardium.

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Effects of Dietary Supplementation of Saturated Fatty Acids and of n-6 or n-3 Polyunsaturated Fatty Acids on Plasma and Red Blood Cell Membrane Phospholipids and Deformability in Weanling Guinea Pigs

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ABSTRACT: The fatty acid composition of plasma cholesteryl esters, plasma phospholipids, red blood cell (RBC) membrane phosphatidylcholine (corresponding to the outer membrane leaflet), and phosphatidylethanolamine (corresponding to the inner membrane leaflet) was investigated in weanling guinea pigs fed with diets of cacao (saturated fatty acids), sunflower oil [n-6 polyunsaturated fatty acids (PUFA)] or fish oil (n-3 PUFA) for 20 wk. RBC deformation was measured by means of a cell-transit analyzer (filtration) and a cone-plate rheoscope. The contents of saturated fatty acids in plasma phospholipids and RBC membrane leaflets were similar in all three groups. Diets with sunflower oil resulted in a high content of linoleic acid in plasma cholesteryl esters and in the outer leaflet of RBC membranes. Fatty acids of fish oil were mainly incorporated in plasma phospholipids and in the inner leaflet of RBC membranes. The arachidonic acid content was high in all groups in the plasma phospholipids and in the inner leaflet. The n-6 and n-3 PUFA were mainly incorporated in the inner leaflet. In all groups the polyunsaturated/saturated fatty acid ratio and the total PUFA content were similar in the inner RBC membrane. The RBC filtration times and the RBC deformation indices were not affected by the dietary treatment.

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Polyunsaturated fatty acids (PUFA) such as arachidonic acid (AA), docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), which are present in cell membrane phospholipids, are important for many biochemical and physiological functions (1,2). Synthesis of AA and DHA occurs *in vivo* by desaturation and elongation of linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), respectively (3). A number of animal studies have demonstrated a close association between perinatal supply of essential fatty acids and long-chain PUFA contents of neural lipids (4,5). Human milk fatty acids usually contain sufficient amounts of essential fatty acids and other PUFA (6), whereas formula-fed infants have lower levels of PUFA (3,7). Addition of long-chain fatty acids to formula increases their concentration in the plasma and RBC membranes of preterm and full-term infants (3,8). Since phospholipids and cholesteryl esters of the plasma and phosphatidylcholine (PC) and phosphatidylethanolamine (PE) of the erythrocyte membranes correlate with the lipid composition of other cell membranes, the effect of dietary fatty acid supplementation may be studied by lipid analyses of plasma and RBC membranes (8,9).

Lipids compose about 45% by weight of the RBC membrane, and phospholipids make up about 60% of the RBC lipids (10). The phospholipids are distributed over the outer and inner leaflet of the lipid bilayer in an asymmetric fashion: The outer monolayer contains all the glycolipids and about 75% of PC, whereas 80% of PE are found in the inner monolayer (10,11). Thus, the PC fraction of the lipid analysis represents the outer part and the PE fraction the inner part of the RBC membrane (12).

Diets with saturated fatty acids or n-3 or n-6 PUFA result in incorporation of the different fatty acids into RBC membranes and may influence RBC deformability. Several studies reported improved RBC deformability (13–15) or recovery of disturbed RBC deformability to normal levels (16,17) after supplemetation of diets with n-3 PUFA. In contrast, other studies showed no effect of n-3 PUFA on RBC deformability (18–21). These contradictary results may be due to different amounts of fish oil having been consumed and to different methods of RBC deformability measurements, i.e., filtration (13–18) or constant shear methods such as the rheoscope (19).

Effects of saturated fatty acid or n-3 and n-6 PUFA diets on RBC deformability have not been studied.

The aim of this study was to investigate the incorporation of saturated fatty acids (cacao), n-6 PUFA (sunflower oil) or n-3 PUFA (fish oil) in plasma and RBC membrane lipids.

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Abbreviations: AA, arachidonic acid 20:4n-6; CE, plasma cholesteryl esters; CTA, cell-transit analyzer; DHA, docosahexaenoic acid 22:6n-3; EPA, eicosapentaenoic acid 20:5n-3; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, plasma phospholipids; P/S ratio, polyunsaturated/saturated fatty acid ratio; PUFA, polyunsaturated fatty acids; RBC, red blood cells.

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Furthermore, we studied the effect of the different diets on RBC deformability measured by means of a cone-plate rheoscope and by means of a cell-transit analyzer (filtration technique). The guinea pig was chosen because its fatty acid metabolism, especially the Δ5-desaturase enzyme activity, is similar to that of humans (2,22).

MATERIALS AND METHODS

Animals and diets. Twenty-four male weanling Duncan Hartley guinea pigs (Thomae Company, Biberach, Germany) were randomized into three feeding groups having similar body mass (250 ± 20 g). They were caged individually and provided with tap water *ad libitum*. They were fed for 2 wk on a ground standard laboratory diet (Unilever Co., Vlaardingen, The Netherlands) before shifting to fat diets containing 20.7% casein, 52.9% starch, 9.2% cellulose, 6.7% mineral mix, 0.5% vitamin mix, and 10% fat (weight %). The fat moieties (Table 1) were made by 8.7% cacao and 1.3% sunflower oil (saturated fatty acid group), 10% sunflower oil (n-6 group), 4.3% fish oil and 5.7% sunflower oil (n-3 group). Sunflower oil (5.7%) was added to fish oil to avoid linoleic acid deficiency. The diets were prepared in pellet form every 14 d (Unilever Co.), supplemented with vitamin E (200 mg/kg) and stored in polyethylene bags at −20°C. Menhaden was the source of fish oil. After 20 wk of dietary treatment (daily intake around 30–50 g) the weight gain was similar in the three groups, and the guinea pigs were sacrificed by cervical dislocation and blood was taken by cardiac puncture using EDTA as anticoagulant.

Blood samples. Blood was collected *via* cardiac puncture into EDTA. RBC, plasma, and buffy coat were separated by centrifugation. The plasma was taken for lipid analyses. The RBC were washed twice in an isotonic phosphate-buffered saline (PBS; 0.005 mol/L KH_2PO_4 and Na_2HPO_4, 0.153 mol/L NaCl, pH 7.4, 300 mosm/kg). RBC-PBS solution (20 μL) was used for RBC deformation studies.

Lipid analysis. Plasma and RBC were separated after centrifugation and the RBC pellet was washed three times with cold saline. The plasma lipid fractions (plasma phospholipids PL; and plasma cholesteryl esters, CE) were separated and analyzed according to a modification of the Folch *et al.* method (23), as described elsewhere (24). RBC lipids (PC and PE) were extracted and processed according to the Laryea *et al.* (25) method. This method avoided formation of alkenylether in the lipid fraction. Separation of the various lipid fractions was achieved by one-dimensional thin-layer chromatography on silica gel plate using chloroform/methanol/water (65:25:1, by vol) for separation of the plasma lipids. For RBC lipid separation chloroform/methanol/acetic acid/water (15:15:5:1, by vol) was used. The fatty acid composition of all lipid fractions was determined by using a Fisons VEGA 6000, series 2GC (Fisons Instruments, Mainz, Germany) capillary gas chromatograph equipped with a CP Sil 88 capillary column (Chrompack, Middleburg, The Netherlands; length 50 m, diameter 0.32 mm) and a flame-ionization detector. Helium was used as carrier gas. Identification of substances and regular calculations of appropriate response factors for each fatty acid were achieved by comparison with commercially available external standards GLC-68a (Nu-Chek-Prep. Inc., Elysian, MN). Data are presented as molar percentages of all fatty acids determined in each lipid fraction.

Red blood cell deformation. (i) Rheoscope studies. For the rheoscope deformability measurements 5 μL of the RBC suspension was diluted 1:50 in PBS containing 20 g/dL of dextran T-2000 (Sigma, Munich, Germany). The osmolality of this dextran solution was 300 ± 10 mosm/kg and the viscosity was 21 mPa·s. The deformability of single RBC was observed and measured by a counterrotating cone-plate rheoscope (26) (Effenberger, Munich, Germany), as described elsewhere (27). Four shear stresses of 1, 2, 4, and 6 Pa were applied, and the elongation of RBC was recorded on video. Length (L) and width (W) of 40 RBC were measured in each sample using a computerized micrometer system (MOP-Videoplan, Kontron Electronics, Eching-Munich, Germany). The RBC deformation index DI is defined as $DI = (L - W)/(L + W)$. DI increases with increasing cell elongation.

(ii) Red blood cell filtration. RBC filtration was determined by means of the cell-transit analyzer (CTA; ABX International, Levallois, France) (28). The CTA system employs a polycarbonate filter placed vertically between two reservoirs and an AC conductimeter. The oligofilter has 30 cylindrical pores with 5 μm diameter and 14 μm length. Pore transit times were measured at a pressure gradient of 4 cm H_2O. Transit times of at least 1000 RBC during 25.4 ms or 100 ms were measured for each sample. The pore transit times are inversely related to cellular deformability, but also dependent on RBC size. Results are given as mean passage time of the 1000 cells during the measuring time interval.

Miscellaneous methods. RBC count, white blood cell

TABLE 1
Fatty Acid Composition of the Diets (mol %)[a]

Fatty acids	Cacao	Sunflower oil	Fish oil
16:0	24.1	8.8	15.2
18:0	31.9	6.34	5.8
Saturated FA	58.2	16.9	26.7
16:1n-7	0.33	0.22	5.2
18:1n-9	30.6	23.2	19.1
20:1n-9)	0.1	0.18	0.65
Monounsaturated FA	31.0	23.6	25.3
18:2n-6	10.8	59.3	39.9
20:3n-6	N.D.	N.D.	N.D.
20:4n-6	0.01	0.04	0.23
n-6 PUFA	10.9	59.9	40.3
18:3n-3	0.00	0.02	0.07
20:5n-3	0.00	0.07	5.01
22:6n-3	0.03	0.03	3.03
n-3 PUFA	0.03	0.15	9.57
Total PUFA	11.8	59.5	48.3
P/S ratio	0.2	3.5	2.7

[a]Values are means of three determinations, corresponding to four weekly preparations of the diets. Abbreviations: FA, fatty acids; PUFA, polyunsaturated FA; P/S, PUFA/saturated FA ratio; N.D., not detected.

count, hemoglobin concentration, and the RBC indices mean cellular volume and mean corpuscular hemoglobin concentration (MCHC) were determined using a cell counter (Contraves, Zürich, Switzerland). The hematocrit was measured by means of a microcentrifuge.

Statistical analyses. Statistical evaluation of differences between the groups was performend by nonparametric test procedures (Kruskal-Wallis test). Comparison of the means was made using analysis of variance.

RESULTS

The three diets–cacao, sunflower oil, and fish oil–contained different percentages of saturated fatty acids, with the highest content in the cacao diet (Table 1). Nevertheless, the three groups showed similar amounts of total saturated fatty acids in PL and in RBC membrane PC and PE fractions (Tables 2–4).

Monounsaturated fatty acids, especially 18:1n-9 fatty acid, were highest in the cacao diet and in the plasma lipids of the cacao-fed animals when compared to the other groups. PUFA was lowest in cacao diet (12%), higher in fish oil (48%), and highest in sunflower oil (59%). These differences were roughly reflected by the PUFA content of the PL and CE. The polyunsaturated/saturated fatty acid ratio (P/S-ratio) of the diets was extremely low in cacao (0.2), higher in fish oil (2.7), and highest in sunflower oil (3.5). However, there were much smaller differences of P/S-ratio in PL and CE among the three groups (Table 2). The DHA and EPA were much higher in fish oil than in the other two diets (Table 1). This corresponded to the PL more than to the CE of fish oil-fed animals (Table 2).

Tables 3 and 4 show the fatty acid composition of the PC and the PE fraction in the RBC membrane of the three groups. In spite of the marked differences in the saturated fatty acid content of the three diets, both the total amount and the composition of the saturated fatty acids in the RBC membranes of the three groups of animals were quite similar. In all three animal groups the percentages of saturated fatty acids averaged about 50% in PC and 36–39% in PE.

The monounsaturated fatty acids in the RBC membranes corresponded to the contents of the different diets (i.e., in the RBC-PC fraction, 22, 10, or 13% and in the RBC-PE fraction, 9.7, 5.6, or 5.8% in the animals fed cacao, sunflower, or fish oil).

The different amounts of total PUFA in the three diets (Table 1) were in part reflected in the PUFA content of PC (Table 3), whereas total PUFA in PE was similar in the three diet groups (Table 4). The n-3 PUFA (EPA and DHA) showed high concentrations in both RBC leaflets of fish oil-fed animals, whereas RBC in the other groups contained very little EPA and DHA. However, the concentration of EPA was five times and that of DHA was four times higher in the PE fraction than in the PC fraction of fish oil-fed animals.

TABLE 2
Fatty Acid Composition (mol %) of the Phospholipids and Cholesteryl Esters in the Plasma of 22-wk-old Guinea Pigs Fed Different Diets[a]

Fatty acids	Cacao (C)	Sunflower oil (S)	Fish oil (F)	Significant differences among three groups ($P < 0.05$)
Phospholipids				
Saturated FA (S)	51.3 ± 1.2	48.1 ± 1.5	49.1 ± 1.1	C > S = F
18:1n-9	15.5 ± 0.23	5.41 ± 0.15	4.51 ± 0.1	C > S > F
18:2n-6	23.6 ± 0.7	36.3 ± 0.8	21.9 ± 0.3	S > C > F
20:3n-6	0.28 ± 0.02	0.23 ± 0.03	0.28 ± 0.02	C = F > S
20:4n-6	1.97 ± 0.15	2.08 ± 0.18	4.66 ± 0.3	F > C = F
18:3n-3	0.13 ± 0.03	0.08 ± 0.01	0.15 ± 0.02	C = F > S
20:5n-3	0.03 ± 0.01	0.02 ± 0.01	2.90 ± 0.6	F > C = S
22:6n-3	0.09 ± 0.1	N.D.	6.43 ± 0.34	F > C > S
PUFA (P)	26.1 ± 0.7	38.7 ± 0.59	36.3 ± 0.35	F = S > C
P/S ratio	0.51 ± 0.03	0.81 ± 0.06	0.74 ± 0.01	S = F > C
Cholesteryl esters				
Saturated FA (S)	19.3 ± 0.4	16.8 ± 0.9	26.9 ± 0.5	F > C > S
18:1n-9	25.3 ± 0.6	7.34 ± 0.4	6.90 ± 0.31	C > S > F
18:2n-6	50.1 ± 1.7	71.4 ± 1.2	54.8 ± 1.9	S > F > C
20:3n-6	0.05 ± 0.02	0.07 ± 0.03	0.08 ± 0.02	S = F > C
20:4n-6	0.69 ± 0.04	0.57 ± 0.05	1.88 ± 0.12	F > C > S
18:3n-3	0.32 ± 0.04	0.11 ± 0.02	0.15 ± 0.04	C > F = S
20:5n-3	N.D.	N.D.	2.41 ± 0.52	F > C = S
22:6n-3	N.D.	N.D.	0.56 ± 0.5	F > C = S
PUFA (P)	51.1 ± 2.3	72.1 ± 0.9	59.9 ± 1	S > F > C
P/S ratio	2.6 ± 0.3	4.3 ± 0.2	2.23 ± 0.1	S > C > F

[a]$P < 0.05$ compared with each group, made up of eight guinea pigs. Values are mean ± SE. For abbreviations see Table 1.

TABLE 3
Fatty Acid Composition (mol %) of the Phosphatidylcholine Fraction of the Red Blood Cell Membrane in Guinea Pigs Fed Different Diets[a]

Fatty acids	Cacao (C)	Sunflower oil (S)	Fish oil (F)	Significant differences among three groups ($P < 0.05$)
16:0	21.5 ± 0.25	19.1 ± 0.4	25.1 ± 0.18	F > C > S
18:0	27.4 ± 0.14	26.8 ± 1.0	24.2 ± 0.21	C = S > F
Saturated FA	53.1 ± 0.50	49.7 ± 1.1	54.1 ± 0.24	C = F > S
16:1n-7	0.34 ± 0.02	0.30 ± 0.04	0.95 ± 0.02	F > C > S
18:1n-9	18.3 ± 0.43	7.54 ± 0.38	7.15 ± 0.11	C > S > F
20:1n-9	1.16 ± 0.03	0.47 ± 0.03	0.54 ± 0.01	C > S = F
24:1n-9	0.91 ± 0.07	0.50 ± 0.1	0.94 ± 0.04	C > S = F
Monounsat. FA	21.7 ± 0.43	10.1 ± 0.26	13.1 ± 0.12	C > F > S
18:2n-6	20.5 ± 0.64	34.3 ± 0.46	22.7 ± 0.49	S > F > C
20:2n-6	0.69 ± 0.07	1.81 ± 0.04	0.83 ± 0.02	S > C = F
20:3n-6	0.25 ± 0.01	0.25 ± 0.01	0.25 ± 0.01	C = S = F
20:4n-6	2.47 ± 0.24	2.36 ± 0.1	4.09 ± 0.10	F > C = S
22:4n-6	0.91 ± 0.08	1.28 ± 0.05	0.37 ± 0.02	S > C > F
n-6 PUFA	24.9 ± 0.48	40.2 ± 0.47	28.5 ± 0.53	S > F > C
18:3n-3	0.17 ± 0.03	0.05 ± 0.01	0.17 ± 0.01	C = F > S
20:5n-3	0.31 ± 0.09	N.D.	2.51 ± 0.43	F > C > S
22:6n-3	0.01 ± 0.01	N.D.	2.91 ± 0.21	F > C = S
n-3 PUFA	0.55 ± 0.08	0.11 ± 0.02	6.90 ± 0.46	F > C > S
n-3/n-6	0.02	0.003	0.24	
EPA/AA	0.12	—	0.61	
Total PUFA	25.2 ± 0.5	40.3 ± 0.46	35.9 ± 0.25	S > F > C
P/S ratio	0.47 ± 0.01	0.81 ± 0.01	0.66± 0.01	S > F > C

[a]$P < 0.05$ compared with each group made up of eight guinea pigs. Values are mean ± SE. AA, arachidonic acid. For other abbreviations see Table 1.

There were no influences of the different diets on RBC filtration time measured by CTA. The mean transit times during 25.4 ms and 101.6 ms measuring time were 1.43 ± 0.09 and 1.33 ± 0.08 ms in the cacao group, 1.48 ± 0.14 and 1.33 ± 0.16 ms in the sunflower group, and 1.43 ± 0.05 and 1.30 ± 0.07 ms in the fish oil group.

The RBC deformation measured by means of the cone-plate rheoscope was similar in the three diet groups at shear stresses of 1 to 6 Pa (Fig. 1). When going from a shear stress of 1 to 6 Pa, RBC deformation increased by 18% in the cacao group, by 19% in the sunflower oil group, and by 16% in the fish oil group.

The RBC indices mean cellular volume, mean corpuscular hemoglobin concentration, mean cellular hemoglobin, and RBC count were not significantly different among the three groups.

DISCUSSION

This study demonstrates that feeding of weanling guinea pigs for 20 wk with different diets enriched either with saturated and monounsaturated fatty acids (cacao), with n-6 PUFA (sunflower oil), or with n-3 PUFA (fish oil) influences incorporation of the fatty acids in plasma lipids and RBC membranes. Schick *et al.* (29) showed that feeding guinea pigs with marine oil-enriched diets resulted in a maximal change in lipid composition of platelets within 10 d. After 5 wk no further changes in lipid composition occurred. We studied RBC after dietary supplementation for 20 wk to achieve an equilibrium between plasma and RBC membrane. PL mainly consist of saturated and monounsaturated fatty acids, whereas CE have a higher content of PUFA.

PE, largely located in the inner leaflet of the membrane, is thought to be renewed by acylation of lysophospholipid, which is apparently a slow process (30). PC, located mainly in the outer leaflet, is renewed by exchange of PC between plasma lipoproteins and erythrocyte membrane (30). Of these fractions, PC in the outer leaflet is believed to be associated with the flexibility of the membrane. Persson *et al.* (12) studied membrane composition and RBC deformability in diabetic patients before and after 4 mon of treatment with insulin. They found no correlation between RBC deformability and saturated fatty acids or PUFA in the inner PE fraction. However, RBC filterability improved with increasing palmitic fatty acid (16:0) content and decreased with increasing stearic acid (18:0) content in the outer membrane PC fraction.

Studies of membrane lipid fluidity measured by fluorescence or electron spin resonance techniques have shown that

TABLE 4
Fatty Acid Composition (mol %) of the Phosphatidylethanolamine Fraction of the Red Blood Cell Membrane in Guinea Pigs Fed Different Diets[a]

Fatty acids	Cacao (C)	Sunflower oil (S)	Fish oil (F)	Significant differences among three groups ($P < 0.05$)
16:0	7.26 ± 0.18	6.17 ± 0.1	9.06 ± 0.28	F > S > C
18:0	33.2 ± 0.40	32.2 ± 0.4	31.8 ± 1.08	C = S > F
Saturated FA	41.3 ± 0.53	39.1 ± 0.38	41.6 ± 1.48	C = F > S
16:1n-7	0.45 ± 0.02	0.30 ± 0.03	0.80 ± 0.07	F > C > S
18:1n-9	7.63 ± 0.17	4.53 ± 0.05	3.80 ± 0.12	C > S > F
20:1n-9	1.20 ± 0.02	0.35 ± 0.01	0.34 ± 0.02	C > S > F
24:1n-9	0.10 ± 0.05	N.D.	N.D.	C > S = F
Monounsat. FA	9.71 ± 0.21	5.66 ± 0.10	5.85 ± 0.22	C > S = F
18:2n-6	10.26 ± 0.2	18.7 ± 0.48	7.96 ± 0.21	S > C > F
20:2n-6	0.70 ± 0.03	1.70 ± 0.08	0.63 ± 0.03	S > C > F
20:3n-6	0.23 ± 0.01	0.36 ± 0.01	0.22 ± 0.01	S > C = F
20:4n-6	24.9 ± 0.48	21.1 ± 0.33	20.0 ± 0.42	C > S > F
22:4n-6	7.86 ± 0.25	10.1 ± 0.17	1.64 ± 0.10	S > C > F
n-6 PUFA	47.3 ± 0.54	54.5 ± 0.45	31.1 ± 0.46	S > C > F
18:3n-3	0.29 ± 0.01	0.14 ± 0.01	0.19 ± 0.02	C > F > S
20:5n-3	1.45 ± 0.04	0.07 ± 0.01	7.83 ± 0.64	F > C > S
22:6n-3	0.52 ± 0.06	0.29 ± 0.04	13.0 ± 0.79	F > C > S
n-3 PUFA	3.11 ± 0.14	0.73 ± 0.08	26.2 ± 1.5	F > C > S
n-3/n-6	0.07	0.01	0.84	
EPA/AA	0.06	0.003	0.4	
Total PUFA	50.5 ± 0.6	55.3 ± 0.4	57.5 ± 1.6	F = S > C
P/S ratio	1.22 ± 0.03	1.4 ± 0.05	1.38 ± 0.11	F = S > C

[a]$P < 0.05$ compared with each group made up of eight guinea pigs. Values are mean ± SE. AA, arachidonic acid. For other abbreviations see Table 1.

saturated fatty acid components of the lipid bilayer decrease the fluidity of the RBC membrane (31,32), whereas PUFA increase fluidity (33,34). Furthermore, the cholesterol content in membranes, which was not measured in our study, may influence membrane lipid fluidity. However, the parameter lipid fluidity characterizes molecular motion, not the mechanical behavior of the entire membrane, as exemplified by deformation (35). We showed that incorporation of saturated fatty acids (lipid A from bacteria) in RBC membranes of humans resulted in decreased RBC deformation (36,37), whereas feeding rats with DHA-rich diet improved RBC deformation without influencing the cholesterol/phospholipid ratio (19).

In the present study, the different diets had no effect on RBC deformability. The fatty acid composition of the PE fraction (i.e., inner leaflet) was markedly influenced by the monounsaturated fatty acids and PUFA. However, the total PUFA and P/S ratio in PE showed only small differences among the three groups (Table 4). Although the n-3/n-6 ratio increased from the sunflower group to the cacao and to the fish oil group, the total PUFA was not influenced. Previously, we adjusted the total amount of n-3 and n-6 PUFA in the different diets for rats to differentiate the effect of the single PUFA (19) and found a correlation between improved RBC deformation and DHA content in the RBC membrane. After

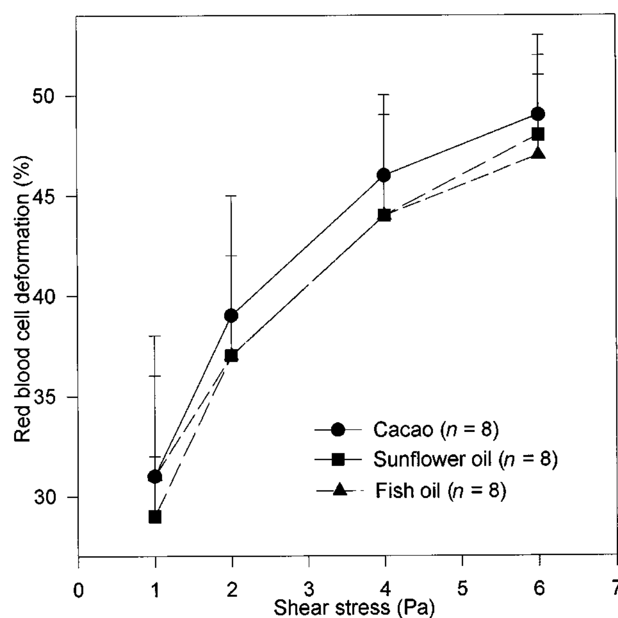

FIG. 1. Red blood cell deformation at shear stresses from 1 to 6 Pa measured in 22-wk-old guinea pigs fed with different diets. Mean ± 1 SD; each group comprised eight animals. The values were not significantly different among the three groups.

dietary supplementation with n-3 and n-6 PUFA, we expected changes in RBC deformation as shown in several studies (13,14,30), but this was not our finding. Thus, similar RBC deformability in the three groups suggests that the total amount of PUFA in the inner RBC leaflet rather than the percentage of individual fatty acids as DHA, EPA, or AA may determine RBC deformability.

The fatty acid composition of the outer PC fraction represents the fatty acid composition in the plasma. The phospholipid renewal occurs by a direct exchange of the phospholipids between the membrane and plasma (38). Our data clearly demonstrate that the saturated fatty acid composition of the PL and the PC fraction was identical in the three groups, although the cacao diet was much richer in saturated fatty acids than the other diets. This suggests either that the saturated fatty acid may be cleared from the plasma before incorporation into the PC fraction or that high saturated acid content in diets decreases their intestinal absorption, as shown in rats (39). The total PUFA and the P/S ratio were not different in the three groups between PL and PC fraction. However, the total PUFA and P/S ratio were significantly lower in the cacao group according to the lower PUFA content in the cacao diet.

The RBC deformability was not affected by the three diets. We suppose that the constant saturated fatty acid content in the two leaflets and the total PUFA content in the inner leaflet may be responsible for the unchanged RBC deformability.

We conclude that the three diets—cacao, sunflower oil, and fish oil—result in similar amounts of total PUFA in the inner RBC leaflet and similar amounts of saturated fatty acids in the outer leaflet. This results in similar RBC deformability.

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The Effects of Dietary α-Linolenic Acid Compared with Docosahexaenoic Acid on Brain, Retina, Liver, and Heart in the Guinea Pig

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ABSTRACT: The aim of this study was to compare two different strategies to elevate brain, retina, liver, and heart docosahexaenoic acid (DHA) levels in guinea pigs. First, we used an increasing dose of α-linolenic acid (ALA) relative to a constant linoleic acid (LA) intake, and second, we used two levels of dietary DHA provided in conjunction with dietary arachidonic acid (AA). The percentage DHA and AA of total phospholipids in retina, liver, and heart, and in the brain phosphatidylethanolamine and phosphatidylcholine was studied in female pigmented guinea pigs (3 wk old) fed one of five semisynthetic diets containing 10% (w/w) lipid for 12 wk. The LA content in the diets was constant (17% of total fatty acids), with the ALA content varying from 0.05% (diet SFO), to 1% (diet Mix), and to 7% (diet CNO). Two other diets (LCP1 and LCP3) had a constant LA/ALA ratio (17.5:1) but varied in the levels of dietary AA and DHA supplementation. Diet LCP1 was structured to closely replicate the principal long chain polyunsaturated fatty acids (PUFA) found in human breast milk and contained 0.9% AA and 0.6% DHA (% of total fatty acids) whereas diet LCP3 contained 2.7% AA and 1.8% DHA. At the end of the study, animals were sacrificed and tissues taken for fatty acid analyses. We found no significant effects of diets on the growth of guinea pigs. Diets containing ALA had profoundly different effects on tissue fatty acid compositions compared with diets which contained the long chain PUFA (DHA and AA). In the retina and brain phospholipids, high-ALA diets or dietary DHA supplementation produced moderate relative increases in DHA levels. There was no change in retinal or brain AA proportions following dietary AA supplementation, even at the highest level. This was in contrast to liver and heart where tissue DHA proportions were low and AA predominated. In these latter tissues, dietary ALA had little effect on tissue DHA proportions although the proportion of AA was slightly depressed at the highest dietary ALA intake, but dietary DHA and AA supplements led to large increases (up to 10-fold) in the proportions of these PUFA. Tissue uptake of dietary AA and DHA appeared maximal for the LCP1 diet (replicate of breast milk) in the heart. There were no significant changes in the plasma levels of 11-dehydrothromboxane B_2 (a thromboxane A_2 metabolite) for any diet. The data confirm that dietary ALA is less effective than dietary DHA supplementation (on a gram/gram basis) in increasing tissue DHA levels and that tissues vary greatly in their response to exogenous AA and DHA, with the levels of these long chain metabolites being most resistant to change in the retina and brain compared with liver and heart. Dietary DHA markedly increased tissue DHA proportions in both liver and heart, whereas the major effect of dietary AA was in the liver. Future studies of the effects of dietary DHA and AA supplementation should examine a variety of tissues rather than focusing only on neural tissue.

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Human breast milk contains arachidonic acid (AA) and docosahexaenoic acid (DHA). Until recently, most infant formulas have lacked these fatty acids (1,2). Interest in the appropriate or "ideal" composition of infant formula is growing since it has been shown that formula-fed infants have reduced visual acuity (3,4) and altered cognitive development (5). These data might be explained by reports that brain DHA levels in infants fed formula are significantly lower than those receiving breast milk (6,7). In animals fed diets deficient in α-linolenic acid (ALA), large reductions in brain and retinal DHA proportions are associated with impairments in the visual function in primates (8), rats (9), and guinea pigs (10). These observations beg the question of how can we improve and maintain optimal tissue levels of AA and DHA.

It is possible to increase tissue DHA in one of two ways: by increasing either dietary ALA or dietary DHA. Human studies have indicated that increasing the ALA in an infant formula can result in significantly increased DHA levels in erythrocytes; however, the DHA levels are still below those found in breast-fed infants (2,11). One outcome from such a strategy is that high intakes of ALA can lead to reductions in plasma AA levels, which is considered an undesirable outcome (2,11).

The other way to increase tissue DHA levels is to supplement the diet with DHA. In particular, if DHA and AA are both included in the diet then the undesirable reduction of tissue AA levels can be avoided. This approach has been used for both term and preterm infants and has been shown to produce blood DHA and AA levels similar to that found with breast-fed infants (12,13). Whether the increased blood DHA and AA levels are reflected in tissues such as retina and brain is not known. For example, it has been reported that rats fed

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Abbreviations: ALA, α-linolenic acid; AA, arachidonic acid; DHA, docosahexaenoic acid, 22:6n-3; LA, linoleic acid; PUFA, polyunsaturated fatty acids.

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dietary AA supplements show significant increases in the plasma level of AA but not in the brain (14). Clearly, such issues can only be addressed easily with animal studies.

In this paper we report the effect of two strategies aimed at increasing brain and retinal DHA levels in guinea pigs. First, we used an increasing dose of ALA relative to a constant linoleic acid (LA) intake, and second, we used two levels of dietary DHA supplements provided in conjunction with dietary AA. The hypotheses being tested were that addition of dietary AA and DHA will increase tissue DHA values beyond those obtained by diets high in ALA, and that the combined addition of dietary DHA plus AA will not decrease tissue AA levels. The novelty of this study is that it focuses on the response of a variety of tissues to fatty acid supplementation and shows that the tissue response to AA and DHA supplementation is not the same.

TABLE 1
Fatty Acid Composition of the Diets[a]

Fatty acid	Diet group SFO	CNO	Mix	LCP1	LCP3
8:0	6.1	1.9	1.6	1.4	1.1
10:0	5.0	1.6	1.4	1.2	1.0
12:0	36.4	11.5	9.8	8.5	6.9
14:0	14.0	4.6	4.2	3.9	3.7
16:0	8.9	6.1	20.7	20.4	19.9
16:1	0.1	0.2	0.1	0.2	0.2
18:0	8.9	4.6	5.7	5.6	5.5
18:1	3.1	42.7	35.9	36.7	35.7
18:2n-6	16.2	16.1	17.3	17.2	17.3
18:3n-3	0.1	7.1	1.0	1.0	1.0
20:4n-6	0	0	0	0.9	2.7
22:6n-3	0	0	0	0.6	1.8
18:2/18:3	323:1	2.3:1	17.5:1	17.7:1	17.4:1

[a]Results expressed as % of total fatty acids. SFO, safflower oil; CNO, canola oil; Mix, mixed vegetable oil; LCP1, mixed vegetable oil plus 0.9% arachidonic acid (AA) and 0.6% docosahexaenoic acid (DHA); LCP3, mixed vegetable oil plus 2.7% AA and 1.8% DHA.

MATERIALS AND METHODS

Animals and diets. Seventy pigmented female guinea pigs (English Shorthair) were randomly divided into five groups of 14 animals at 3 wk of age. The guinea pigs were fed one of five different semisynthetic diets *ad libitum* for 12 wk and were supplemented with fresh carrots and drinking water containing ascorbic acid (400 mg/L). The macronutrient composition of the diets (g/kg of diet) was: casein 300, sucrose 100, glucose 70, starch 200, solkafloc (cellulose) 100, kaolin 30, L-arginine 3, DL-methionine 2, mineral mix 68, and vitamin mix 27, as described by Weisinger *et al.* (10). The diets contained 10% (w/w) lipid, supplied by mixed vegetable oils, and each diet was designed to provide 17% LA (as % of total fatty acids).

In all diets, the lipids were based on mixed vegetable oils. In diet SFO, the main lipid was provided from safflower oil and the LA/ALA ratio was 323:1 (ALA 0.05% total fatty acids). In diet CNO, the main lipid was provided from canola oil with an LA/ALA ratio of 2.3:1 (ALA 7% total fatty acids). Diet Mix was based on mixed vegetable oils, with an LA/ALA ratio of 17.5:1 (ALA 1% total fatty acids). Diets LCP1 and LCP3 were similar to diet Mix, but they were designed to contain supplementary levels of AA (1%) and DHA (0.7%) (mimicking the levels in human breast milk) or 3% AA plus 2.1% DHA, respectively. AA was obtained from ARASCO oil (Martek Bioscience, Columbia, MD), processed from a common soil fungus widely distributed in nature, and DHA was obtained from DHASCO oil (Martek Bioscience), processed from a microalga.

Fatty acid assays of the five diets showed that the LA levels achieved the desired range of between 16.1 and 17.2% (% total fatty acids; Table 1). Assays of diet LCP1 gave AA and DHA values of 0.87 and 0.59%, respectively, whereas diet LCP3 gave 2.74 and 1.80% of AA and DHA, respectively.

At the end of the 12 wk of feeding, animals were sacrificed by CO_2 asphyxiation. The brain, retina, heart, and liver were removed for processing. The brain, heart, and liver were washed in saline, blotted dry, and stored at −70°C for fatty acid analysis. Each retina was removed and washed in ice-cold phosphate buffered saline and stored in 10 mL of chloroform/methanol (2:1,vol/vol) containing butylated hydroxytoluene (10 mg/L) as an antioxidant. Blood was drawn by cardiac puncture and 5 mL was transferred to a lithium heparin vacutainer tube. This was centrifuged for 10 min (1000 rpm, at 18°C), and the platelet-rich plasma was then stored at −70°C. The concentration of plasma 11-dehydrothromboxane B_2 was measured by competitive enzyme immunoassay (Caymen Chemical Company, Ann Arbor, MI) and the resulting plates were read at 410 nm.

Lipid analyses. Following lipid extraction from retina, heart, and liver by chloroform/methanol (2:1), the total phospholipids were separated from the neutral lipids by thin-layer chromatography (TLC) (15). There was insufficient retinal tissue to allow separation of the individual phospholipids, but brain lipids were separated by TLC (16) and the phosphatidylethanolamine (PE) and phosphatidylcholine (PC) fractions were scraped into tubes and the methyl esters of the phospholipid fatty acids were formed by saponification using KOH followed by transesterification in BF_3 in methanol (15). The fatty acid methyl esters were separated by capillary gas– liquid chromatography using a 50 m × 0.32 mm (i.d.) fused-silica bonded phase column (BPX70; SGE, Melbourne, Australia). The column oven was programmed to rise after 3 min at 125°C to 220°C at 8°C/min with a helium flow rate of 43 cm/s as the carrier gas. 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).

Statistical analyses. Significant differences between dietary groups were tested using a one-way analysis of variance for each type of fatty acid. *Post hoc* comparisons were made using the Tukey test with a significance level of 0.05.

RESULTS

There was no significant difference in the body weights of animals at the start or finish of the experiment. The mean increase in body weight across all groups was 359 ± 46 g. The

proportion of total polyunsaturated fatty acids (PUFA) in the retinal lipids ranged between 41–45% regardless of diet. However, the fatty acid profiles varied as a function of diet group. The main retinal phospholipid fatty acids were 16:0, 18:0, 18:1n-9, AA, 22:5n-6, and DHA (Table 2). There were significant increases in the proportions of 22:5n-3, DHA, and 24:6n-3 and significant decreases of LA, AA, 22:4n-6, 24:4n-6, 24:5n-6, and 22:5n-6 as the ALA content of the diet increased (Table 2: diet SFO vs. diet CNO). The additions of AA and DHA supplements (diets LCP1 and LCP3) were associated with significant decreases in 22:4n-6, 22:5n-6, and 24:4n-6 and significant increases in 22:5n-3 and DHA (Table 2). Diet LCP3 resulted in significantly more DHA and less 22:5n-6 than did diet LCP1 or diet Mix (Table 2). Supplementing diets with DHA and AA resulted in significant increases in the proportions of the 24-carbon n-3 PUFA and significant decreases in the 24-carbon n-6 PUFA.

Table 3 shows that the main fatty acids of the brain PE fraction were 18:0, 18:1n-9, AA, 22:4n-6, 22:5n-6, and DHA. There was no difference in fatty acid composition between diets SFO or Mix. Diet CNO contained a significantly higher proportion of DHA and lower proportion of 22:5n-6 than found with diets SFO or Mix. Addition of dietary AA and DHA (diets LCP1 and LCP3) was associated with a significant decline in the 22:5n-6 proportion (from 9.2% in diet Mix to 4.0% in diet LCP3) and an increase in DHA (from 8.3% in diet Mix to 15.0% in diet LCP3). The highest DHA and AA diet (Diet LCP3) led to further significant changes in the proportions of 22:5n-6 and DHA compared with diet LCP1. There were no significant differences in the proportion of AA or 22:4n-6 in any of the dietary groups.

The major fatty acids of the brain PC were 16:0, 18:0, and 18:1 which accounted for 76–79% of the total (data not shown). In contrast to the brain PE fraction, brain PC fractions showed minor changes in PUFA as a result of dietary manipulations. Supplementation of the diet with AA and DHA did not significantly change the proportion of AA and DHA in this fraction.

Table 4 shows the effect of diet on the fatty acid composi-

TABLE 2
Polyunsaturated Fatty Acid Composition of Retinal Phospholipids from Guinea Pigs Fed Diets Containing Different Levels of n-3 PUFA[a]

	Diet Group				
Fatty acid	SFO (n = 12)	CNO (n = 14)	Mix (n = 14)	LCP1 (n = 14)	LCP3 (n = 14)
n-6 PUFA					
18:2n-6	1.37 ± 0.15[a]	1.38 ± 0.16[a]	1.50 ± 0.18[a]	1.10 ± 0.12[b]	0.91 ± 0.13[c]
20:3n-6	0.88 ± 0.24[a]	0.74 ± 0.03[a,b]	0.73 ± 0.03[b]	0.76 ± 0.03[a,b]	0.70 ± 0.01[b,c]
20:4n-6	9.09 ± 0.33[a]	8.67 ± 0.22[b]	9.06 ± 0.27[a]	9.21 ± 0.32[a]	8.81 ± 0.39[a,b]
22:4n-6	3.50 ± 0.10[a]	2.70 ± 0.10[b]	3.49 ± 0.14[a]	2.85 ± 0.16[b]	2.14 ± 0.12[c]
22:5n-6	17.35 ± 0.67[a]	9.28 ± 0.96[b]	15.16 ± 0.65[c]	9.27 ± 0.73[b]	3.43 ± 0.56[d]
24:4n-6	2.59 ± 0.22[a]	2.02 ± 0.10[b]	2.34 ± 0.16[a]	2.11 ± 0.13[b]	1.31 ± 0.12[c]
24:5n-6	0.31 ± 0.04[a]	0.20 ± 0.01[b]	0.24 ± 0.02[b]	0.23 ± 0.02[b]	0.16 ± 0.02[c]
n-3 PUFA					
20:5n-3	0.09 ± 0.07[a]	0.07 ± 0.0[a]	0.08 ± 0.01[a]	0.07 ± 0.01[a]	0.13 ± 0.01[b]
22:5n-3	0.47 ± 0.05[a]	1.25 ± 0.11[b]	0.65 ± 0.06[c,d]	0.59 ± 0.08[c]	0.82 ± 0.08[d]
22:6n-3	8.70 ± 0.76[a]	16.35 ± 1.09[b]	9.64 ± 0.63[c]	17.60 ± 1.05[b]	25.45 ± 0.51[d]
24:5n-3	0.17 ± 0.03[a]	0.65 ± 0.05[b]	0.22 ± 0.02[c]	0.25 ± 0.03[c]	0.41 ± 0.03[d]
24:6n-3	0	0.13 ± 0.01[a]	0.05 ± 0.00[b]	0.14 ± 0.02[a]	0.35 ± 0.05[c]

[a]Results expressed as % of total phospholipid fatty acids, mean ± SD. Different roman supercript letters indicate significant-differences between the diets, at $P < 0.05$. PUFA, polyunsaturated fatty acid. For other abbreviations see Table 1.

TABLE 3
Polyunsaturated Fatty Acid Composition of Brain PE from Guinea Pigs Fed Diets Containing Different Levels of n-3 PUFA[a]

	Diet Group				
Fatty acid	SFO (n = 12)	CNO (n = 14)	Mix (n = 14)	LCP1 (n = 14)	LCP3 (n = 14)
n-6 PUFA					
18:2n-6	0.59 ± 0.04[a]	0.53 ± 0.06[a]	0.58 ± 0.05[a]	0.54 ± 0.05[a]	0.42 ± 0.05[b]
20:3n-6	0.95 ± 0.07	1.00 ± 0.15	0.91 ± 0.07	0.77 ± 0.26	0.82 ± 0.09
20:4n-6	13.07 ± 0.54	12.96 ± 0.80	12.46 ± 0.85	12.68 ± 0.65	12.86 ± 0.46
22:4n-6	7.51 ± 0.26	7.45 ± 0.56	7.27 ± 0.82	7.31 ± 0.47	7.42 ± 0.33
22:5n-6	9.59 ± 1.02[a]	6.93 ± 0.98[b]	9.21 ± 0.90[a]	6.92 ± 1.39[b]	3.96 ± 0.55[c]
n-3 PUFA					
22:5n-3	0.21 ± 0.03[a]	0.54 ± 0.04[b]	0.29 ± 0.07[c]	0.24 ± 0.05[a,c]	0.21 ± 0.02[a]
22:6n-3	8.63 ± 0.34[a]	11.47 ± 1.10[b]	8.32 ± 0.79[a]	11.51 ± 1.24[b]	15.01 ± 1.52[c]

[a]Results expressed as % of total phosphatidylethanolamine (PE) fatty acids, mean ± SD. Different supercript letters indicate significant differences between the diets, at $P < 0.05$. For abbreviations see Tables 1 and 2.

tion of the liver total phospholipids, with the main fatty acids in this tissue being 16:0, 18:0, 18:1n-9, LA, and AA. The diets significantly affected all of these fatty acids. Increasing the ALA content significantly decreased the LA and AA proportions and significantly increased the proportions of ALA, 22:5n-3, and DHA; however, these changes in the long chain n-3 PUFA were minor. The most significant changes occurred with DHA and AA supplementation, particularly in the proportion of LA, AA, and DHA. Supplementing the diet with 0.6% DHA led to more than a 10-fold increase in the proportion of DHA in the liver, whereas supplementing at 1.8% DHA increased tissue proportions even further (from 6.1% on diet LCP1 to 14.6% on diet LCP3). Supplementing the diet with 0.9% AA produced a doubling in liver AA proportion, and a further significant increase was evident with 2.7% AA supplementation. There were marked decreases in liver phospholipid LA proportions in the diets which contained AA and DHA.

The main fatty acids in heart phospholipids were the same as those in the liver (Table 5), however, there was more than twice as much AA in heart compared with liver and a reduced proportion of LA. The most significant changes due to diet occurred with DHA and AA supplementation, particularly in the LA, AA, and DHA proportions. Supplementing the diet with 0.6% DHA led to more than a 10-fold increase in tissue DHA proportion, but supplementing at 1.8% DHA did not increase tissue proportion any further. Supplementing the diet with 0.9% AA produced a small increase in tissue AA, and the 2.7% AA supplementation produced little extra increase in tissue AA proportion. The increased DHA and AA proportions found with diets LCP1 and LCP3 were accompanied by substantial decreases in the proportions of LA.

The effects that diets have on the tissue levels of the principal metabolites of the n-3 (DHA) or n-6 (AA) families are shown in Figure 1, which indicates how the proportions of DHA and AA vary between the tissues. Retina and brain have moderate levels of DHA and AA whereas liver and heart have low levels of DHA and moderate to high levels of AA. Increasing the dietary ALA intake resulted in increasing DHA levels in the retina and brain with little effect being evident for the liver and heart. This DHA increase was associated with decreasing AA levels in liver and heart but stable AA levels in retina and brain. The change in retina and brain DHA was particularly evident with diet CNO. Supplementation with DHA (diets LCP1 and LCP3) resulted in further increases of tissue DHA in all cases although the heart showed a plateau in this effect beyond diet LCP1. Supplementation of AA (diets LCP1 and LCP3) appeared to have no effect on retina and brain levels of AA but showed a substantial increase for liver and heart, with heart levels stabilizing beyond diet LCP1.

In order to consider the more subtle aspects of these

TABLE 4
PUFA Composition of Liver Phospholipids from Guinea Pigs Fed Diets Containing Different Levels of n-3 PUFA[a]

	Diet group				
Fatty acid	SFO (n = 12)	CNO (n = 14)	Mix (n = 14)	LCP1 (n = 14)	LCP3 (n = 14)
n-6 PUFA					
18:2n-6	36.02 ± 1.10[a]	32.90 ± 0.90[b]	33.71 ± 1.74[b]	23.82 ± 0.80[c]	12.97 ± 1.29[d]
20:3n-6	0.84 ± 0.08[a]	0.64 ± 0.04[b]	0.58 ± 0.10[b,c]	0.57 ± 0.08[b,c]	0.42 ± 0.06[c]
20:4n-6	8.92 ± 0.63[a]	7.29 ± 0.66[b]	7.6 ± 0.52[b]	15.58 ± 0.74[c]	19.6 ± 0.69[d]
22:4n-6	0.86 ± 0.08[a]	0.52 ± 0.09[b]	0.71 ± 0.13[a]	0.80 ± 0.18[a]	0.69 ± 0.12[a,b]
22:5n-6	0.78 ± 0.10[a]	0.45 ± 0.08[b]	0.63 ± 0.09[c]	0.53 ± 0.11[b]	0.32 ± 0.06[c]
n-3 PUFA					
18:3n-3	0.32 ± 0.01[a]	1.25 ± 0.17[b]	0.34 ± 0.04[a]	0.18 ± 0.06[d]	0.11 ± 0.02[d]
22:5n-3	0.38 ± 0.09[a]	0.97 ± 0.13[b]	0.43 ± 0.06[a]	0.31 ± 0.05[a]	0.27 ± 0.04[a]
22:6n-3	0.28 ± 0.04[a]	0.84 ± 0.12[b]	0.42 ± 0.26[a,b]	6.06 ± 0.83[c]	14.55 ± 1.55[d]

[a]Results expressed as % of total phospholipid fatty acids, mean ± SD. Different roman supercript letters indicate significant differences between the diets, at $P < 0.05$. For abbreviations see Tables 1 and 2.

TABLE 5
PUFA Composition of Heart Phospholipids from Guinea Pigs Fed Diets Containing Different Levels of n-3 PUFA[a]

	Diet group				
Fatty acid	SFO (n = 12)	CNO (n = 14)	Mix (n = 14)	LCP1 (n = 14)	LCP3 (n = 14)
n-6 PUFA					
18:2n-6	24.63 ± 0.99[a]	24.50 ± 0.93[a]	24.86 ± 0.94[a]	16.66 ± 2.90[b]	16.02 ± 2.70[b]
20:4n-6	23.87 ± 0.53[a]	20.48 ± 0.64[b]	22.08 ± 0.81[c]	25.28 ± 1.07[d]	25.45 ± 1.11[d]
22:4n-6	0.87 ± 0.09[a]	0.39 ± 0.04[b]	0.64 ± 0.07[c]	0.43 ± 0.02[b]	0.44 ± 0.03[b]
22:5n-6	1.17 ± 0.11[a]	0.49 ± 0.05[b]	0.86 ± 0.13[c]	0.26 ± 0.14[d]	0.29 ± 0.11[d]
n-3 PUFA					
22:5n-3	0.82 ± 0.13[a]	1.87 ± 0.16[b]	0.99 ± 0.10[a]	0.46 ± 0.10[c]	0.42 ± 0.09[c]
22:6n-3	0.30 ± 0.04[a]	0.75 ± 0.09[b]	0.35 ± 0.06[a]	5.67 ± 2.09[c]	5.60 ± 1.49[c]

[a]Results expressed as % of total phospholipid fatty acids, mean ± SD. Different roman supercript letters indicate significant differences between the diets, at $P < 0.05$. For abbreviations see Tables 1 and 2.

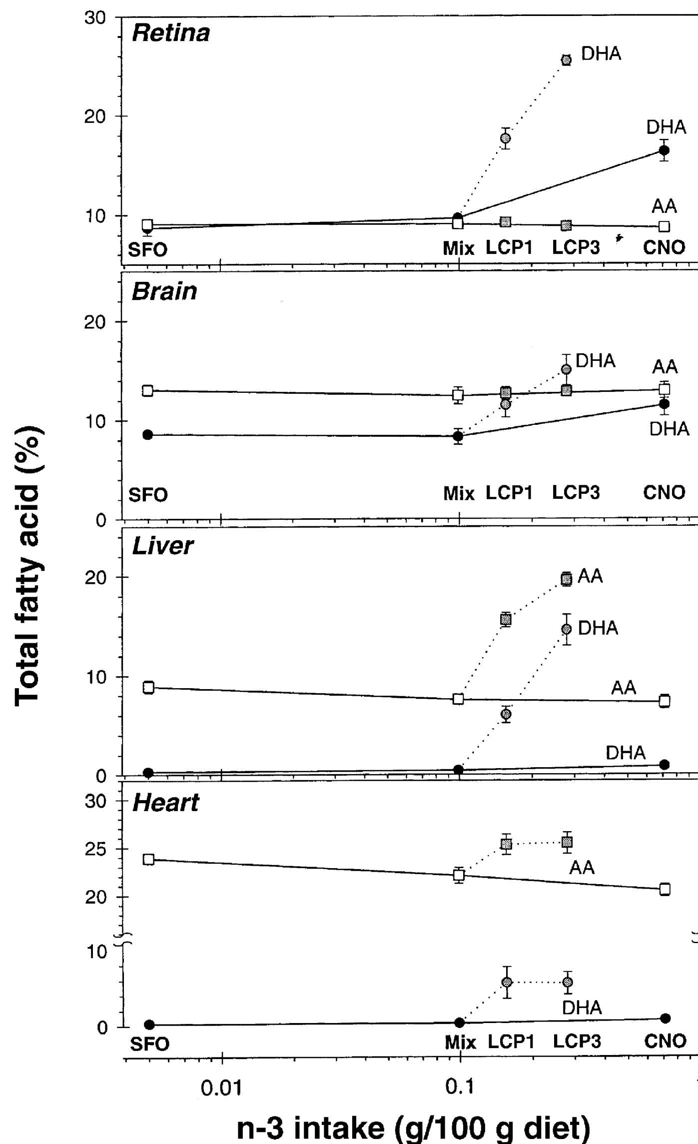

FIG. 1. The effects of diets containing α-linolenic acid (ALA) or docosahexaenoic acid (DHA) plus arachidonic acid (AA) on DHA and AA proportions in the phospholipids of guinea pig tissues. The dietary level of n-3 fatty acids is shown on the *x* axis in g/100 g diet, whereas the AA and DHA values are shown as percentage of total fatty acids in the tissue lipid fraction examined. DHA values are shown with circles (○) and AA as squares (□), and the effect of ALA on DHA levels is shown as a solid line whereas the effects of AA or of DHA are shown as a dashed line. Subsets of DMA or AA from diets with ALA are solid symbols; subsets from diets with ALA + DHA are gray symbols. The letters SFO (safflower oil), Mix (mixed vegetable oil), LCP1 (mixed vegetable oil plus 0.9% AA and 0.6% DHA), LCP3 (mixed vegetable oil plus 2.7% AA and 1.8% DHA) and CNO (canola oil) represent the five diets used in this study.

changes, Figure 2 shows them normalized to the baseline values found for diet SFO: values >1 indicate an increase in tissue levels compared with those found for diet SFO. Figure 2A shows the relative changes found for tissue AA levels and Figure 2B gives these for DHA. In essence, Figure 2 confirms the trends shown in Figure 1. It is evident that adding n-3 PUFA to diets results in improved DHA tissue proportions in all tissues (Fig. 2B) although the retina and brain showed the smallest relative change. Adding dietary DHA supplements also gave the smallest benefits for the central nervous system (CNS) as their starting DHA levels were moderate. On the other hand, dietary DHA supplementation showed an enormous increase in DHA levels for heart and liver (>10-fold) where the initial proportions were low. AA proportions were robust to changes regardless of diet (Fig. 2). However, subtle trends were evident in this data, with the heart and liver showing decreased tissue levels of AA as the n-3 intake increased, which was not seen in the CNS. Supplementation with dietary AA had no effect on the retina and brain, gave minor improvements in the AA levels of the heart, and a large (>2 fold) increase in liver AA content.

Determination of the plasma concentrations of 11-dehy-

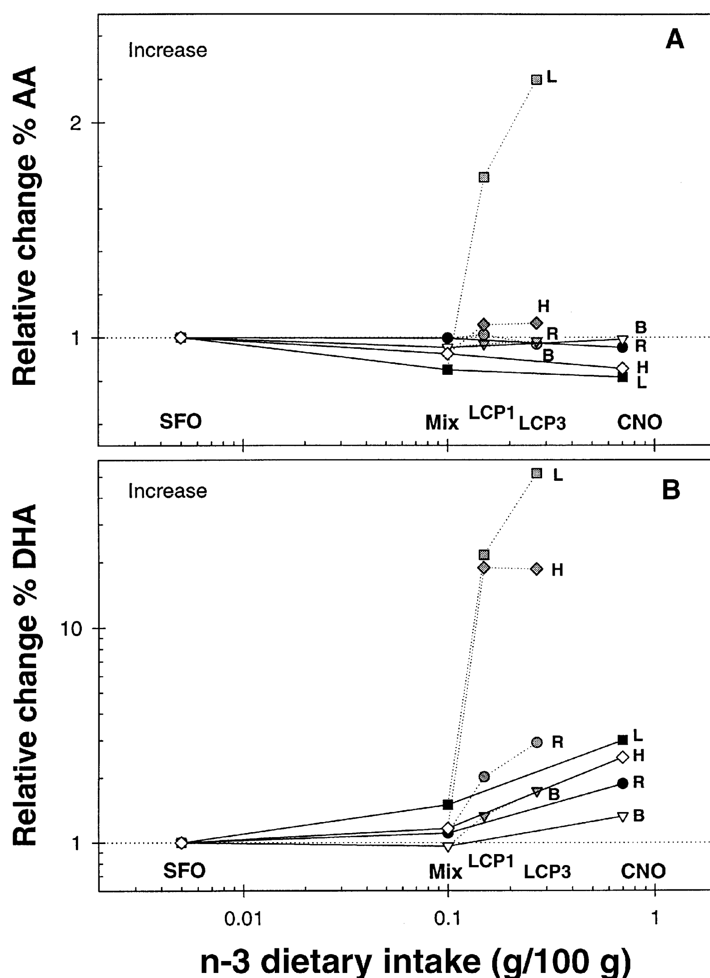

FIG. 2. The relative effects of diets containing ALA or DHA plus AA on tissue DHA and AA levels in the guinea pig, normalized to baseline values found in diet SFO (n-3 deficient diet). The dietary level of n-3 fatty acids is shown on the x axis in g /100 g diet, whereas the AA and DHA values are shown as relative change in DHA or AA in the tissue lipid fraction examined. Values greater than 1 indicate an increase in tissue levels compared with diet SFO. (A) Effects on AA levels in tissue; (B) effects on DHA tissue levels. The effect of ALA on tissue DHA and AA levels is shown with a solid line, whereas the effects of dietary AA and DHA are shown with a dashed line. The tissues are represented by different symbols and a letter, B = brain phosphatidylethanolamine, R = retina phospholipids, L = liver phospholipids, and H = heart phospholipids. See Figure 1 for diets and other abbreviations.

drothromboxane B_2, a metabolite of thromboxane A_2, revealed no significant difference between the five dietary groups. The concentrations were 61 ± 39 (n = 12) for diet SFO, 43 ± 28 (n = 12) for diet Mix, 47 ± 25 (n = 13) for diet CNO, 39 ± 22 (n = 12) for diet LCP 1, and 47 ± 30 (n = 12) pg/mL of plasma for diet LCP3.

DISCUSSION

Lipid nutrition during pregnancy and early postnatal life is important because rapid brain growth and phospholipid accumulation occur during this period. Animal studies have shown that extreme modifications of intake of LA and ALA can lead to alterations in the PUFA composition and function of the developing brain and retina (8–10,17). The present study compared dietary ALA with a combined supplement of DHA plus AA as strategies for elevating brain and retinal DHA levels. At the highest level of ALA (7% dietary fatty acids) increases in DHA were found in all tissues with no decrease in the proportion of AA in neural tissues, but there were significant declines in AA levels in heart and liver. The DHA increase in brain and retina on the high-ALA diet was quite marked (as a percentage of the total fatty acids), however, in the heart and liver the increase was small since the proportion of DHA in the phospholipid fraction of these tissues was less than 1% even at the highest ALA intake.

The other strategy to raise tissue DHA levels, which involved using dietary DHA plus AA, showed a completely different result. First, there were significant increases in neural DHA levels without alteration to the neural AA level. In the liver and heart tissues, however, there were two- to ten fold increases in both DHA and AA. These results clearly show

the diverse responses by different tissues and highlight the importance of examining more than just neural tissues in experiments that undertake dietary manipulation. That is, dietary DHA and AA supplements have the potential to influence the phospholipid fatty acid composition of all tissues in the body and also to influence physiological function in a number of these tissues through changes in eicosanoid production (18), membrane protein function (19), or ion flux through membranes (20). Diets containing ALA have markedly different effects on tissue fatty acid compositions compared with diets which contain the long chain PUFA (DHA and AA) in this species.

In the retinal lipids, there was very little alteration in the proportion of retinal AA in this experiment. However, the proportions of 22:5n-6 varied considerably and were inversely related to the proportion of DHA. This suggests that DHA might be an inhibitor of 22:5n-6 synthesis or, alternatively, a better substrate for reacylation of the phospholipids. Craig-Schmidt *et al.* (21) also reported that diets with DHA alone or with DHA plus AA did not lead to differences in the AA levels in the retinal PC or PE fractions in the piglet. Studies in the rat show that low dietary LA/ALA ratios (<1:1) can lead to significant decreases in retinal AA in animals fed diets with low LA intakes and high ALA levels (22). Other studies in piglets, rats, and monkeys have reported that increasing either ALA or DHA can increase the retinal DHA level (21,23–25), but none of these studies examined the relative effects of ALA vs. DHA. In this study we found that the diet containing 7.1% ALA gave the same retinal and brain PE DHA values as a diet which contained 0.6% DHA and 1% ALA, indicating the potent effect of dietary DHA as a source of neural DHA. Thus, as has been shown many times, dietary DHA is a more effective precursor of tissue DHA than equivalent amounts of ALA, perhaps because only a small fraction of ingested dietary ALA is converted to DHA (26).

The changes in the brain PE fraction were similar to those described for the retina, however, there was little effect of dietary n-3 PUFA on the brain PC fatty acid composition. This contrasts with data from piglet retinal PE and PC where changes were observed in both fractions following feeding with DHA (21).

The liver and heart data in the guinea pig are dissimilar to those reported for these tissues in the rat and the piglet. In rats and piglets, diets containing relatively high levels of ALA are associated with DHA values in liver and heart phospholipids of the order of 7–8% of fatty acids compared with values of less than 1% for the guinea pigs (27–29). These large differences in heart n-3 fatty acid levels in apparently normal animals suggest that the guinea pig might be an interesting animal model with which to study the role of these fatty acids in this tissue. The liver AA levels in the guinea pig were also substantially lower than in the rat or piglet (24, 29). This has been reported previously by Horrobin *et al.* (30) and is perhaps due to a reduced activity of the Δ-5 desaturase enzymes in the guinea pig, as reported by Willis (31).

This study has focused on tissue PUFA changes rather than functional changes such as retinal electrophysiology, behavior, or changes in enzyme activity. In addition to changes in function, there has been much concern about the effect of altering tissue PUFA levels because of the possible effects on eicosanoid synthesis, particularly thromboxane. Craig-Schmidt and Huang (18) have reported that there was reduced lung biosynthesis of prostacyclin in piglets fed DHA alone compared with diets containing AA alone or AA plus DHA; in contrast, thromboxane synthesis was reduced by DHA either alone or in combination with AA relative to the AA-fed group. In this study, we could not find any effect of diet on the plasma level of 11-dehydrothromboxane B_2 which is a marker of the systemic production of thromboxane A_2. Takasaki *et al.* (32) have reported that the plasma level of 11-dehydrothromboxane B_2 is a useful marker of *in vivo* thromboxane formation in rabbits.

In terms of animal models, it needs to be recognized that some mammals provide better models of the nutritional requirements in human retinal development than others. Mammalian species vary in their retinal development, where growth can be classified as prenatal, perinatal, or postnatal with respect to the major retinal growth spurt. Precocial mammals such as humans, primates and guinea pigs have both pre- and postnatal retinal growth spurts (33,34). In contrast, altricial species such as rats and dogs have the greater part of retinal development occurring after birth (33). As a consequence of these differences in retinogenesis, the development of retinal function has been reported as more similar in man and guinea pigs than it is in rats (34–36). Therefore, the effects that neonatal dietary manipulations have on function may be expected to vary between species. Moreover, the rate of conversion of 18-carbon PUFA to long chain metabolites is slower in guinea pigs than rats, and may be more closely related to the rate of conversion seen for humans (31,36). For these reasons, we believe that the guinea pig is a useful animal model for examining the effect that dietary manipulation of n-3 fatty acids can have on tissue DHA levels and physiological function.

In conclusion, this study in guinea pigs has highlighted the difference between the use of diets containing LA and ALA with those containing LA, ALA, AA, and DHA on tissue fatty acid composition. The effects observed in neural tissue are not the same as those in other tissues like liver and heart, and the potential that differences in long chain PUFA levels may have distinguishable effects on physiological function (eicosanoid production, enzyme activity, ion flux, etc.) should be explored in future studies.

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Dietary Supplementation with Arachidonic and Docosahexaenoic Acids Has No Effect on Pulmonary Surfactant in Artificially Reared Infant Rats

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ABSTRACT: Despite the potential use of long-chain polyunsaturated fatty acid (LCPUFA) supplementation to promote growth and neural development of the infant, little is known about potential harmful effects of the supplementation. The present study determined whether supplementation with arachidonic acid (AA) and/or docosahexaenoic acid (DHA) in rat milk formula (RMF) affects saturation of pulmonary surfactant phospholipids (PL). Beginning at 7 d of age, infant rats were artificially fed for 10 d with RMF supplemented with AA at 0, 0.5, and 1.0% of total fatty acid, or supplemented with DHA at 0, 0.5, and 1.0%, or cosupplemented with AA and DHA at levels of 0:0, 0.5:0.3, and 1.0:0.6% of the fat blend. Lung tissue PL contained 43 weight percent palmitate (16:0) of total fatty acids in infant rats fed the unsupplemented RMF. The supplementation with AA at both 0.5 and 1.0% decreased the weight percentage of 16:0 and stearate (18:0), indicating a decrease in saturation of PL. The observed decreases were accompanied by increases in AA and linoleic acid (18:2n-6). Surfactant phosphatidylcholine (PC) consisted of 71 weight percent 16:0 in the unsupplemented group, and this highly saturated PC was not altered by the cosupplementation with AA and DHA although there was a slight increase in DHA. Similarly, the cosupplementation did not change fatty acid composition of surfactant PL when compared with the unsupplemented group. The cosupplementation slightly decreased the weight percentage of 16:0 with a proportional increase in 18:0 leading to an unchanged weight percentage of total saturated fatty acids. These results suggest that, unlike lung tissue PL, the composition of saturated fatty acids in surfactant PL, particularly PC, is resistant to change by dietary AA and DHA supplementation. This, together with the unchanged concentration of total fatty acids in surfactant PC, indicates that LCPUFA cosupplementation causes no effect on pulmonary surfactant.

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Pulmonary surfactant is composed of lipoprotein complexes rich in phospholipids (PL), especially phosphatidylcholine (PC) (1). Among lipids, disaturated phosphatidylcholine (DSPC) accounts for approximately two-thirds of the total PC and is the major metabolically active component of lung surfactant (2). Surfactant lipids stabilize pulmonary alveoli and prevent alveolar collapse by reducing surface tension at the air-liquid interface (3). An insufficient amount of surfactant causes respiratory distress syndrome in both newborns and adults (4,5).

Surfactant content in alveoli is regulated by its synthesis and secretion by alveolar type II cells (6,7). The synthesis of PL, on the other hand, is dependent on the availability of fatty acids (8,9). Although fatty acid synthesized *de novo* contributes about 40% of DSPC production (8), dietary fatty acids presented to tissue *via* the circulation play an important role in modulating the quantity and composition of surfactant lipids (10). Similar to tissues such as liver, heart, kidney and brain, lung tissue fatty acid content is responsive to dietary lipids (11–16). Thus, it is not surprising that supplementation with long-chain polyunsaturated fatty acids (LCPUFA) of the n-6 and n-3 families—including arachidonic acid (AA, 20:4n-6), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3)—increased the corresponding fatty acids and increased the level of total unsaturated fatty acids of lung tissue PL (11–14). Despite the extensive studies with lung tissue little is known about the impact of dietary LCPUFA on surfactant PL.

DHA and AA are integral components of retina and neural tissue and are considered to be essential for growth and development in humans and animals (17,18). Accordingly, supplementation of LCPUFA has been studied to determine if dietary DHA and AA benefit neurodevelopment of human infants (19–24). In these studies, such supplementation increased LCPUFA concentrations in plasma and erythrocytes of human infants (19–24). Animal studies, on the other hand, showed that aside from an increase in LCPUFA in blood, the supplementation enhanced accretion of the fatty acids in the brain and liver leading to increased unsaturation of PL (16,25,26). Since increased unsaturation of surfactant PC could potentially perturb the respiratory function of pul-

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Abbreviations: AA, arachidonic acid; AR, artificial rearing; DHA, docosahexaenoic acid; DSPC, disaturated phosphatidylcholine; EPA, eicosapentaenoic acid; LCPUFA, long-chain polyunsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; RMF, rat milk formula.

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monary surfactant (1), the present experiments were undertaken to determine whether LCPUFA supplementation in milk formula alters fatty acid composition and the degree of saturation of surfactant PL. To this end infant rats were artificially fed milk formula *via* an intragastric tube as described previously (16). The results showed that, unlike lung tissue PL, the fatty acid composition of surfactant PC and other PL is resistant to change by dietary AA and DHA.

MATERIALS AND METHODS

Animals. Pregnant Sprague-Dawley rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) were housed in individual plastic containers. Water and food (Purina Rat Chow, Ralston Purina, St. Louis, MO) were available to the dams at all times. For each experiment, newborns from four pregnant rats were delivered naturally, culled to 10 pups per dam within 24 h postpartum, and were nourished by their dams until the beginning of artificial rearing (AR). At 7 d of age, one or two weight-matched rat pups from each litter were assigned to one of three AR groups. Thus, each group consisted of rat pups derived from four dams. In the first series of experiments, three groups of infant rats were fed rat milk formulas (RMF) which contained 0, 0.5, and 1.0% AA as a percentage of the total fatty acids. The second experiment featured three RMF that contained 0, 0.5, and 1.0% DHA. In the third series of experiments infant rats were fed RMF cosupplemented with AA and DHA at concentrations of 0:0, 0.5:0.3, or 1.0:0.6% for AA and DHA, respectively.

AR system. Artificially reared infant rats were used throughout the study. The detailed account of AR has been previously described (16). Briefly, at the age of 7 d infant rats were permanently implanted with intragastric cannulae, connected to a semiautomatic feeding pump, and fed intragastrically for 15 min each hour. The intragastric cannulation on day 7 after birth was essential to ensure the survival of the pups during the course of AR. The pups were weighed daily between 0930 and 1100 in the animal care room. All animal care and surgical procedures were in accordance with protocols established by the Institutional Animal Care and Use Committee of The Pennsylvania State University.

The RMF were adopted from that of Auestad *et al.* (27). As described in the previous study (16), all formulas contained 11.8% fat (w/w) consisting of fat from milk formula base (3.4%), medium-chain triglycerides (2.8%), and corn oil plus microbial oil (6.4%). A fungal source of AA (i.e., Microbial oil A) and an algal source of DHA (i.e., Microbial oil B) were employed (Martek Bioscience, Columbia, MD). Microbial oil A consisted of 23.4% 18:1n-9, 17.7% AA, 16.3% 16:0, 15.1% 18:0, 11.5% 18:2n-6, but no DHA and EPA. Microbial oil B contained 41.9% DHA, 19.1% 16:0, 15.5% 14:0, 13.8% 18:1n-9, but no AA and EPA. All RMF contained micro- and macronutrients comparable to those found in rat milk (27). Upon the completion of the preparation, RMF were degassed with nitrogen and allocated into 100-mL plastic bottles for storage at −20°C. The formulas were rehomogenized daily immediately prior to feeding.

Tissue preparation and lipid analysis. The AR system provides a useful model to investigate the effects of nutrients on postnatal development of the rat. In a series of studies we evaluated the effects of exogenous LCPUFA on brain lipid metabolism (16). In addition, lung tissues were obtained from the same study to assess whether highly unsaturated fatty acids provided in the diet alter the degree of saturation of lung tissue and surfactant PL. Artificial feeding was terminated at the age of 17 d (i.e., 10 d after AR began) when the rat is known to have reached the peak of brain myelination (28). On the day of termination the brain, liver, and lung were excised from the rat pups under anesthetic state after intraperitoneal injection of pentobarbital (5 mg/100 g body weight). Immediately after the removal, tissues were rinsed in 0.9% saline, blot-dried, frozen in liquid nitrogen, and then stored at −80°C until subsequent analyses. The brain and liver were analyzed for fatty acids as reported elsewhere (16). In the present study, lipids were extracted from lung tissues with chloroform/methanol (2:1, vol/vol) by the procedure modified from that of Folch *et al.* (29). In a series of experiments, surfactant isolated from lung tissue was used for lipid extraction. Lung tissues were homogenized using a Ten-Broek homogenizer, and surfactant was isolated by the method of Frosolono *et al.* (30) as modified by Sanders and Longmore (31). The extracted lipids were separated into lipid classes by thin-layer chromatography using Silica Gel H plates and a solvent system consisting of hexane/ethyl ether/glacial acetic acid (80:20:1; by vol) (32). After the plates had dried, they were exposed to iodine vapor to identify total PL with standards. For analysis of PL species, the lipid extracts were separated into PC and phosphatidylethanolamine (PE) as described elsewhere (16). The isolated PL and PL species were scraped into ampules and immediately transmethylated with 12% boron trifluoride in methanol (wt/vol). Heptadecanoate (100 µg) was added to the transmethylation ampule as an internal standard. The sealed ampules were heated at 100°C in a heating block for 30 min. The methylated fatty acids were analyzed by using a gas chromatograph (Model 5890 Series II; Hewlett-Packard, Palo Alto, CA) equipped with a fused-silica capillary column (SP-2330, 30 m × 0.25 mm, 20 µm film; Supelco, Inc., Bellefonte, PA). The gas chromatographic analyses were performed under the following conditions: column temperature, 150°C; injector temperature, 220°C; detector temperature, 250°C; flow rate of the carrier gas (helium), 21.4 mL/min; and a split ratio of 76:1. Appropriate methyl ester standards (Supelco, Inc., Bellefonte, PA) were used to identify fatty acids. Fatty acid composition was expressed as weight percentage (wt%) of total fatty acids or µg fatty acid/g tissue.

Data and statistical analysis. All statistical analyses were performed on an IBM PS/2 computer using Minitab 10.1 (Minitab, Inc., State College, PA). Data were expressed as mean ± SD, and significance was assigned at $P < 0.05$. Difference in values among experimental groups was analyzed by one-way analysis of variance, and Tukey's pairwise com-

parisons were used to identify where the difference existed (33).

RESULTS

Rat milk formulas were analyzed for fatty acids. As expected, the formulas supplemented with AA, or DHA, or AA plus DHA were enriched with the respective fatty acids (Table 1). The actual wt% of AA (i.e., 0.4 and 1.1%) and of DHA (i.e., 0.5 and 0.9%) were close to the targeted 0.5 and 1.0%, respectively (Table 1). Similarly, the AA to DHA ratios of 0.5:0.3 and 0.9:0.5 in AA- and DHA-supplemented formula were the same as or close to the expected target ratio of 0.5:0.3 and 1.0:0.6. There was no significant difference in other fatty acids except a decrease in 18:2n-6 in AA-supplemented formula, and a slight reduction in 18:1n-9 in 1.0% DHA-supplemented formula. The supplementation did not affect the sum of saturated fatty acids and of polyunsaturated fatty acids.

At 7 d of age infant rats were weight-matched and randomly assigned to three groups for AR. After 10 d of feeding of RMF supplemented with or without AA plus DHA, the body weights ranging from 32.6 to 34.6 g, and lung weights ranging from 0.37 to 0.38 g were comparable among the three groups (Table 2). These values resulted in unaltered lung to body weight ratios. Comparable body weights were also noted in the experiments in which infant rats were fed diets supplemented with or without AA, or DHA alone (data not shown).

Total PL of lung tissue contained 43% palmitate (16:0) as a portion of the total fatty acids in infant rats fed the unsupplemented formula (Table 3). The supplementation with AA at 0.5 and 1.0% equally lowered the weight percentage of 16:0 and stearate (18:0) by 12–16% and 14–16%, respectively. Conversely, the AA supplementation resulted in higher weight percentages of AA and linoleate (18:2n-6) by 38–72% and 25–33%, respectively. These changes led to decreased total saturated fatty acids and increased total polyunsaturated fatty acids. The supplementation with DHA, on the other hand, did not alter fatty acids composition of total PL except that the weight percentage of DHA was higher in the supplemented groups than in the unsupplemented group (Table 3). Despite the small net change in weight percentage of DHA, total saturated and polyunsaturated fatty acids remained unchanged.

Table 4 shows the fatty acid composition of total PL and PL species isolated from pulmonary surfactant. The weight percentage of 16:0 was the highest in PC (71%), and the lowest in total PL (49%) in rat pups fed the unsupplemented RMF (Table 4). Conversely, the highest weight percentage of 18:0 was found in PE (24%) and the lowest in PC (5.5%). The cosupplementation with AA and DHA at either the 0.5:0.3 or the 1.0:0.6 ratio did not alter fatty acid composition of total PL. Similarly the cosupplementation did not change the composition of PC despite a slight increase of AA in the group supplemented with a high ratio of AA to DHA. The weight percentage of 16:0 in PE was lower in the cosupplemented groups by 11–12% than that of the unsupplemented counterparts. Also, the weight percentage of 20:2n-6 in PE was lower in the cosupplemented group than in the unsupplemented group. The observed reduction in 16:0 and 20:2n-6 was accompanied by an increase in 18:0. The sum of all saturated fatty acids was unchanged by dietary AA and DHA supplementation. The determination of the fatty acid concentration in surfactant isolated from 1 g of lung tissue disclosed that total fatty acids in PC were comparable in all three groups of infant rats (i.e., 400 ± 66 vs. 403 ± 12 vs. 415 ± 32 µg/g tissues). Similarly, fatty acid concentrations in PE and total PL remained unchanged by the AA and DHA cosupplementation.

DISCUSSION

Dietary fat is the most important determinant of tissue lipid both quantitatively and qualitatively. For example, a number of studies have shown that lipid composition of brain tissue

TABLE 1
Fatty Acid Composition of Rat Milk Formulas Supplemented with Arachidonate and/or Docosahexaenoate[a]

	AA (%)			DHA (%)			AA/DHA (%/%)		
Fatty acid	0	0.5	1.0	0	0.5	1.0	0:0	0.5:0.3	1.0:0.6
8:0	9.2 ± 0.4	9.7 ± 0.4	9.9 ± 0.4	4.9 ± 1.4[a]	5.9 ± 1.0[a,b]	7.0 ± 0.8[b]	8.1 ± 0.4	8.3 ± 0.6	8.3 ± 0.7
10:0	5.7 ± 0.1	5.9 ± 0.1	5.8 ± 0.2	4.6 ± 0.3	5.0 ± 0.5	4.7 ± 0.2	5.1 ± 0.2	5.4 ± 0.3	5.0 ± 0.4
12:0	0.9 ± 0.1	1.0 ± 0.01	1.0 ± 0.02	1.0 ± 0.3	1.0 ± 1.0	1.1 ± 0.1	0.9 ± 0.03	0.9 ± 0.02	1.0 ± 0.04
14:0	3.1 ± 0.3	3.2 ± 0.1	3.2 ± 0.1	3.4 ± 0.6	3.5 ± 0.2	3.6 ± 0.5	3.0 ± 0.1	3.1 ± 0.03	3.3 ± 0.3
16:0	15.3 ± 0.6	15.5 ± 0.1	16.3 ± 0.2	16.2 ± 1.9	16.1 ± 0.4	16.0 ± 1.0	14.9 ± 0.1	14.9 ± 0.1	15.3 ± 0.9
18:0	5.0 ± 0.3	5.3 ± 0.1	6.2 ± 0.1	5.3 ± 1.1	5.2 ± 0.2	5.2 ± 0.2	5.0 ± 0.01	5.1 ± 0.07	5.7 ± 0.3
18:1n-9	22.2 ± 0.2	22.1 ± 0.2	22.2 ± 0.2	23.6 ± 0.3[a]	23.1 ± 0.6[a,b]	22.8 ± 0.3[b]	23.1 ± 0.2	22.5 ± 0.3	23.1 ± 0.5
18:2n-6	37.2 ± 1.2[a]	35.5 ± 0.2[a]	32.5 ± 0.3[b]	39.8 ± 3.9	38.6 ± 1.1	37.4 ± 2.2	38.2 ± 0.6	36.3 ± 0.6	35.3 ± 0.7
18:3n-6	0.7 ± 0.01	0.6 ± 0.01	0.6 ± 0.01	0.7 ± 0.05	0.7 ± 0.01	0.7 ± 0.01	0.7 ± 0.01	0.7 ± 0.01	0.7 ± 0.01
20:4n-6	—	0.4 ± 0.01	1.1 ± 0.01	—	—	—	—	0.5 ± 0.02	0.9 ± 0.11
22:6n-3	—	—	—	—	0.56 ± 0.01	0.9 ± 0.05	—	0.3 ± 0.01	0.5 ± 0.01
Sum SFA	39.1 ± 1.0	40.5 ± 0.4	42.4 ± 0.5	35.4 ± 3.9	36.7 ± 1.5	37.6 ± 2.1	37.3 ± 0.9	39.0 ± 0.8	38.8 ± 0.7
Sum PUFA	37.9 ± 1.2[a]	36.5 ± 0.2[a]	34.4 ± 0.3[b]	40.5 ± 3.9	39.7 ± 1.1	38.9 ± 2.2	38.8 ± 0.6	37.7 ± 0.7	37.3 ± 0.6

[a]Values expressed as weight percentage of total fatty acids are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each supplement with different superscript roman letter in the same row are significantly different at $P < 0.05$. AA, arachidonic acid; DHA, docosahexaenoic acid; SFA, saturated fatty acids; PUFA, polyunsaturated fatty acids.

TABLE 2
Body and Lung Weights of Infant Rats Fed Milk Formulas Containing Arachidonate and Docosahexaenoate[a]

	AA/DHA (%/%)		
	0:0	0.5:0.3	1.0:0.6
Body wt (g)	32.8 ± 1.9	34.6 ± 0.1	32.6 ± 1.1
Lung wt (g)	0.37 ± 0.05	0.38 ± .01	0.38 ± 0.02
Lung/body (%)	1.20 ± 0.11	1.10 ± 0.01	1.15 ± 0.07

[a]Values are means ± SD for four rats. Lung/body (%) = lung (g)/body weight (g) × 100. For abbreviations see Table 1.

(15,34,35), cells (36), and subcellular organelles (25,36) of infant rats reflected that of maternal diets. Specifically, the tissue and cellular levels of n-6 and n-3 LCPUFA were effectively enriched by the corresponding fatty acids presented in maternal diets (15,25,26,34–36). These fatty acids supplemented in rat milk formulas also were rapidly incorporated into various tissues (e.g., liver and brain) in artificially reared infant rats (16,25). The present study further determined the role of dietary fat in lung tissue and surfactant during postnatal development in AR model. Aside from many advantages of AR described elsewhere (16), the model permits evaluation of the direct impact of dietary nutrient on neonates without interference of maternal involvement.

Consistent with earlier studies by other investigators (11–14), the results of the present study showed that dietary AA increased the accretion of AA with a proportional decrease in saturated fatty acids, i.e., 16:0 and 18:0 in PL isolated from lung tissue. These changes led to a reduction in PL saturation as indicated by the decreased weight percentage of total saturated fatty acids and increased weight percentage of total polyunsaturated fatty acids. However, the supplementation of DHA had no such effect on saturation of lung tissue PL despite a slight increase in DHA accretion. Although the effect of cosupplementation with AA and DHA on lung tissue PL was not determined, the fatty acid composition and the level of saturation of surfactant PL was not altered by simultaneous supplementation with the two LCPUFA. An analysis of individual PL species of surfactant revealed that the cosupplementation with AA and DHA did not reduce the degree of saturation of PE or PC, which is the major component of surfactant lipids. This finding was consistent with our previous study which showed that fish oil rich in DHA and EPA did not decrease the proportion of saturated fatty acids

TABLE 3
Fatty Acids of Lung Phospholipids in Infant Rats Fed Formulas Supplemented with Arachidonate or Docosahexaenoate[a]

	AA (%)			DHA (%)		
Fatty acid	0	0.5	1.0	0	0.5	1.0
16:0	43.4 ± 2.6[a]	37.4 ± 1.3[b]	38.1 ± 0.7[b]	42.7 ± 2.2	43.5 ± 1.9	41.5 ± 0.4
18:0	17.7 ± 0.8[a]	14.9 ± 0.5[b]	15.2 ± 0.9[b]	13.8 ± 0.8	12.6 ± 0.5	14.1 ± 0.3
18:1n-9	13.8 ± 0.3	14.3 ± 0.3	13.6 ± 0.5	13.9 ± 0.5	13.6 ± 0.2	13.9 ± 0.2
18:2n-6	6.3 ± 1.1[a]	7.9 ± 0.02[b]	8.4 ± 0.7[b]	9.8 ± 0.5	9.4 ± 0.5	10.1 ± 0.3
20:2n-6	0.9 ± 0.2[a,b]	0.6 ± 0.02[b]	0.7 ± 0.1[a]	0.7 ± 0.02	0.7 ± 0.01	0.7 ± 0.03
20:4n-6	7.1 ± 1.7[a]	12.6 ± 0.6[b]	11.9 ± 0.6[b]	9.4 ± 0.8	8.6 ± 1.0	9.5 ± 0.5
22:6n-3	0.5 ± 0.1	0.7 ± 0.1	0.7 ± 0.1	0.8 ± 0.1[a]	1.6 ± 0.3[b]	2.5 ± 0.1[b]
Sum SFA	66.5 ± 3.3[a]	57.5 ± 0.7[b]	58.8 ± 1.1[b]	59.4 ± 1.7	59.5 ± 1.6	57.6 ± 0.4
Sum PUFA	15.5 ± 2.9[a]	22.9 ± 0.8[b]	22.8 ± 1.4[b]	25.6 ± 1.5	25.6 ± 1.4	27.4 ± 0.4

[a]Values expressed as weight percentage of total fatty acids are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each supplement with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

TABLE 4
Fatty Acids of Pulmonary Surfactant Phospholipids and Phospholipid Species in Infant Rats Fed Formulas Supplemented with Arachidonate and Docosahexaenoate[a]

	Phospholipids [AA/DHA (%/%)]			Phosphatidylcholine [AA/DHA (%/%)]			Phosphatidylethanolamine [AA/DHA (%/%)]		
Fatty acid	0:0	0.5:0.3	1.0:0.6	0:0	0.5:0.3	1.0:0.6	0:0	0.5:0.3	1.0:0.6
16:0	49.2 ± 2.7	51.9 ± 2.6	48.3 ± 2.0	70.8 ± 0.5	71.0 ± 1.6	70.9 ± 1.2	32.2 ± 1.7[a]	28.6 ± 1.6[b]	28.2 ± 1.3[b]
18:0	13.4 ± 1.2	13.2 ± 1.4	15.1 ± 1.5	5.5 ± 0.7	6.2 ± 0.7	5.9 ± 0.6	23.9 ± 1.5[a]	29.7 ± 1.7[b]	28.1 ± 1.1[b]
18:1n-9	17.4 ± 1.4	16.0 ± 1.8	18.6 ± 1.4	12.7 ± 0.7	13.5 ± 0.8	12.6 ± 0.9	30.3 ± 1.7	31.5 ± 2.9	33.0 ± 1.9
18:2n-6	3.4 ± 0.3	3.7 ± 0.3	3.5 ± 0.5	2.1 ± 0.2	1.7 ± 0.3	2.0 ± 0.3	1.8 ± 0.5	1.0 ± 0.7	1.4 ± 0.6
20:2n-6	1.1 ± 0.5	1.2 ± 0.7	0.9 ± 0.5	0.1 ± 0.07	0.1 ± 0.02	0.1 ± 0.04	2.4 ± 0.8[a]	1.7 ± 0.3[b]	1.5 ± 0.3[b]
20:4n-6	6.5 ± 1.3	6.2 ± 1.6	7.2 ± 0.7	0.7 ± 0.1[a]	0.6 ± 0.2[a]	1.0 ± 0.2[b]	4.1 ± 0.7	3.4 ± 0.6	3.5 ± 0.6
22:6n-3	1.0 ± 0.5	1.1 ± 0.4	1.2 ± 0.2	0.02 ± 0.001	0.02 ± 0.01	0.05 ± 0.02	—	—	—
Sum SFA	66.3 ± 2.4	68.0 ± 2.7	65.7 ± 2.0	81.7 ± 0.4	81.7 ± 1.4	84.4 ± 1.7	57.1 ± 2.7	57.1 ± 3.1	56.9 ± 3.9
Sum PUFA	14.9 ± 1.9	14.8 ± 1.7	15.0 ± 1.3	3.1 ± 0.3	2.7 ± 0.5	3.2 ± 0.6	9.9 ± 2.7	7.6 ± 2.2	8.0 ± 2.9

[a]Values expressed as weight percentage of fatty acids are means ± SD for four samples. Fatty acids included only major saturated and unsaturated (n-9, n-6, and n-3) species, and hence did not add up to 100%. Values for each phospholipid with different superscript roman letters in the same row are significantly different at $P < 0.05$. For abbreviations see Table 1.

in PL of alveolar type II cells (37). Clearly, unlike lung tissue PL, surfactant PL, especially PC, is resistant to modification by dietary AA and DHA. The reason for the differential effects on lung tissue and surfactant is not completely understood but may be attributed to the complex cellular organization of the lung, which is known to consist of more than 40 cell types (38). Surfactant is synthesized by alveolar type II cells which constitute only 14% of the total cell mass in the lung (38). It is reasonable to speculate that the lack of modification of surfactant PC by dietary LCPUFA may stem in part from preferential incorporation of saturated fatty acids into PC by alveolar type II cells. In support of such speculation, we showed that PL of alveolar type II cells were able to maintain the level of saturated fatty acids without increasing the sum of n-3 and n-6 LCPUFA by fish oil supplementation (37). Alternatively, the maintenance of PC saturation under the present experimental conditions may be achieved by the deacylation-reacylation mechanism by remodeling of *de novo* synthesized unsaturated PC (1).

The function of alveoli depends not only on the composition of surfactant PL but also on the amount of surfactant secreted onto alveolar space (39). It is therefore important to determine whether dietary AA and DHA influence the amount of surfactant present in the lung. Although no attempt was made in the present study to quantify surfactant, the constant concentration of total fatty acids measured in surfactant PC among three groups of infant rats strongly suggests that dietary LCPUFA did not reduce the quantity of lung surfactant. However, it is not known whether the findings of lung surfactant reflect that of alveolar surfactant. In an earlier study we demonstrated that fish oil fed to adult rats had no effect on mass of DSPC and unsaturated PC in alveolar surfactant isolated from lung lavage (37). This, together with the fact that tissue surfactant isolated in the present study represents extracellular and intracellular materials (31), led us to speculate that saturation of surfactant PC on the alveolar space remains unchanged by AA and DHA supplementation.

In summary, the neutral effect of the LCPUFA supplementation on lung surfactant observed in the present study lend support for n-6 and n-3 fatty acid supplementation to promote growth and development of infants (17,18).

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Lipophilic Aldehydes and Related Carbonyl Compounds in Rat and Human Urine

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ABSTRACT: Rat and human urine samples were analyzed for lipophilic aldehydes and other carbonyl products of lipid peroxidation. The following compounds were identified as their 2,4-dinitrophenyl hydrazones by cochromatography with pure standards using three solvent systems: butanal, butan-2-one, pentan-2-one, hex-2-enal, hexanal, hepta-2,4-dienal, hept-2-enal, octanal, non-2-enal, deca-2,4-dienal, 4-hydroxyhex-2-enal, and 4-hydroxynon-2-enal. In general, fasted rats excreted less of these compounds than fed rats, indicating they were partially of dietary origin or that the endogenous compounds were excreted in a form not susceptible to hydrazone formation. The compounds excreted in human urine were similar to those excreted in rat urine but were present in lower concentrations. Identification of the conjugated forms of the lipophilic aldehydes and related carbonyl compounds excreted in urine may be a source of information about their reactions *in vivo*.

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The decomposition of lipid hydroperoxides in biological systems is accompanied by the formation of a variety of aldehydes and other carbonyl compounds. The main mechanism for their formation is the so-called β-cleavage reaction of lipid alkoxyl radicals (1). Recent evidence indicates that some saturated and unsaturated aldehydes, including malondialdehyde (MDA), hydroxyaldehydes and other short chain carbonyl compounds, contribute to peroxidative cell damage by reacting with sensitive biomolecules (2). Low molecular weight aldehydes such as 4-hydroxyalkenals and MDA are sufficiently long-lived to damage target molecules distant from the site of their formation (3,4). These aldehydes are capable of impairing protein function, inhibiting protein synthesis, causing cell lysis, and affecting cellular reproductive integrity (5–7). Recently, a lysine-4-hydroxynonenal adduct has been identified in an *in vitro* study on the modification of glucose-6-phosphate dehydrogenase by 4-hydroxynon-*trans*-2-enal (HNE) (8). Thus 4-hydroxyalkenals and other reactive carbonyl compounds formed by lipid peroxidation may be seen as secondary toxic messengers emanating from the initial free-radical event (9,10).

Studies on the formation of aldehydes other than MDA have concentrated on their *in vitro* production in whole cells (11,12), subcellular fractions (13,14), plasma and tissues (15) in response to prooxidant stimuli. A great variety of carbonyl compounds, including alkanals, alk-2-enals, 4-hydroxyalk-2-enals, alk-2,4-dienals, and alk-2-ones from ADP-Fe^{2+}-stimulated peroxidation of rat liver microsomes, has been characterized by Esterbauer and colleagues (16).

Few studies have been carried out on the *in vivo* formation of aldehydes other than MDA. The formation of HNE in rat liver was shown to be increased following administration of the prooxidants carbon tetrachloride, bromobenzene, or bromotrichloromethane (17,18), and in the renal cortex by administration of celiptium (19). Yoshino *et al*. (20) found that the concentrations of 4-HNE and hexanal in plasma and of HNE, hexanal, and pentanal in the liver of rats were significantly increased by vitamin E deficiency. Similarly, Dratz *et al*. (21) found elevated amounts of 4-hydroxyalkenals in the retinas of vitamin E-deficient rats and dogs. Goldring *et al*. (22) found 4-HNE in normal human plasma and Strohmaier *et al.* (23) in the plasma of humans with lung injury. Recently several endogenous saturated and unsaturated aldehydes such as 2-butenal, 2,4-pentadienal, hexanal, heptanal, octanal, 2-octenal, 2-nonenal, and decanal were found in significantly elevated levels in diabetic rat and human sera (24). Some of these compounds have been shown to be potent inhibitors of glycolytic enzymes such as phosphofructokinase and glyceraldehyde-3-phosphate dehydrogenase. Thiobarbituric acid-reactive substances in human urine include lipophilic alkadienals and/or alkanals as well as MDA (25). Based on the above observations, we see that a variety of aldehydes of medium chain length are produced as degradation products of lipid peroxidation *in vivo*. These aldehydes range from 4 to 10 carbons, may be saturated or unsaturated, and may be hydroxylated. The cell pathology of these lipophilic compounds is largely unknown. Identification of the free and conjugated forms of these aldehydes and carbonyl compounds excreted in the urine may, as in the case of MDA ([26]), provide information on the nature of their reactions *in vivo*.

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Abbreviations: DNPH, 2,4-dinitrophenylhydrazine; DNP-hydrazones, 2,4-dinitrophenylhydrazones; HNE, 4-hydroxynon-*trans*-2-enal; HPLC, high-performance liquid chromatography; MDA, malondialdehyde; MeOH, methanol; NONPOL, nonpolar carbonyl compounds; POL, polar carbonyl compounds; TLC, thin layer chromatography.

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In our previous studies, analysis of rat urine by high-performance liquid chromatography (HPLC) revealed the presence of several carbonyl compounds besides free and conjugated MDA and showed that the excretion of some of these compounds was increased in vitamin E deficiency (27). The objective of this study was to identify the lipophilic aldehydes and related carbonyl compounds excreted into urine of normal rats and human subjects.

MATERIALS AND METHODS

Chemicals. 2,4-Dinitrophenylhydrazine (DNPH) and hexanal were obtained from Sigma Chemical Company (St. Louis, MO); pentan-2-one (97%), hept-2-enal (97%), hepta-2,4-dienal (90%), decanal, and deca-2,4-dienal from Aldrich Chemical Co. (Milwaukee, WI); and hydrochloric acid and HPLC-grade acetone, methanol, dichloromethane, hexane, and water from EM Science (Gibbstown, NJ). DNPH derivatives of butanal, butanone, hexanal, octanal, non-2-enal, 4-hydroxyhex-2-enal, and 4-hydroxynon-2-enal were a gift from Dr. Hermann Esterbauer, University of Graz (Graz, Austria). Pentan-2-one, hept-2-enal, hepta-2,4-dienal, and deca-2,4-dienal DNPH derivatives were synthesized from pure standards and purified by repeated recrystallization from methanol. Silica gel thin-layer chromatography (TLC) plates (Silica gel 60, aluminum backed, 20 × 20 cm, 0.2-mm thickness) were purchased from Alltech Associates Inc. (Deerfield, IL).

Instrumentation. The HPLC system consisted of an Altex Model 110A solvent metering pump, an Altex Model 110A sample injector (Beckman Instruments, Berkeley, CA), a Spectra-Physics Model SP8400 ultraviolet/visible detector, and a Spectra-Physics Model SP4100 computing integrator (Spectra-Physics, Arlington, IL). The HPLC separations were performed on an Ultrasphere ODS C_{18} reversed-phase column (25 cm × 4.6 mm i.d., 5 µm particle size) (Altex, Berkeley, CA) with a 2 cm × 2 mm i.d. guard column (Chrom Tech, Apple Valley, MN). Disposable syringes were equipped with a 0.2 µm PUDF filter (Chrom Tech).

Animals, diets, and urine collections. Seven male Wistar rats (Harlan, Indianapolis, IN) weighing 165–190 g were fed standard AIN-76 semipurified diet (28). After 7 wk, urine was collected for 24 h without fasting. Nine Sprague-Dawley female weanling rats (Harlan, Indianapolis, IN) were fed a normal purified diet for 19 wk (29). They were then housed individually in stainless steel metabolic cages and fasted for 48 h with access to water *ad libitum*. Urine was collected during the second 24 h of fasting and stored at −70°C until analysis. Urine samples from nonfasting humans were collected from five healthy female subjects aged 20 to 40 yr.

Measurement of urinary lipophilic aldehydes and carbonyl compounds: Preparation of DNPH reagent. The reagent was prepared daily by a modification of the method of Esterbauer *et al*. (16). DNPH reagent (12.5 mg), recrystallized three times, was mixed with 25 mL of 1 N hydrochloric acid and the suspension was kept at 50°C for about 1 h. After cooling it was extracted four times with 20 mL of hexane to remove impurities. The purified DNPH reagent was used immediately.

Synthesis of 2,4-dinitrophenylhydrazones (DNP-hydrazones). Pentan-2-one, hexanal, hept-2-enal, hepta-2,4-dienal, decanal, and deca-2,4-dienal were synthesized by a method modified from Cheronis and Entrikin (30). DNPH (80 mg), 8 mL warm methanol, 0.2 mL 6 N hydrochloric acid, and 100 µL of an aldehyde standard were mixed and heated in a water bath at 60°C for 10 min. After cooling overnight at 4°C, 10 drops of water was added to induce crystallization. The fine crystals were filtered using a Büchner funnel and recrystallized from 20 mL of methanol.

Ultrafiltration of urine. Four milliliter aliquots of urine samples were filtered using an Amicon cell equipped with a YC05 Diaflo Ultrafilter (Amicon Corp., Beverly, MA) to remove compounds with a molecular mass larger than 500 daltons. The Amicon cell was pressurized to 50 psi with N_2 gas.

Isolation of DNP-hydrazones of urinary aldehydes and related compounds. The isolation and identification of the DNP-hydrazones was accomplished using a modification of the method of Esterbauer *et al*. (16). Three milliters of ultrafiltered urine samples was reacted with an equal volume of DNPH reagent. The reaction mixtures were mixed and incubated in the dark at room temperature overnight. The reagent blank and the acetone-DNPH standard were prepared as described above but instead of urine 3 mL DNPH reagent or 3 mL acetone/water (1:99 vol/vol) were used, respectively.

The urinary DNPH derivatives, the reagent blank, and the acetone-DNPH standard were each extracted three times with 3 mL of dichloromethane, and the organic phase was separated by centrifugation at 1360 × *g* for 10 min. The pooled extracts were evaporated to 500 µL under N_2 gas and applied to TLC plates.

The total dichloromethane extract (500 µL) was applied to a silica gel TLC plate, which was developed with dichloromethane for a preliminary separation. The nonpolar and polar carbonyl compounds were separated and located by comparison to the R_f values of acetone-DNPH (0.55) and purified DNPH reagent (0.23). The nonpolar carbonyl compounds (NONPOL) such as alkanals, alkenals, ketones and dienals were located between R_f 0.55 and the solvent front. Osazones were separated from NONPOL and polar carbonyl compounds (POL) on TLC between acetone-DNPH (R_f 0.55) and DPNH reagent (R_f 0.23) reference bands. POL, containing hydroxyl groups, were located between the origin and R_f 0.23. The nonpolar and polar regions were individually cut from the plates and eluted three times with 5 mL of methanol. The pooled methanol extracts of NONPOL and POL samples were centrifuged at 1360 × *g* for 20 min. The clear supernatant fractions were concentrated to about 300 µL under N_2 gas and made up to exactly 600 µL with methanol/water (50:50 vol/vol). Aliquots of the NONPOL and POL DNPH derivatives were then analyzed separately by HPLC using two different solvent systems.

Identification of DNPH derivatives. Aliquots (100 µL) of NONPOL-DNPH derivatives were injected onto an HPLC re-

versed-phase C_{18} column, equipped with a guard column, using isocratic elution for 10 min with methanol/water (75:25 vol/vol), followed by a linear gradient of methanol/water (75:25 vol/vol) to methanol for 30 min at a flow rate of 0.8 mL/min. Operating conditions for the HPLC separation of POL-DNPH derivatives were identical with those for NONPOL-DNPH derivatives except that methanol/water (50:50 vol/vol) was used as the starting eluent and the flow rate was 1.0 mL/min. Absorbance was monitored at 378 nm. Hexanal, hept-2-enal, and decanal DNP-hydrazones were used as standards to measure the reproducibility of the HPLC system before the application of samples.

Identification of individual urinary NONPOL and POL compounds was accomplished by cochromatography in HPLC solvent systems of different polarities. The retention times of the DNPH derivatives of the carbonyl standards were established in three different solvent systems according to their polarity (Table 1). The DNPH derivatives of the urinary compounds were tentatively identified by comparing their retention times with those of standards. Further evidence of identity was obtained by cochromatography using three solvent systems. The quantity of standard added was selected so as not to increase the peak area of the DNPH derivatives more than two times. Hexanal DNP-hydrazone standard was used to estimate the recovery of the urinary NONPOL and POL. To 3 mL of urine 7.6 µg of pure hexanal-DNPH standard was added and the percentage recovery was calculated from the resulting increase in peak area.

TABLE 1
Solvent Systems Used for Cochromatography of Lipophilic Carbonyl Compounds[a]

	Mobile phases					
	65% MeOH 35% water	68% MeOH 32% water	70% MeOH 30% water	75% MeOH 25% water	80% MeOH 20% water	85% MeOH 15% water
I. Nonpolar carbonyl compounds						
Butanal	*		*	*		
Butan-2-one		*	*	*		
Pentan-2-one	*		*	*		
Hexanal	*		*	*		
Hept-2-enal	*		*	*		
Hepta-2,4-dienal		*	*	*		
Octanal				*	*	*
Non-2-enal			*	*	*	
Deca-2,4-dienal			*	*	*	

	Mobile phases			
	45% MeOH 55% water	50% MeOH 50% water	55% MeOH 45% water	60% MeOH 40% water
II. Polar carbonyl compounds				
4-Hydroxyhex-2-enal		*	*	*
4-Hydroxynon-2-enal	*	*	*	

[a]The three solvent systems used for cochromatography of the nonpolar and polar carbonyl compounds are indicated by an asterisk.

Statistical analysis. Student's unpaired, two-sided *t*-test was used to test the significance of differences between the values obtained in nonfasted female and fasted male animals. The data were normally distributed and showed equality of variances and therefore were expressed as the mean ± SEM. The level of statistical significance employed was $P < 0.05$.

RESULTS AND DISCUSSION

Typical HPLC chromatograms obtained for the DNPH derivatives of the nonpolar and polar compounds in rat urine are presented in Figures 1 and 2, respectively. The NONPOL and POL DNPH derivatives were well separated on the C_{18} reversed-phase column. The results of cochromatography of standard hexanal-DNPH and HNE-DNPH with their urinary counterparts are illustrated in Figures 3 and 4. The retention times of the standards (Figs. 3A and 4C) corresponded exactly with those of the DNPH derivatives of urinary origin (Figs. 3C and 4B). Mixing the derivatives of the urinary compounds with the standards resulted in coelution (Figs. 3B and 4A). Similar results were obtained using three solvent systems of different polarity (Table 1). In each case, the peak area for the mixture of compounds approximated the sum of the peak areas for the standards and the urinary metabolites (Table 2). By using these criteria the following urinary NONPOL were tentatively identified: butanal, butan-2-one, pen-

TABLE 2
Comparison of the Areas of Urinary Compounds and Standards Together with the Sum of the Areas of the Urinary Compounds and Standards Measured Separately[a]

	Mobile phases					
	65% MeOH 35% water	68% MeOH 32% water	70% MeOH 30% water	75% MeOH 25% water	80% MeOH 20% water	85% MeOH 15% water
I. Nonpolar carbonyl compounds						
Butanal	0.88		0.97	1.04		
Butan-2-one		0.98	0.80	0.92		
Pentan-2-one	0.92		1.03	0.91		
Hexanal	1.04		1.37	1.10		
Hept-2-enal	1.07		0.95	1.13		
Hepta-2,4-dienal		1.11	0.86	0.85		
Octanal				0.94	0.88	0.92
Non-2-enal			0.98	1.05	0.92	
Deca-2,4-dienal			0.91	0.96	1.01	

	Mobile phases			
	45% MeOH 55% water	50% MeOH 50% water	55% MeOH 45% water	60% MeOH 40% water
II. Polar carbonyl compounds				
4-Hydroxyhex-2-enal		0.96	0.91	1.06
4-Hydroxynon-2-enal	0.94	1.04	0.90	

[1]Calculation: $a/(b + c)$, where a = area from the cochromatography of urinary compound plus standard; b = area of urinary compound; c = area of standard. Values represent mean of duplicate samples.

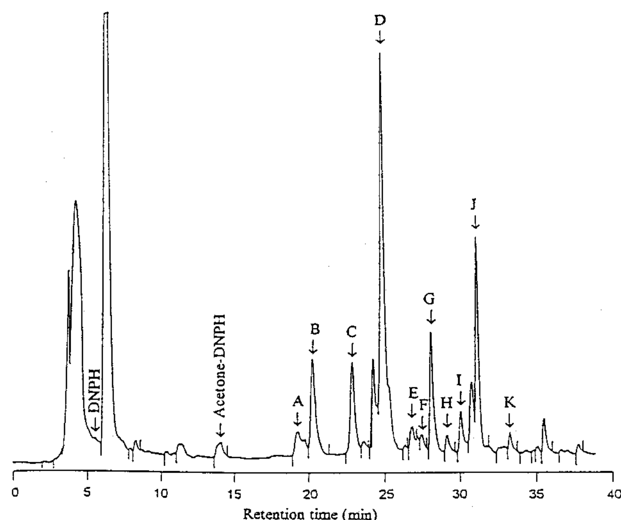

FIG. 1. High-performance liquid chromatography (HPLC) separation of 2,4-dinitrophenylhydrazine (DNPH) derivatives of urinary nonpolar aldehydes and carbonyl compounds from fasted male rats. A, butanal; B, butan-2-one; D, pentan-2-one; F, hex-2-enal; G, hexanal; H, hepta-2,4-dienal; I, hept-2-enal; K, octanal; C, E and J, unidentified. Absorbance was monitored at 378 nm.

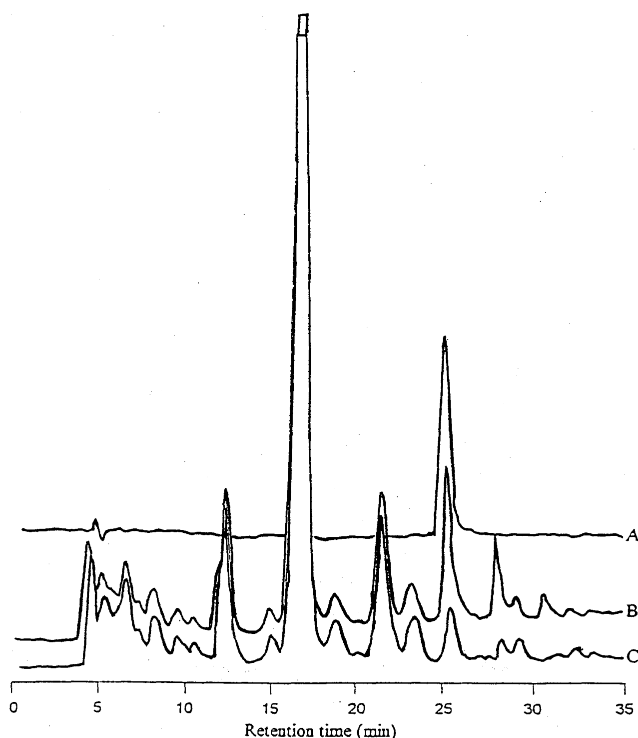

FIG. 3. Cochromatography of DNPH derivatives of urinary hexanal with hexanal standard. A, hexanal standard; B, cochromatogram of urinary nonpolar carbonyl compounds with hexanal standard; C, urinary nonpolar carbonyl compounds. Absorbance was monitored at 378 nm. For abbreviation see Figure 1.

tan-2-one, hexanal, hex-2-enal, hept-2-enal, hepta-2,4-dienal, octanal, non-2-enal, and deca-2,4-dienal. The hydroxyaldehydes 4-hydroxyhex-*trans*-2-enal and HNE also were tentatively identified. Some nonpolar and polar compounds were not identified because of a lack of appropriate standards.

In general, nonfasted male animals excreted higher levels of NONPOL and POL than fasted females (Figs. 5 and 6). Although these two groups were of opposite sexes, several studies suggest sex is unlikely to have influenced the excretion of NONPOL and POL. Rats chronically fed ethanol had greatly elevated hepatic MDA concentrations; however, these concentrations were not influenced by sex (31). Similarly, in a

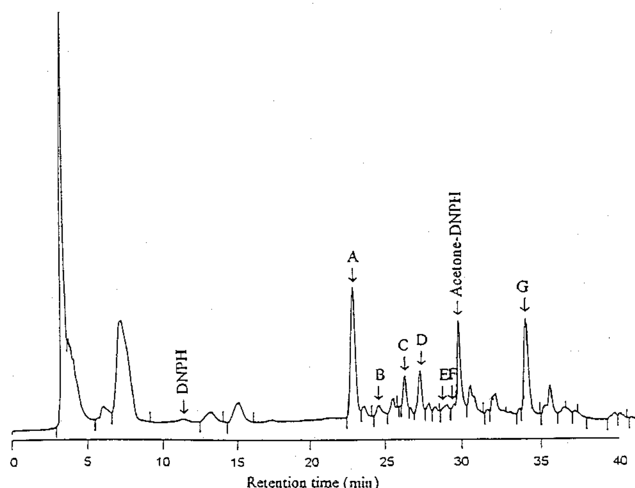

FIG. 2. HPLC separation of DNPH derivatives of urinary polar aldehydes and carbonyl compounds from fasted male rats. E, 4-hydroxyhex-2-enal; A, B, C, D, F and G, unidentified. Absorbance was monitored at 378 nm. For abbreviations see Figure 1.

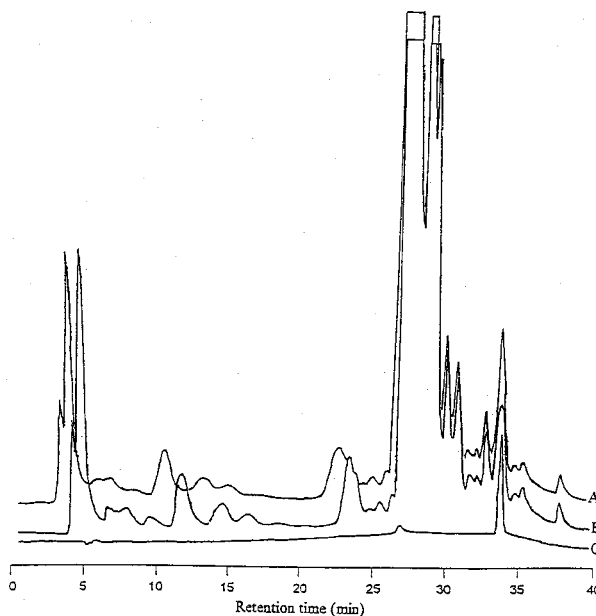

FIG. 4. Cochromatography of DNPH derivatives of nonfasted female rat urinary 4-hydroxynon-2-enal with 4-hydroxynon-2-enal standard. A, cochromatogram of urinary polar compounds with 4-hydroxynon-2-enal standard; B, urinary polar carbonyl compounds; C, 4-hydroxynon-2-enal standard. Absorbance was monitored at 378 nm. For abbreviation see Figure 1.

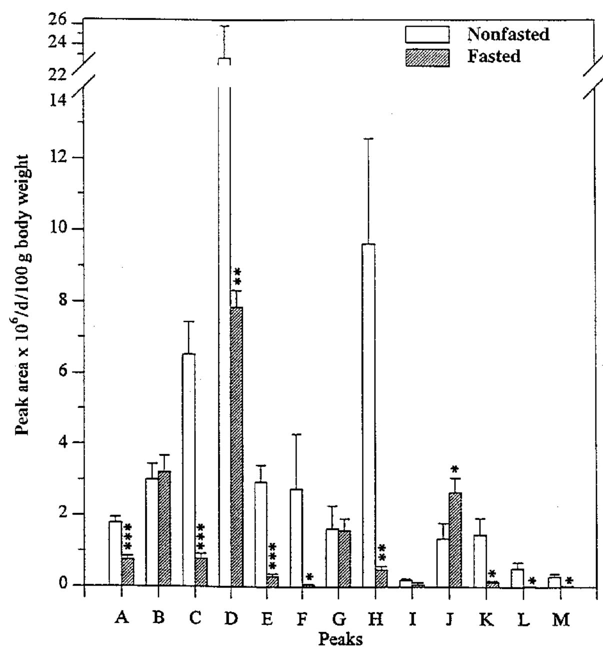

FIG. 5. Urinary excretion of nonpolar carbonyl compounds in nonfasted female and fasted male rats measured as DNPH derivatives. A, butanal; B, butan-2-one; F, hex-2-enal; G, hexanal; H, hepta-2,4-dienal; I, hept-2-enal; K, octanal; L, non-2-enal; M, deca-2,4-dienal; C, E and J, unidentified. Values represent mean ± SEM for $n = 7$ and $n = 9$, respectively, in nonfasted and fasted animals. Statistical significance of difference is determined by Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.005$, and $^{***}P < 0.0001$. For abbreviation see Figure 1.

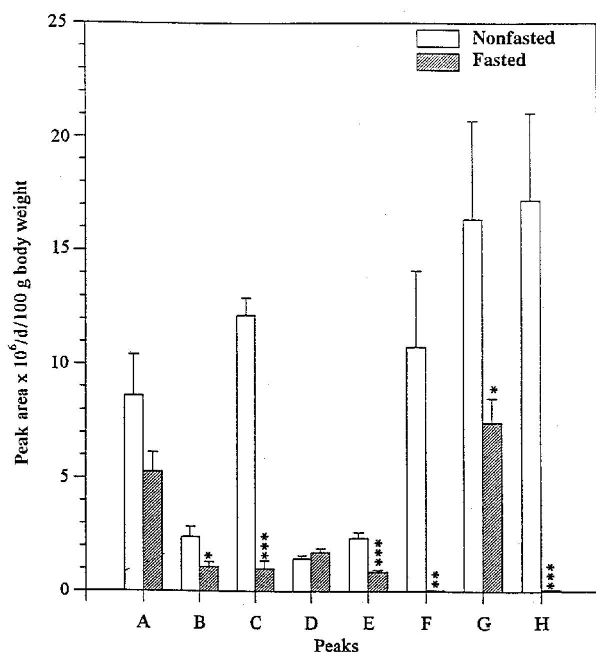

FIG. 6. Urinary excretion of polar carbonyl compounds in nonfasted female and fasted male rats measured as DNPH derivatives. E, 4-hydroxyhex-2-enal; H, 4-hydroxynon-2-enal; A, B, C, D, F, and G, unidentified. Values represent mean ± SEM of 7 and 9 animals in Experiments I and II, respectively. Statistical significance of difference is determined by Student's *t*-test; $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$. For abbreviation see Figure 1.

study of thiobarbituric acid reactivity in human urine, no differences due to sex were noted (32). Significant differences in NONPOL excretion were found for A, butanal; D, pentan-2-one; F, hex-2-enal; H, hept-2-enal; K, octanal; L, non-2-enal; M, deca-2,4-dienal and the unidentified compounds C, E, and J. No significant differences were found for B, butan-2-one; G, hexanal; and I, hept-2-enal (Fig. 5). The unidentified compound J was the only NONPOL compound that was significantly higher in the urine of fasted animals. Significant differences in the excretion of the following POL were found between nonfasted male and fasted female animals: E, 4-hydroxyhex-*trans*-2-enal; H, HNE and the unidentified compounds B, C, F, and G. Unidentified compound A tended to be lower in fasted animals but the difference was not significant (Fig. 6). The source of the increased urinary POL and NONPOL in the fed animals is possibly dietary fat. It has been shown in rats that secondary lipid oxidation products of linoleic acid are largely excreted in the urine (33).

The virtual disappearance of HNE in the urine of the fasted animals is noteworthy, as it is unlikely that none is formed *in vivo*. This observation suggests that HNE is excreted in a form that is not susceptible to hydrazone formation. This possibility is supported by the findings of Alary *et al.* (34), who showed that HNE is conjugated with glutathione in the rat and excreted as mercapturic acid conjugates. It is also possible that HNE is bound firmly to proteins and not released into the urine. The preponderance of stable Michael-type addition products to proteins over the easily hydrolyzable Schiff base adducts of α-β unsaturated aldehydes such as HNE was demonstrated by Bruenner *et al.* (35). Uchida and Stadtman (36) reported a similar type of stable addition of HNE to histidine. Szweda *et al.* (37) described the formation of a stable secondary lysine-HNE adduct between the ε-amino group of a lysine residue, in glucose 6-phosphate, and the double bond of HNE.

When the urinary NONPOL and POL were summed, highly significant differences were found between the results for nonfasted male and fasted female animals (Fig. 7). This difference indicates that some of the POL and NONPOL found in the urine may have originated in the diet. In both experiments, significantly greater amounts of POL than NONPOL were excreted in the urine ($P < 0.05$ for both nonfasted and fasted animals).

The chromatographic patterns for NONPOL and POL in human urine were similar to those for rat urine (Figs. 8 and 9) except for a relatively higher level of the peak coeluting with the hexanal-DPNH standard and a lower level of the one corresponding to pentan-2-one. These differences may be due to the differences in the aldehydes and carbonyls coming from dietary fats. The distribution of lipophilic compounds found for rat and human urine is similar to that reported by Esterbauer *et al.* (16) for microsomes peroxidized by treatment with ADP-Fe^{2+} (Table 3). Similar lipophilic POL and NONPOL have been detected *in vitro* by other investigators (11,13). The formation of HNE and 4-hydroxy-*trans*-2-hexenal in peroxidizing tissues has been documented by several investigators (38,39). 4-Hydroxyoctenal, 4-hydroxydece-

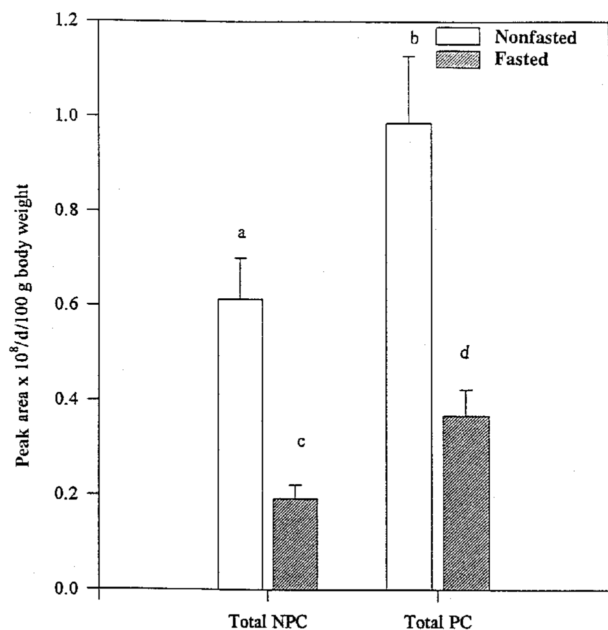

FIG. 7. Urinary excretion of total, HPLC separated, nonpolar (NPC) and total polar (PC) carbonyl compounds in nonfasted female and fasted male rats measured as DNPH derivatives. Values represent mean ± SEM of $n = 7$ and $n = 9$, respectively, in nonfasted and fasted animals. Statistical significance of difference is determined by Student's *t*-test; a vs. b, $P < 0.05$; c vs. d, $P < 0.05$; a vs. c, $P < 0.001$; b vs. d, $P < 0.001$. For other abbreviations see Figure 1.

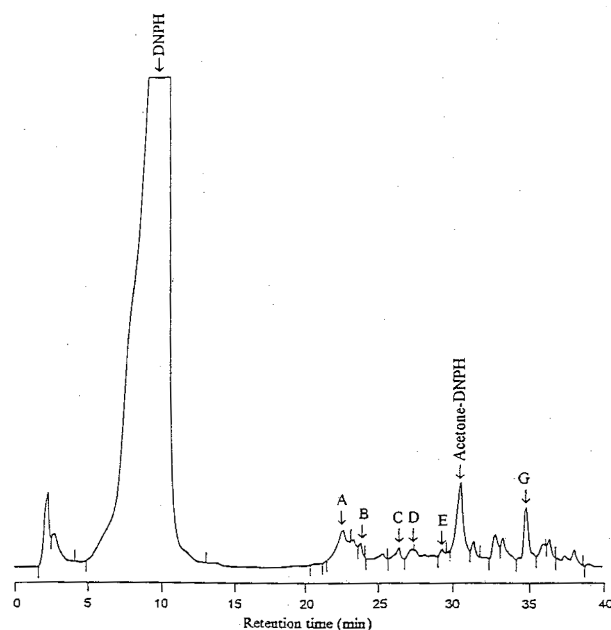

FIG. 9. HPLC separation of DNPH derivatives of urinary polar aldehydes and carbonyl compounds from a representative nonfasted human subject. E, 4-hydroxyhex-2-enal; A, B, C, D, and G, unidentified. Absorbance was monitored at 378 nm. For abbreviations see Figure 1.

nal, and 4-hydroxyundecenal have been detected in peroxidizing liver microsomes (40) and 5-hydroxypentanal in plasma and liver of normal rats (41). In addition to hydroxyaldehydes, hexanal is formed in foods and animal tissues as a product of the peroxidation of n-6 polyunsaturated fatty acids (12,15) along with various alkanals, alkenals, and alkadienals as products of n-6 and n-3 PUFA oxidation (42,43). Recently the α-hydroxyaldehydes 2-hydroxyheptenal and 2-hydroxyhexanal were identified in bovine lipids (44). Kaneko *et al.* (45) found that the lethal effect of hydroxyaldehydes is enhanced with increases in chain length and unsaturation.

The present findings indicate that there is a general similarity between the lipophilic products of lipid peroxidation formed *in vivo* and those formed *in vitro*. Identification of the conjugates of the lipophilic carbonyl compounds excreted in urine may provide valuable information on the nature of their reactions *in vivo*, as has been the case with MDA (26).

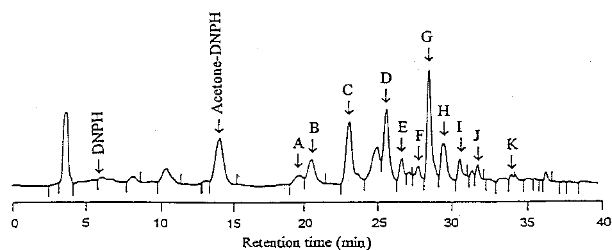

FIG. 8. HPLC separation of DNPH derivatives of urinary nonpolar aldehydes and carbonyl compounds from a representative nonfasted human subject: A, butanal; B, butan-2-one; D, pentan-2-one; F, hex-2-enal; G, hexanal; H, hepta-2,4-dienal; I, hept-2-enal; K, octanal; C, E, and J, unidentified. Absorbance was monitored at 378 nm. For abbreviations see Figure 1.

TABLE 3
DNPH Derivatives of Lipophilic Aldehydes Identified *In Vivo* and *In Vitro*

	In vivo			*In vitro*[a]
	Rat urine			
Lipophilic aldehydes	Nonfasted group	Fasted group	Human urine	Rat liver
Butanal	*	*	*	*
Butan-2-one	*	*	*	
Pentanal				*
Pent-2-enal				*
Pentan-2-one	*	*	*	
Hex-2-enal	*	*	*	*
Hexanal	*	*	*	*
Hepta-2,4-dienal	*	*	*	
Hept-2-enal	*	*	*	*
Oct-2-enal				*
Octanal	*	*	*	
Non-2-enal	*	*		
Deca-2,4-dienal	*	*		
4-Hydroxyhex-2-enal	*	*	*	
4-Hydroxyoct-2-enal				*
4-Hydroxynon-2-enal	*	*		*
4-Hydroxyundec-2-enal				*

[a]From Reference 16. Lipid peroxidation of liver microsomes was induced by ADP-Fe^{2+}. DNPH, 2,4-dinitrophenylhydrazine.

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Fenofibrate Protects Lipoproteins from Lipid Peroxidation: Synergistic Interaction with α-Tocopherol

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ABSTRACT: One of the earliest steps of atherosclerotic plaque formation is an increase of circulating apolipoprotein B-containing lipoproteins which, after infiltrating the subendothelial space, undergo oxidative modification. Fenofibrate is an effective cholesterol- and triglyceride-lowering agent which has been shown to be beneficial in the treatment of atherosclerosis. Vitamin E, or α-tocopherol, is a powerful antioxidant which has been shown in a variety of studies to prevent lipoprotein peroxidation. The purpose of the present study was to investigate the effect of fenofibrate treatment, either alone or in combination with α-tocopherol, in reducing the susceptibility of lipoproteins to oxidative modification. Rats fed a normal diet were treated for up to 27 d with fenofibrate, either alone or in combination with equimolar doses of α-tocopherol. Combined VLDL (very low density lipoproteins) and LDL (low density lipoproteins) isolated after fenofibrate treatment were more resistant to copper-mediated oxidation, as assessed by conjugated diene formation. Lag time was prolonged up to 3.2-fold, while the maximal rate of diene production was significantly decreased by up to 2.2-fold. Treatment of rats with α-tocopherol alone at the selected dose had no significant effect on lag time, while the propagation rate was slightly decreased. Coadministration of fenofibrate with α-tocopherol prolonged the lag phase to a greater extent than fenofibrate alone, showing a synergistic interaction between the two compounds. Finally, the combination of fenofibrate and α-tocopherol was significantly more effective in modifying lipoprotein oxidation parameters than what was observed with α-tocopherol and bezafibrate or gemfibrozil. Thus, in addition to its well-established effects on lipoprotein concentrations and atherogenic parameters, fenofibrate reduces the susceptibility of VLDL and LDL to oxidative modification and exerts its action synergistically with α-tocopherol.

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Atherosclerosis is a multifactorial disease, the incidence of which is still increasing in Western society, along with being a marker disease of emerging affluence for developing countries (1). In accordance with metabolic diseases of aging, characterized by slow progression and irreversible oxidative alteration of biological parameters, the role of low density lipoprotein (LDL) modification in atherogenesis has been evoked (2). While the antioxidant defense mechanisms present in serum are sufficient under most conditions to prevent modification of circulating lipoproteins, LDL having diffused to the subendothelium may no longer be resistant to oxidation. Furthermore, alterations in LDL metabolism may alter its half-life and resident time in the arterial lumen (2,3).

Fenofibrate is a member of the fibrate class of lipid-lowering drugs (4) and its mechanism of action derives from activation of the peroxisome proliferator-activated receptor α (PPARα) (5). PPARα, as well as its congener PPARγ, are members of the steroid hormone nuclear receptor super family and have the potential to interact with several proteins, including RXRα, NF-κB and HSP72, leading to the modification of the expression of a wide variety of genes of intermediate metabolism (5–7). PPARα differs from PPARγ by its pattern of tissue expression as well as the nature of its endogenous ligands (5,8,9).

Hepatic and vascular expression of PPARα has the potential to favorably alter several aspects of atherogenesis (5,10). In the liver, activated PPARα modifies a panel of genes linking fatty acid, cholesterol, and glucose metabolism (5). In macrophages and smooth muscle cells, PPARα activators, including fenofibrate, have been shown to have an antiinflammatory effect and promote apoptosis (10–12). The role of PPARγ in atherogenesis is still uncertain as both pro- and antiatherogenic functions have been suggested (7,13).

In addition to lowering apolipoprotein β-containing lipoproteins in man, fenofibrate has been shown to alter the quantity of small, dense LDL (14). This latter subfraction of LDL is particularly susceptible to oxidative modification and is present in increased concentration in Type II diabetics (15). While essentially all lipid and protein components of LDL are subject to peroxidative modification, it is currently unknown which molecular components of LDL confer antioxidant protection or susceptibility (16). The effects of fenofibrate on liver apolipoprotein as well as macrophage and smooth muscle cell antiinflammatory gene expression have been well characterized, as have the effects on the quantity of small, dense LDL. Little is known concerning the ability of fenofibrate to qualitatively modify the intrinsic antioxidant potential of lipoproteins. In an initial study to explore the utility of associating a lipid-lowering drug with a known antioxidant, we attempted to determine the potential of fenofibrate

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Abbreviations: LDL, low density lipoproteins; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acids; VLDL, very low density lipoproteins.

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to retard the extent and rate of lipoprotein modification when administered alone or with vitamin E. We next determined whether effects were strictly fibrate class effects or dependent upon the individual fibrate, by comparing fenofibrate with bezafibrate and gemfibrozil.

EXPERIMENTAL PROCEDURES

Animals and treatments. Male Sprague-Dawley rats weighing approximately 230–250 g were obtained from Iffa-Credo (L'Arbresle, France). Animals were fed a standard pelleted chow (Ets Piètrement, Provins, France) with tap water *ad libitum*, and were housed under light and dark cycles of 12 h each.

Rats were treated for 3, 8, 15, or 27 d with equimolar doses of fenofibrate (43 mg/kg/d, Laboratoires Fournier, Daix, France), *d,l*-α-tocopherol acetate (55 mg/kg/d; Sigma Chemical Co., St. Louis, MO), or both. Drugs were administered once daily by oral gavage in soybean oil. Control rats received drug vehicle alone (2 mL/kg/d).

In a second experiment, groups of rats were given *d,l*-α-tocopherol acetate (55 mg/kg/d) either alone or in combination with equimolar doses of fenofibrate (43 mg/kg/d), bezafibrate (42 mg/kg/d, Sigma Chemical Co.) or gemfibrozil (29 mg/kg/d, Sigma Chemical Co.) in soybean oil for 15 d by gastric intubation. Control animals received vehicle alone (2 mL/kg/d).

At the end of the experiments, rats were fasted for 5 h and then anesthetized with sodium pentobarbital. Blood was collected from the abdominal aorta on EDTA (1 mg/mL). Plasmas were separated by low-speed centrifugation and pooled by two in order to carry out all the measurements foreseen.

Lipoprotein isolation. Combined very low density lipoproteins (VLDL) and LDL were isolated from plasma by density gradient ultracentrifugation. Briefly, 4 mL plasma was adjusted to density 1.050 g/mL with solid KBr and overlaid with 4 mL of a solution containing NaCl (9 mg/mL), EDTA (1 mg/mL) and KBr at a density of 1.050 g/mL. Samples were centrifuged [22 h, 15°C, 134,000 × *g* ($r_{av.}$ 5.91 cm)] in a Beckman 50Ti fixed-angle rotor in a Beckman L8-M ultracentrifuge (Beckman Instruments Inc., Palo Alto, CA). The top layer of each tube was harvested by aspiration using a Pasteur pipette and kept at 4°C in the dark in a nitrogen atmosphere until use.

Lipoprotein oxidation. The isolated VLDL + LDL fraction was dialyzed at 4°C in the dark against three changes of Dulbecco's phosphate-buffered saline solution without Ca^{2+} and Mg^{2+}. EDTA was present during the two first changes at a concentration of 10 μM. Lipoproteins from the various treatments were diluted in phosphate-buffered saline to a common protein concentration of 20 μg/mL, and oxidation was initiated by the addition of 5 μM $CuSO_4$. Conjugated diene formation was monitored by recording the absorbance at 234 nm at 37°C at 10-min intervals over 10 h with a Shimadzu UV-160A spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Lag time and maximal rate of conjugated diene production were calculated as previously described by Esterbauer *et al.* (17). The length of the lag phase was defined as the intercept with the time scale axis of the absorbance curve during the propagation phase, while the maximal rate of conjugated diene formation was calculated from the slope of the absorbance curve during the propagation phase. Lag phase was expressed in minutes, and propagation rate as nanomoles of conjugated dienes formed per minute per milligram protein, using a molar extinction coefficient for conjugated dienes of $2.95 \times 10^4\ M^{-1}\ cm^{-1}$ (18).

Biochemical analysis. Total cholesterol, triglyceride, and phospholipid levels in plasma and isolated lipoproteins were determined using assay kits from Biomérieux (Marcy-l'Etoile, France). Proteins were measured by the method of Lowry, as modified by Markwell *et al.* (19).

Statistical analysis. Results were expressed as mean ± standard deviation. Statistical differences between treatment groups were established by variance analysis followed by Student's *t*-test, using Statistical Analysis System software (SAS Institute Inc., Cary, NC). Differences were considered significant if $P \leq 0.05$.

RESULTS

Effect of fenofibrate and α-tocopherol on plasma lipid levels in rats. Fenofibrate and α-tocopherol acetate were administered either individually or in combination to rats for 3, 8, 15, or 27 d. There was no evidence of altered growth rate in rats as measured by body weight after 27 d of treatment compared to controls (data not shown). As expected, plasma total cholesterol levels were significantly reduced after fenofibrate administration. Modifications varied from −31 to −40%, and as is routinely seen in rats (20), reductions were in high density lipoprotein levels and not apolipoprotein B-containing lipoproteins (results not shown), and were already maximal after 3 d of treatment (Table 1). Phospholipid levels were initially decreased by the drugs at 3 and 8 d of treatment, but returned to control values by 15 d of drug administration. Triglyceride concentrations were not significantly affected by treatment. Administration of α-tocopherol alone had no effect on plasma lipid parameters while coadministration of α-tocopherol and fenofibrate gave similar modifications of plasma parameters as observed with fenofibrate alone.

A β-lipoprotein fraction, containing VLDL and LDL (VLDL + LDL) was isolated. Mean levels of total cholesterol and triglyceride in this fraction were not modified by fenofibrate, when administered either alone or in combination with α-tocopherol (data not shown). No modification of the above parameters could be observed after administration of α-tocopherol alone.

Effect of fenofibrate and α-tocopherol on VLDL + LDL oxidation in vitro. The kinetics of oxidation of the VLDL + LDL fraction were studied by monitoring the formation of fatty acid conjugated dienes at 234 nm. VLDL + LDL lipoproteins from the fenofibrate group exhibited an increased lag time and a much slower rate of oxidation, compared with controls (Fig. 1). The lag phase was 2.7-fold

TABLE 1
Influence of Fenofibrate, α-Tocopherol, and Their Combination on Plasma Lipid Levels[a]

Treatment	Days	Total cholesterol	Phospholipids	Triglycerides
Control	3	0.81 ± 0.07	1.33 ± 0.07	0.65 ± 0.24
α-Tocopherol		0.74 ± 0.07[b]	1.28 ± 0.10	0.72 ± 0.24
Fenofibrate		0.49 ± 0.05[b]	0.97 ± 0.05[b]	0.44 ± 0.07
Fenofibrate + α-tocopherol		0.52 ± 0.05[b]	1.02 ± 0.02[b]	0.51 ± 0.07
Control	8	0.67 ± 0.05	1.25 ± 0.10	0.83 ± 0.22
α-Tocopherol		0.69 ± 0.05	1.18 ± 0.12[b]	0.69 ± 0.24
Fenofibrate		0.46 ± 0.05[b]	1.09 ± 0.05[b]	0.43 ± 0.12
Fenofibrate + α-tocopherol		0.43 ± 0.02[b]	1.06 ± 0.05	0.57 ± 0.24
Control	15	0.66 ± 0.10	1.26 ± 0.10	0.82 ± 0.29
α-Tocopherol		0.65 ± 0.10	1.30 ± 0.15	0.86 ± 0.20
Fenofibrate		0.41 ± 0.05[b]	1.23 ± 0.05	0.69 ± 0.12
Fenofibrate + α-tocopherol		0.39 ± 0.05[b]	1.23 ± 0.10	0.71 ± 0.20
Control	27	0.70 ± 0.10	1.46 ± 0.12	1.16 ± 0.12
α-Tocopherol		0.65 ± 0.07	1.35 ± 0.12	0.94 ± 0.27
Fenofibrate		0.43 ± 0.05[b]	1.29 ± 0.10	1.24 ± 0.56
Fenofibrate + α-tocopherol		0.41 ± 0.12[b]	1.30 ± 0.17	0.99 ± 0.27

[a]Rats were given co-micronized fenofibrate (43 mg/kg/d), α-tocopherol acetate (55 mg/kg/d) or their combination for 3, 8, 15, or 27 d. Control animals received soybean oil alone. At the end of treatment, blood was collected and six pools of two plasmas were obtained for each group. Results are expressed as g/L and are mean ± standard deviation of six values.

[b]Significantly different from control group with $P \leq 0.05$.

longer after fenofibrate administration for 8 d, and results obtained after 8, 15, and 27 d of treatment were comparable. The propagation rate was significantly decreased as early as 3 d of treatment. Decreases were between 36 and 55% compared to controls while no clear time-effect was noted. No statistically significant increase in (VLDL + LDL) lag time was noted with administration of α-tocopherol alone, though the propagation rate was slightly decreased (from −12 to −20%). Coadministration of fenofibrate and α-tocopherol prolonged the lag time to a greater extent than with fenofibrate alone, whatever the duration of treatment and in spite of a lack of effect of α-tocopherol alone. Statistical analysis demonstrated a significant interaction between the two drugs, meaning that the efficacy of one drug differed depending on the presence of the other.

Effect of the combined administration of α-tocopherol with fenofibrate, bezafibrate, or gemfibrozil on VLDL + LDL oxidation. Combined treatments with α-tocopherol and either fenofibrate, bezafibrate, or gemfibrozil were compared in a second experiment. All compounds were administered at equimolar doses for 15 d. Coadministration of α-tocopherol with fenofibrate or bezafibrate similarly decreased plasma total cholesterol levels by 44 and 38%, respectively, while gemfibrozil was less effective, with a 13% reduction (data not shown). α-Tocopherol alone was again ineffective, consistent with the results from the first experiment. Lag time as well as propagation rate was significantly modified by all three combinations, compared to controls (Fig. 2), but the association between fenofibrate and α-tocopherol was more effective than the association with the other two fibrates. Coadministration of fenofibrate along with α-tocopherol induced a 4.2-fold increase in lag phase duration and a 2.6-fold decrease in propagation rate, while these parameters were modified 3.3, and 1.9-fold, respectively, after bezafibrate + α-tocopherol treatment. Gemfibrozil + α-tocopherol were the least effective, and results did not differ significantly from those obtained after administration of α-tocopherol alone.

DISCUSSION

Intraluminal oxidative modification of β-lipoproteins increases their uptake by monocyte-macrophages, thus initiating a cascade of events leading to fatty streak formation and atherogenesis (2). In circulating blood, lipoproteins are resistant to peroxidation. The passage of lipoproteins into the subendothelium may prolong their half-lifes while exposing lipoproteins to an environment depleted of antioxidant defense mechanisms. While pharmacological modification of this cascade could target (i) reduction in β-lipoproteins (LDL and/or VLDL), (ii) increased lipoprotein antioxidant reserves, (iii) decreased percentage of polyunsaturated fatty acids (PUFA), or, eventually (iv) decreased lipoprotein penetration into the subendothelium, the factors controlling *in vivo* lipoprotein modification are still incompletely understood (3).

Fenofibrate belongs to the fibrate class of lipid-lowering drugs whose mechanism of action is largely dependent upon the nuclear receptor PPARα (5). PPARα mediates a plethora of key genes overlapping fatty acid, glucose, and cholesterol

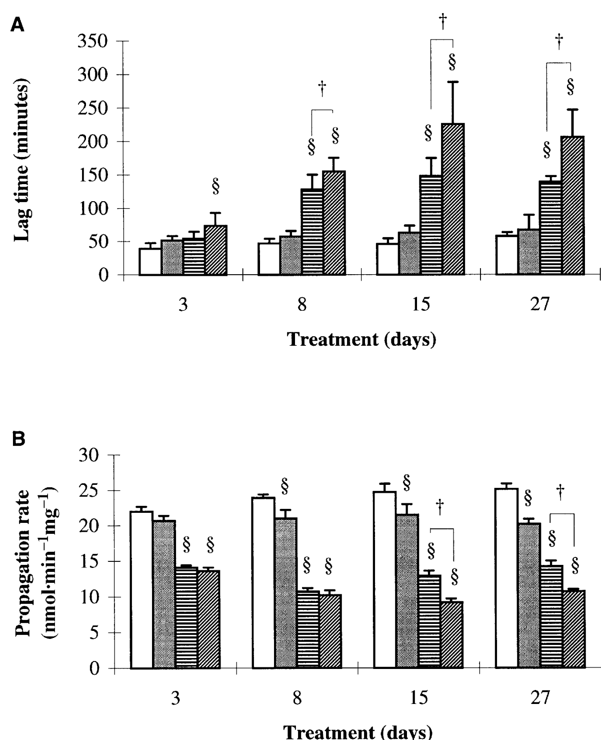

FIG. 1. Bar graphs showing the effect of drug administration on combined very low density lipoprotein (VLDL) and low density lipoprotein (LDL) susceptibility to *in vitro* oxidation. (VLDL + LDL)-containing samples were isolated from rats treated with vehicle (open bars), α-tocopherol (shaded bars), fenofibrate (horizontally striped bars) or fenofibrate + α-tocopherol (diagonally striped bars) for 3, 8, 15, or 27 d, and incubated in phosphate-buffered saline with 5 μM $CuSO_4$ at 37°C. Oxidation profiles were recorded at 234 nm. (A) Lag phase duration (min). (B) Propagation rate (nmol/min/mg proteins). Results are mean ± SD of six values. § Significantly different from control group, $P \leq 0.05$. †, $P \leq 0.05$.

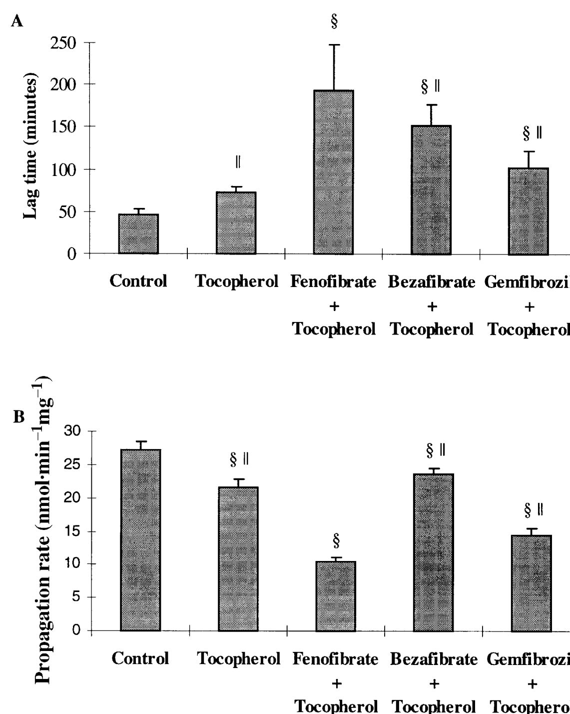

FIG. 2. Bar graphs showing the effect of combined administration of α-tocopherol and fenofibrate, bezafibrate, or gemfibrozil on VLDL + LDL susceptibility to *in vitro* oxidation. (VLDL + LDL)-containing samples were isolated from rats after 15 d of treatment, and incubated in phosphate-buffered saline with 5 μM $CuSO_4$ at 37°C. Oxidation profiles were recorded at 234 nm. (A) Lag phase duration (min). (B) Propagation rate (nmol/min/mg proteins). Results are mean ± SD of six values. §, $P \leq 0.05$ vs. control group. ||, $P \leq 0.05$ vs. fenofibrate + α-tocopherol group. See Figure 1 for other abbreviations.

metabolism. As PPARα activation has the potential to directly and indirectly modify a wide range of metabolic processes, we attempted to determine possible effects of fenofibrate on the various steps of lipoprotein peroxidation. In this initial study, we investigated the utility of combining an antioxidant, vitamin E, with a lipid-lowering drug to prevent lipoprotein peroxidation. We used low, nonsaturating doses of each drug so as to detect possible synergistic effects. We employed the measurement of lipoprotein peroxidation lag time and propagation rate as a surrogate estimate of the antioxidant potential of lipoproteins in the intraluminal space. While the quantification of endogenous antioxidants could give an additional indication of the factors responsible for the observed changes, quantitative modifications in antioxidants alone do not always translate into parallel changes in peroxidative susceptibility of lipoproteins (21,22).

Apolipoprotein B-containing lipoproteins from fenofibrate-treated rats were more resistant to oxidative modification than lipoproteins from control animals. When added *in vitro* to lipoproteins, fenofibrate as well as fenofibric acid, the active metabolite of fenofibrate (23), did not prevent VLDL + LDL oxidation, even at high concentrations (data not shown). Fenofibrate treatment did not alter the total cholesterol, phospholipid, or triglyceride content of the combined fraction of VLDL and LDL. In the experimental conditions described, increases in lag time after α-tocopherol administration to rats did not reach statistical significance. In contrast, the propagation rate of lipid peroxidation was slight but significantly decreased. This latter effect of α-tocopherol was not unexpected, as decreased propagation rate upon vitamin E feeding had already been reported in a number of studies (24–26).

Esterbauer *et al.* (27) observed that the lipid peroxidation process does not enter the propagation phase unless LDL are nearly completely depleted of all their antioxidants, including α-tocopherol. Enrichment of LDL with α-tocopherol, either *in vitro* or through dietary vitamin E supplementation, was shown to delay the onset of lipid peroxidation (26–29). In subjects consuming a normal diet and hence low amounts of α-tocopherol, there is no correlation between resistance of LDL to lipid peroxidation and α-tocopherol content (27,30–32). Thus, a number of factors may alter LDL peroxidation including the ratio of PUFA/α-tocopherol (23), cholesterol content, and other endogenous antioxidants including ubiquinol-10, through

α-tocopherol sparing and/or recycling (34–38). Studies by Tribble *et al.* (39,40) showed that small, dense LDL, which are readily oxidized, exhibit a higher rate of α-tocopherol consumption as compared with large, buoyant LDL, suggesting a lower efficiency of the antioxidant within these particles. An increasingly labile α-tocopherol has been hypothesized to relate to a lower ubiquinol-10 content of the particles, together with an increased PUFA/α-tocopherol ratio, as depletion rates are lower after dietary monounsaturated fatty acid intake. Similarly, experiments in which both dense and buoyant LDL were enriched with vitamin E after dietary supplementation demonstrated a lower efficacy of vitamin E in the dense LDL subfraction (41). Changes in the content of other antioxidants such as ubiquinol-10 cannot be ruled out, while the role of the latter is still a matter of debate due to its low concentration within lipoproteins (18,42). Thus, the quantity of α-tocopherol *per se* may not be a sufficiently accurate parameter to predict either *ex vivo* or *in situ* protection against LDL peroxidation.

The current data demonstrate that fenofibrate affords protection against lipid peroxidation synergistically with α-tocopherol. Simultaneous administration of fenofibrate and α-tocopherol to rats was much more effective in extending the oxidation resistance of lipoproteins than expected from the data obtained for each compound taken separately. The idea of an interaction between these two substances has been suggested by Steinerova *et al.* (43). The explanation for this prolongation of the lag phase could be related to an effect of fenofibrate on the relative amounts of PUFA and monounsaturated fatty acids (44). The synergistic effect observed with fenofibrate plus vitamin E could then be due to a favorable ratio of vitamin E/PUFA whereby the antioxidant defense is bolstered concomitant to a reduction in the free radical-propagating PUFA. In addition to the ability of fenofibrate to modify fatty acid composition, activation of PPARα has recently been shown to be associated with decreased tissue oxidative stress (45) which may be indirectly related to lipoprotein peroxidative stress. The effect of fenofibrate alone and the synergistic effect observed with the addition of vitamin E were more pronounced after 8 d of treatment. The gradual effects could be due to the time necessary for the remodeling of the composition of serum lipoproteins and would reflect the half-lives of the various molecular components.

The combination of fenofibrate and α-tocopherol was significantly more effective than that with bezafibrate, despite comparable hypocholesterolemic activities, suggesting differences between the two fibrates possibly impinging upon other parameters. There are known qualitative as well as quantitative differences between the various members of the fibrate class (4). While fenofibrate and bezafibrate lower fibrinogen in man, gemfibrozil has no effect. Furthermore, fenofibrate alone decreases serum uric acid levels. Although both bezafibrate and fenofibrate lowered serum cholesterol to a comparable degree, the dose employed may have been saturating for both compounds with respect to changes in serum cholesterol, yet less so for bezafibrate with respect to another unknown gene target directly relevant to lipoprotein peroxidation. For example, fenofibrate has a dose-dependent differential effect on apolipoprotein AI and apolipoprotein AII gene expression, although both genes are modified *via* PPARα activation (46).

In conclusion, combined VLDL + LDL from rats treated with fenofibrate were resistant to copper-mediated oxidation. A synergistic effect on the reduction of lipoprotein peroxidation was observed when fenofibrate and vitamin E were coadministered relative to the effect observed with each compound given separately.

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Microemulsion of Seal Oil Markedly Enhances the Transfer of a Hydrophobic Radiopharmaceutical into Acetylated Low Density Lipoprotein

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ABSTRACT: Four different microemulsions differing in their core lipid component (triolein, canola oil, squalene, or seal oil) and containing 1,3-dihydroxypropan-2-one 1,3-diiopanoate (DPIP), a potential radioimaging probe, were prepared by means of ultrasonication. The DPIP microemulsions were incubated with acetylated human low density lipoprotein (AcLDL) and the amount of DPIP transferred into AcLDL was examined. The amount of DPIP in the microemulsions expressed as DPIP/oil (w/w) was dependent on the core lipid component of the microemulsion in the order of seal oil (0.19 ± 0.04, mean ± standard deviation) > squalene (0.15 ± 0.02) > canola oil (0.12 ± 0.02) > triolein (0.07 ± 0.004). With the exception of canola oil, all microemulsions were effective in enhancing the transfer of DPIP into AcLDL in comparison with commonly used methods, i.e., direct diffusion and detergent solubilization. DPIP in seal oil resulted in the highest amount of DPIP transferred into AcLDL [309.16 ± 34.82 vs. 203.19 ± 64.51 using squalene and 151.31 ± 28.54 using triolein (DPIP molecules per AcLDL particle)]. For the first time, oil from harp seals, was studied as a major core lipid component of formulating pharmaceutical microemulsions. DPIP in seal oil resulted in the highest transfer of DPIP into AcLDL which is likely due to the highest DPIP concentration found in this microemulsion as well as the high fluidity of seal oil.

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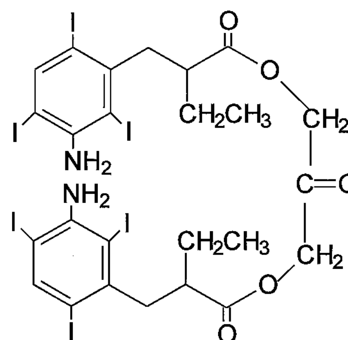
Although the etiology of atherosclerosis is not fully understood, the involvement of monocyte-macrophages in the arterial intima and their subsequent accumulation of modified low density lipoprotein (LDL) has been well elucidated (1). Macrophages express a scavenger receptor for modified LDL such as oxidized LDL and acetylated LDL (AcLDL) (2). This receptor is involved in the formation of macrophage-derived foam cells in human atherosclerotic lesions (3). We therefore proposed to use AcLDL as a carrier to deliver radioimaging probes, such as radiolabeled cholesterol iopanoate (CI), to atherosclerotic lesions for the purpose of early diagnosis, and results have been promising (4). Our group is now investigating a polyiodinated diglyceride analog, 1,3-dihydroxypropan-2-one 1,3-diiopanoate (DPIP, Scheme 1), as another potential radioimaging agent. In order to achieve sufficient accumulation of radioactive DPIP for detection at the sites of atherosclerotic lesions, it is desirable to load a large amount of DPIP into AcLDL. Methods including direct diffusion and detergent solubilization have been reported for loading drug molecules into LDL or AcLDL particles (5–7). In this paper, we report on the transfer of DPIP into AcLDL *via* the preparation of DPIP microemulsions using triolein, canola oil, squalene, and seal oil as the core lipid component. The amount of DPIP transferred from the microemulsions to AcLDL was determined and compared. For the first time, seal oil was used to formulate microemulsions. The harp seal, a marine mammal, lives in an environment that can be extremely cold (−40 to −70°C). In such cold conditions, its circulatory system can still function well and its hydrophobic lipids such as triglycerides, cholesterol and cholesteryl esters remain uncrystallized in the liquid state. Although little research has been done to explain such fascinating phenomena, the unique long chain polyunsaturated fatty acids may contribute to greater fluidity of hydrophobic compounds in harp seal at the extremely low temperatures. In addition seal oil has strong antioxidant property which is useful in formulating stable microemulsions.



SCHEME 1

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Abbreviations: AcLDL, acetylated LDL; CI, cholesterol iopanoate; DPIP, 1,3-dihydroxypropan-2-one 1,3-diiopanoate; EM, electron microscopy; LDL, human low density lipoprotein; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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MATERIALS AND METHODS

Materials. Thin-layer chromatography was carried out on Fisher Scientific silica gel 60, F-254 polyethylene-backed plate and visualized by ultraviolet light. High-performance liquid chromatography analyses were performed using a Beckman system with a Phenomenex Bondclone 10C18, 150 × 3.0 mm column. Radioactivity was measured using a CKB-Wallac 1277 Gammamaster automatic gamma counter. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a Bio-Rad Mini-Protein® II Electrophoresis Cell. A Philips EM 300 was used for electron microscopy (EM) and particles were negatively stained with 1% uranyl acetate. Image analysis was done by Bioquant™ System IV.

Carrier-free aqueous solutions of $Na^{125}I$ were purchased from DuPont NEN Research Products (Boston, MA). Pivalic acid was obtained from Aldrich Chemical Co. Canada. Sephadex® G-25 was purchased from Pharmacia Biotech AB (Uppsala, Sweden). Poly-Prep® chromatography columns were obtained from Bio-Rad Laboratories (Hercules, CA). SDS-PAGE molecular weight standards and Bio-Rad protein assay kits were purchased from Bio-Rad Laboratories (Richmond, CA). L-α-Phosphatidylcholine dipalmitoyl, DL-α-phosphatidylethanolamine dipalmitoyl, and squalene were purchased from Sigma-Aldrich Canada, Oakville, Ontario, Canada. Canola oil was purchased from a local supermarket. Seal oil from harp seal, *Phoca groenlandica*, was obtained from Terra Nova Fishery Ltd., St. John's, Newfoundland, Canada.

Methods. (i) Preparation of DPIP and 125I-DPIP. DPIP was synthesized as reported (8) and radiolabeled with ^{125}I *via* an isotope exchange reaction in a pivalic acid melt (9). The chemical and radiochemical purities of ^{125}I-DPIP were determined by high-performance liquid chromatography and thin-layer chromatography as described before (9).

(ii) Preparation of DPIP microemulsions. DPIP microemulsions were prepared using triolein, canola oil, squalene, and seal oil, respectively, as the core lipid component. L-α-Phosphatidylcholine dipalmitoyl (12 mg), DL-α-phosphatidylethanolamine dipalmitoyl (8 mg), triolein, canola oil, squalene, or seal oil (20 mg), DPIP (10 mg) and ^{125}I-DPIP (0.05 mg) were dissolved in chloroform, dried with a gentle stream of nitrogen, and suspended in 10 mL of saline at 25°C. ^{125}I-DPIP was used as a radioactive tracer for analysis. The suspension was sonicated for 2 h with a Virosonic Cell Disrupter Model 16-850 at 40–50 watts while being cooled in a −10°C salt–ice water bath under a nitrogen stream. The mixture was then centrifuged at 40,000 rpm for 20 h at 4°C using a Beckman SW41 rotor and a Beckman L5-65 ultracentrifuge. The microemulsion that floated to the top of the centrifuge tubes was collected and subjected to EM and image analysis. The amount of DPIP in the individual microemulsions was determined using a CKB-Wallac 1277 Gammamaster automatic gamma counter.

(iii) Preparation of AcLDL. Fresh human plasma was obtained from the Canadian Red Cross Society, St. John's, Newfoundland, Canada. LDL was isolated as previously described (10) by sequential ultracentrifugation of fresh human plasma at 40,000 rpm for 24–40 h at 8°C using a Beckman L8-M ultracentrifuge and a 60Ti rotor. LDL was dialyzed at 4°C overnight against a buffer containing 0.3 mM EDTA, 150 mM NaCl, and 50 mM Tris (pH 7.4). Protein concentrations were determined by the method of Bradford (11). Acetylation of LDL was performed as described by Basu *et al.* (12). The AcLDL was dialyzed and protein concentrations were determined as above.

(iv) Incorporation of DPIP into AcLDL. The ^{125}I-DPIP microemulsions were incubated with AcLDL at 37°C for 24 h at a molar ratio of DPIP to AcLDL of 1000:1. AcLDL loaded with ^{125}I-DPIP (^{125}I-DPIP/AcLDL) was then separated from microemulsions by ultracentrifugation as described (10). The DPIP content in ^{125}I-DPIP/AcLDL was determined by γ-counting.

DPIP was also incorporated into AcLDL using direct diffusion (13) and detergent solubilization (14) with minor modifications. In brief, for the method of direct diffusion, DPIP was dissolved in chloroform in a glass vial and dried under a stream of nitrogen to form a thin film on the wall of the vial. AcLDL was added to the vial and incubated at 37°C for 24 h. For the method of detergent solubilization, DPIP was dissolved in saline in the presence of Tween 20 (<3%) and incubated with AcLDL at 37°C for 24 h. The molar ratio of DPIP to AcLDL used remained the same as above.

(v) Characterization of ^{125}I-DPIP/AcLDL. ^{125}I-DPIP/AcLDL (500 µL), about 1 mg/mL, was loaded onto 1 g of Sephadex® G-25 in a Poly-Prep® chromatography column and eluted with phosphate-buffered saline (pH 7.4). Fractions of 0.5 mL were collected, and radioactivity and protein content of each fraction were measured and plotted.

In order to assess the integrity of the apolipoprotein B-100 of ^{125}I-DPIP/AcLDL, samples were analyzed by SDS-PAGE and EM as described (15).

RESULTS AND DISCUSSION

Our previous studies suggested that AcLDL could be used as a carrier to deliver radiolabeled CI, a cholesterol ester analog, to atherosclerotic lesions in rabbits (4). Compared to CI, DPIP is considered to be a better probe for the detection of atherosclerotic lesions. Through radio-iodination, the six iodine atoms on each DPIP molecule (Scheme 1) provide even higher specific radioactivity than CI. This is desirable for the accumulation of the high radioactivity required for diagnostic purposes. In this report, the loading of DPIP into AcLDL was studied.

Owing to their simplicity, the direct diffusion and detergent solubilization methods have been widely used for loading lipophilic drugs into LDL or AcLDL (16). Microemulsions have also been employed to transfer various drugs into LDL or AcLDL (5–7,17), and triolein has been one of the most commonly used core lipid components. In this study,

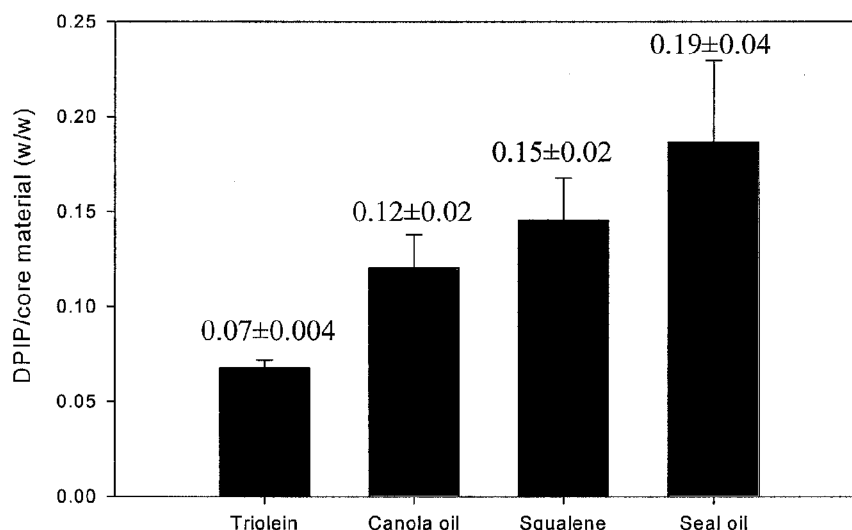

FIG. 1. Comparison of 1,3-dihydroxypropan-2-one 1,3-diiopanoate (DPIP) concentrations in microemulsions using triolein, canola oil, squalene, and seal oil as the core lipid component. The results are expressed as the ratio (mean ± standard deviation) of DPIP to the oil used (w/w).

squalene and seal oil were used as the core lipid components of microemulsions for the first time. Squalene, a highly unsaturated hydrocarbon ($C_{30}H_{50}$), is capable of resupplying the cells with oxygen (18), and is an intermediate product in the synthesis of cholesterol. In recent years, seal oil has been of interest for its potential use in the maintenance of human health (19–22). The n-3 polyunsaturated long chain fatty acids found in seal oil are beneficial in lowering blood pressure (23), reducing triacylglycerols (24), and modulating cell function and cell reactivity to external stimuli (25). In addition, seal oil is very resistant to oxidation, which is desirable for preparing stable microemulsions.

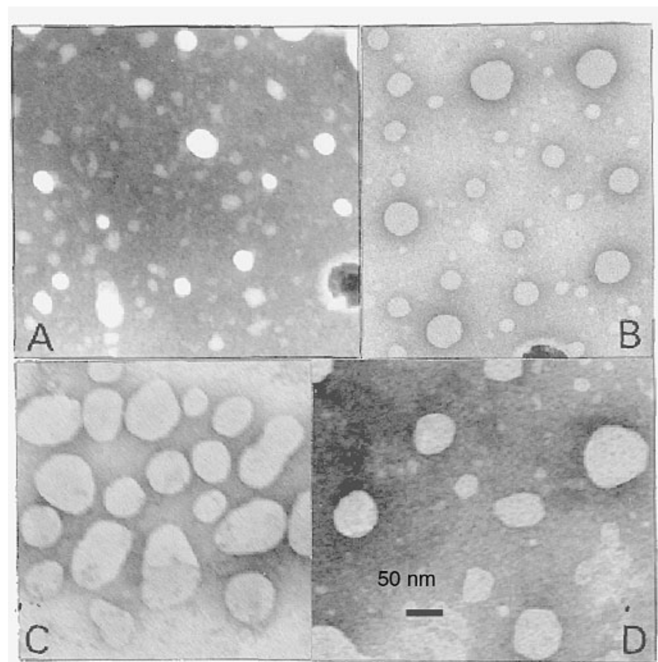

FIG. 2. Electron micrographs of DPIP microemulsions using triolein (A), canola oil (B), squalene (C) and seal oil (D) as the core lipid component. For abbreviation see Figure 1.

Microemulsions were prepared by hydrating a dried film of the lipid mixtures with saline at room temperature, followed by ultrasonication, during which the aqueous dispersions were cooled in a salt–ice bath at −10°C to prevent overheating due to the sonication. Preparation of the microemulsions at a temperature below the gel-liquid crystalline phase transition temperature of the phospholipids would possibly result in limited hydration of the phospholipids. However, electron micrographs show structures that are typical of those observed by Redgrave and Maranhao (26) in preparations of microemulsions formed by sonication at temperatures above the phospholipid transition temperatures. Furthermore, those authors reported microemulsions that were 50–200 nm in diameter. This compares well with our preparations, which had diameters of 30–90 nm. The slightly larger diameters seen by Redgrave and Maranhao could be due to the use of a phosphatidylcholine species containing unsaturated fatty acids that increases the molecular area of the phospholipid compared with the fully saturated phosphatidylcholines in our preparations.

The concentrations of DPIP in microemulsions with triolein, canola oil, squalene, and seal oil as the core lipid component were compared. The amount of DPIP eventually loaded into the microemulsions, expressed as DPIP/oil (w/w), was in the order of seal oil (0.19 ± 0.04, mean ± standard deviation) > squalene (0.15 ± 0.02) > canola oil (0.12 ± 0.02) > triolein (0.07 ± 0.004) as shown in Figure 1. EM (Fig. 2) and image analysis (Fig. 3) results showed that the particle size of the microemulsions is in the same order of seal oil [longest diameter (LD) = 67.8 ± 25.37 nm] > squalene (LD = 58.2 ± 22.01 nm) > canola oil (LD = 52.9 ± 16.05 nm) > triolein (LD = 40.6 ± 13.5 nm).

Upon incubation of the DPIP microemulsions with

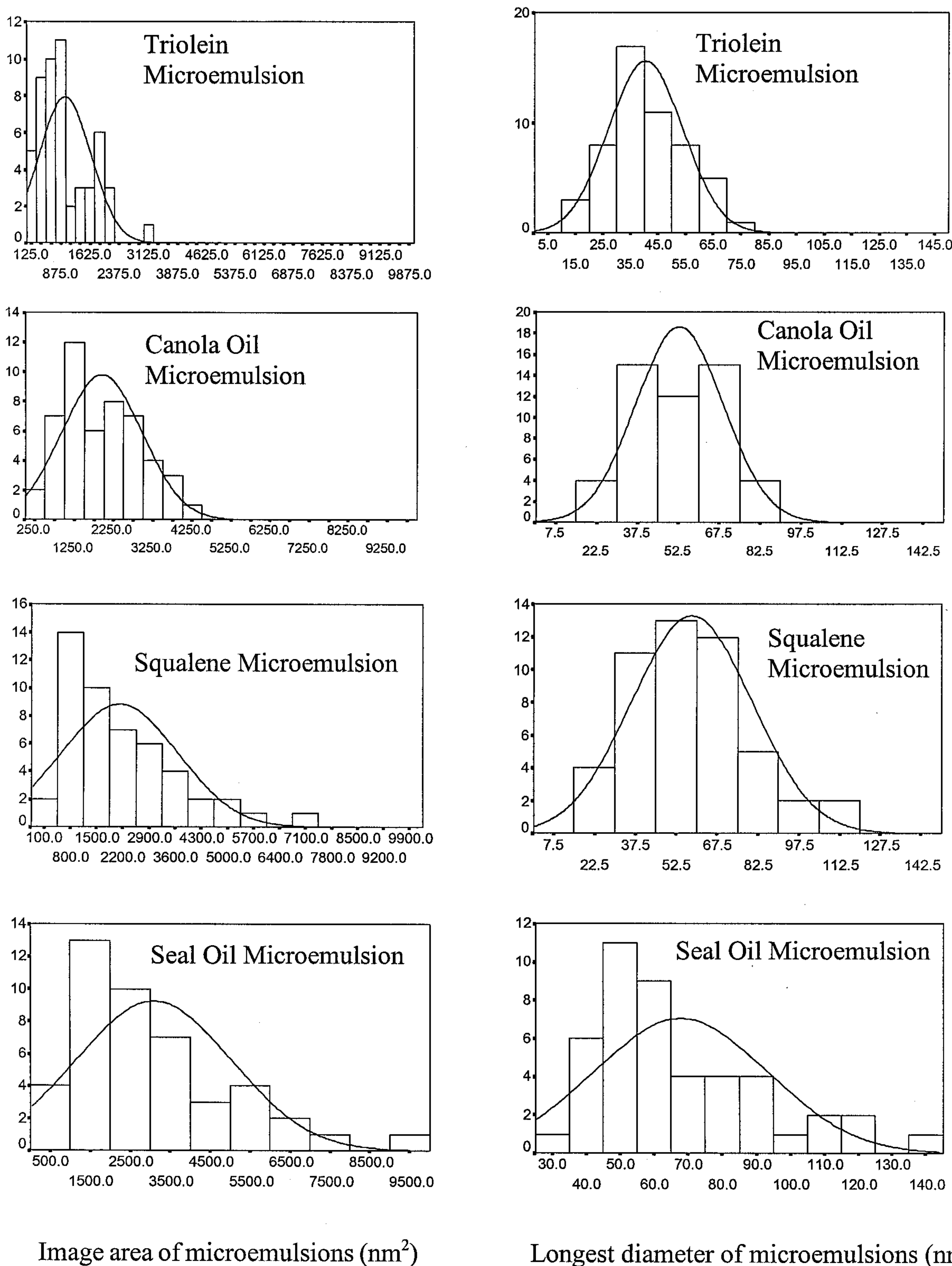

FIG. 3. Image analysis of DPIP microemulsions using triolein, canola oil, squalene, and seal oil as the core lipid component. The left four panels are the distributions of image areas of four microemulsions (nm^2) and the right four panels are the distributions of longest diameters of the four microemulsions (nm). The *y* axes of Figure 3 represent the frequency of the number of tested samples located in the correspondent scale. The curve was fitted by Gaussian normal distribution. For abbreviation see Figure 1.

AcLDL, DPIP was successfully transferred into AcLDL with the exception of the canola oil microemulsion, in which AcLDL was found to be aggregated. DPIP/AcLDL preparations were subjected to gel filtration. The chromatograms of radioactivity and protein contents were plotted. As shown in Figure 4, radioactivity (due to ^{125}I-DPIP) and protein content

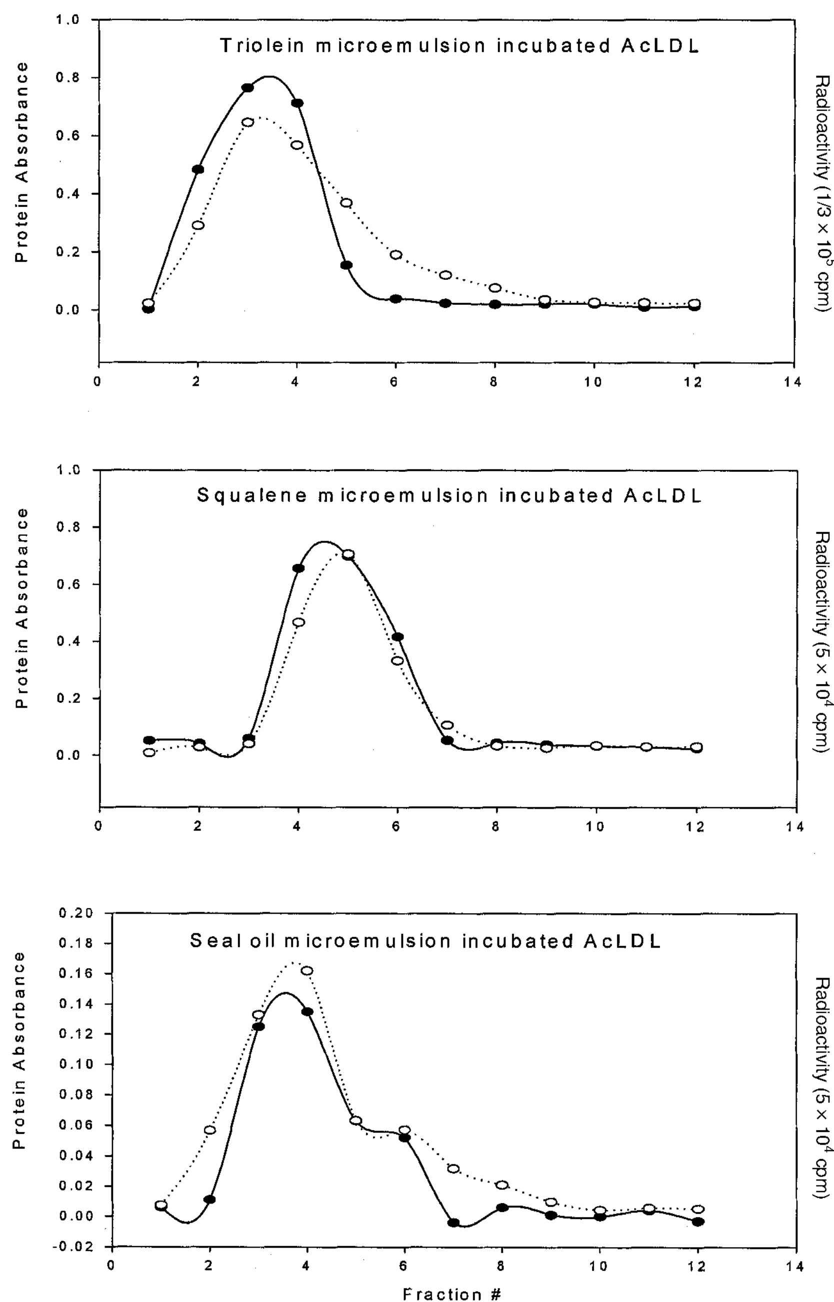

FIG. 4. Confirmation of ^{125}I-DPIP incorporation into acetylated low density lipoprotein (AcLDL) particles by using three different lipid microemulsions. ^{125}I-DPIP/AcLDL conjugates were analyzed by gel filtration chromatography through Sephadex G-25 with phosphate-buffered saline. The radioactivity and protein content of each fraction were determined as described in the Materials and Methods section. For other abbreviations see Figure 1.

were superimposed, suggesting that DPIP was transferred to AcLDL (or DPIP/AcLDL formed). SDS-PAGE results of DPIP/AcLDL indicated that the integrity of apolipoprotein B-100 was intact in comparison with native LDL and AcLDL (data not shown). The poor results obtained with canola oil are possibly due to the presence of preservatives or other additives in the commercial canola oil.

The transfer efficiency of DPIP from the different microemulsions to AcLDL was also compared with direct diffusion and detergent solubilization. As shown in Figure 5, the amount of DPIP transferred into AcLDL through the different DPIP microemulsions was higher than that through direct diffusion (27.70 ± 1.99 DPIP molecules/per AcLDL) and solubilization (66.97 ± 14.81 DPIP molecules/per AcLDL). Among the microemulsions studied, the seal oil microemulsions resulted in the highest amount of DPIP transferred into AcLDL (309.16 ± 34.82 vs. 151.31 ± 28.54 using triolein and 203.19 ± 64.51 using squalene expressed as DPIP mole-

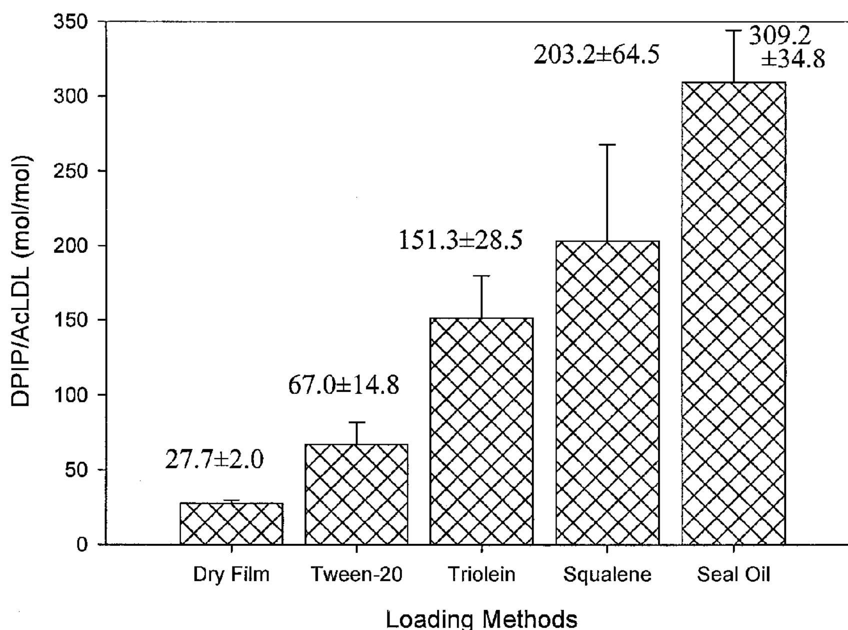

FIG. 5. Comparison of the DPIP loading efficiency into AcLDL using different methods. Results are expressed as DPIP/AcLDL. For abbreviations see Figures 1 and 4.

cules/per AcLDL). The similar trend found in Figures 1 and 5 suggests that the concentration of DPIP found in the microemulsion is the decisive factor for the transfer of the drug into AcLDL.

Possible explanation for these observations may lie in the differences in the physical behavior of the different core lipids. Seal oil contains high levels of highly unsaturated triglycerides. These may allow for solvation of more hydrophobic DPIP than triolein and may also, because of increased fluidity, allow for more rapid transfer of DPIP to the lipoprotein particles.

In summary, we prepared different DPIP microemulsions, using seal oil and squalene for the first time. All DPIP microemulsions resulted in a higher DPIP incorporation into AcLDL than the commonly used methods. More interestingly, DPIP microemulsions using seal oil and squalene loaded more DPIP into AcLDL than that using triolein, which is likely the result of the higher DPIP concentrations found in these microemulsions. Therefore, seal oil and squalene may be appropriate substitutes for triolein in manufacturing microemulsions. Obviously, significant studies, such as safety and stability of those marine mammal oils, are required.

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Cellular Uptake and Retention Measurements of Alkylphosphocholines in the SK-BR-3 Breast Cancer and Molt-4 Leukemia Cell Line Using Capillary Gas Chromatography

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ABSTRACT: The determination of cellular content of octadecylphosphocholine (D-19391) and hexadecylphosphocholine (HePC, D-18506), two anticancer agents of the alkylphosphocholine group, using capillary gas chromatography is described. The compounds' cytotoxicity was first determined by the MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium] assay, being indicative for the concentration used in the uptake and retention measurements. D-19391 was added to the SK-BR-3 breast cancer cell line and HePC to the Molt-4 leukemia cell line in concentrations of, respectively, 18.6 and 15.0 μM, during a 36-h incubation period at 37°C, 5% CO_2. HePC uptake in the leukemia cells was followed by a 24-h reversibility test in drug-free medium. Subsequently, sample clean-up was performed on a weak cation-exchange column. For the quantitative analysis, HePC was used as internal standard for the D-19391 measurements and vice versa. Derivatization of the samples with trimethylsilylbromide was followed by capillary gas chromatographic analysis. From these data we conclude that our uptake results are quite similar with those of a previous study of HePC cellular uptake in the more resistant Caco-2T colon cancer cell line. Without having investigated the mechanism that underlies the cellular uptake results obtained, our study points to no direct correlation between the compounds' cellular uptake and their cytotoxic effects.

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Hexadecylphosphocholine (HePC, D-18506) and octadecylphosphocholine (D-19391) are representatives of the alkylphosphocholine (APC) class, a new group of antitumor agents with, as far as we know, no mutagenic, carcinogenic or immunosuppressive properties like common chemotherapeutics (1). Clinical studies of HePC proved its efficacy for the treatment of skin metastases in patients with breast cancer (2). Other APC and alkyllysophospholipids (ALP) are entering clinical trials. Although promising results were obtained in laboratories and in clinical studies, the mechanism of their antineoplastic and selective action remains unknown (3–5). Based on their lipid-soluble structure, it is generally accepted that the main target of APC/ALP appears to be the plasma membrane: they incorporate into cellular membranes, influencing membrane fluidity (6), inhibiting normal phospholipid metabolism (7) and affecting several membrane proteins including protein kinase C (8) and phospholipase C (9). Moreover, these agents are selectively cytotoxic and anti-invasive (10), they affect the cell cycle progression in tumor cells (11), and they promote apoptosis (12). These findings indicate that a combination of all these molecular events may contribute to the explanation of the antineoplastic activity of these agents on tumor cells. In our laboratory a capillary gas chromatography (cGC) method was established (13) and further optimized (14) to determine APC and ALP in cell cultures and cell culture media. Based on this cGC procedure, the HePC uptake and retention in the human colon cancer cell line Caco-2T (15) and in the human breast cancer cell line SK-BR-3 (14) have already been investigated. Our aim was to analyze the uptake of D-19391 as a HePC structural analog in the same SK-BR-3 cells and to extend this study to the HePC determination in the suspension leukemia cell line Molt-4. Since similar uptake results were obtained for both compounds on the breast cancer cell line, and since these cellular measurements are quite time-consuming, HePC was chosen as representative compound of the APC class to measure the cellular uptake and retention in the Molt-4 suspension cells. By determining the uptake and retention of these compounds in an adherent and a suspension cell line, insight will be gained in the relation between the compound's cytotoxicity and the cellular uptake and retention in these two different types of cell lines. Since the cellular uptake is the initial step finally leading to the compound's antitumor action, this study can contribute to the elucidation of the mechanism of these antineoplastic agents.

MATERIALS AND METHODS

Reagents. HePC (D-18506) and D-19391 (clinical grade) were kindly provided by Dr. Th. Klenner from Asta Medica (Frankfurt am Main, Germany). Trimethylsilylbromide and

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Abbreviations: ALP, alkyllysophospholipid; APC, alkylphosphocholine; CBA, carboxylic acid; cGC, capillary gas chromatography; D-19391, octadecylphosphocholine; FBS, fetal bovine serum; HePC, hexadecylphosphocholine (D-18506); ID_{50}, 50% inhibitory concentration; MTT, 3- (4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium; OD, optical density.

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the MTT reagent [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium] were obtained from Acros Chimica (Kortrijk, Belgium). Bond Elut columns containing a weak cation-exchange sorbent of the carboxylic acid (CBA) type were provided by Varian Belgium N.V./S.A. (Brussels, Belgium). Trypan Blue solution (0.4%) was supplied by Sigma Biosciences (Antwerp, Belgium). *n*-Hexane for spectroscopy was obtained by Merck-Belgolabo (Brussels, Belgium). Other products and solvents were of analytical grade and used without further purification.

Cell culture. The SK-BR-3 human breast cancer cell line was maintained in minimum essential medium supplemented with 11% heat-inactivated fetal bovine serum, 65 IU/ml penicillin/streptomycin solution, and 2 mM L-glutamine. The Molt-4 T-lymphoma cells were cultured in RPMI 1640 supplemented with 10% heat-inactivated fetal bovine serum and 2 mM L-glutamine. Both cell lines were maintained in plastic culture flasks in a humidified incubator at 37°C, 5% CO_2. For subculturing or experiments, the adherent cells were detached from the culture flask by the use of trypsin/EDTA solution, after washing the cells with Hanks' Balanced Salt Solution. Suspension cultures were centrifuged and resuspended in fresh medium. Cell number and cell viability were determined using a cell counting chamber by trypan blue exclusion. All reagents and materials for cell culture were provided by Life Technologies (Paisley, United Kingdom).

Solid-phase extraction. Sample clean-up was performed according to the solid-phase extraction procedure described by Coene *et al.* (13), with minimal alterations (14). Bond Elut columns of the CBA type, containing 500 mg sorbent, were initially conditioned with 2 mL of 100% methanol, 2 mL of distilled water, and 2 mL of 0.1 M phosphate buffer (pH 5.2). Each standard together with the internal standard was buffered with 5 mL 0.1 M phosphate buffer to obtain optimal retention of the APC on the CBA column. For quantitation of the D-19391 uptake measurements in the SK-BR-3 cell line, a standard curve was obtained with standard concentrations ranging from 2.3 to 11.5 µM and 12.3 µM HePC as internal standard. In the same way, standards with a concentration of 1.2 to 12.3 µM HePC, combined with 9.2 µM D-19391 as internal standard, allowed us to determine HePC uptake and retention in the Molt-4 suspension cell line. The buffered standard solution was applied to the column and subsequently washed with 2 mL 0.01 M HCl/0.01 M KCl in water (pH 2.0) and 2 mL 30% methanol in water. These washings were discarded. Finally the APC were eluted with 2 mL 100% methanol and transferred into a 250-mL round-bottomed flask. After evaporation of the solution under a gentle stream of nitrogen, the residue was dissolved in 2 mL dry acetonitrile followed by 5 min sonication making the sample ready for derivatization. Therefore, the flasks were equipped with a condenser and a $CaCl_2$ drying tube after adding 100 µL trimethylsilylbromide. The samples were derivatized at 98°C for 1 h in the absence of light. After derivatization, the samples were transferred into tubes and evaporated under nitrogen. The residue was dissolved in 1 mL *n*-hexane and sonicated for 10 min. To remove impurities, another sample clean-up step was performed by filtration of the obtained solution, followed by evaporation under nitrogen. By dissolving the residue in 100 µL *n*-hexane and sonicating for 10 min, the sample was made ready for cGC injection.

Instrumentation. After derivatization with trimethylsilylbromide, the samples were injected into a Hewlett-Packard (HP) series II gas chromatograph, connected with an HP A/D converter interface to an HP Chemstation. The column was a cross-linked methyl silicone gum Ultra (25 m × 0.32 mm i.d.; film thickness 0.17 µm). Cold on-column injections of 1 µL were done manually in the oven-track mode. The oven temperature was held at 50°C for 1 min following injection, and programmed at 20°C/min to 200°C and then at 10°C/min to 300°C. As carrier gas helium was used (3.0 mL/min), and nitrogen and air were used as make-up gas for flame-ionization detection at 250°C (13). For the performance of the MTT assay, a V_{max} microtiter plate reader (Bio-Tek Instruments, Winooski, VT) and a CO_2 incubator (Precision Scientific, Chicago, IL) at 37°C, 5% CO_2 were used.

MTT cell viability assay. The D-19391 cytotoxic effect on the adherent SK-BR-3 cell line was evaluated by the MTT assay according to Romijn *et al.* (16) and is based on the enzymatic reduction of the tetrazolium salt into formazan crystals. Since dead cells are unable to reduce the tetrazolium salt, this assay allows the discrimination between metabolically active cells and dead cells. By measuring the optical density (OD) of the formazan crystals produced, this colorimetric assay is very useful for quantitating the cytotoxicity induced by a compound. Therefore, 100 µL of a cell suspension with a cell density of approximately 62×10^3 cells/mL was transferred into the wells of rows 3 to 11 of the microplate, sealed with Micropore tape (3M, St. Paul, MN) and incubated for 4 h at 37°C, 5% CO_2. Subsequently, 200 µL SK-BR-3 medium without compound was transferred to the wells in row 2 (blank), 100 µL medium to the wells in row 3 (control), and 100 µL of increasing concentrations of D-19391 prepared in cell medium to the wells in rows 4 to 11. The plate was sealed with Micropore tape and now incubated for 4 d in a humidified incubator at 37°C, 5% CO_2. After this incubation period, 100 µL medium of every well was replaced by 100 µL of a 1 mg/mL MTT solution in phosphate-buffered saline (pH 7.4), followed by a 4-h incubation in the absence of light at 37°C, 5% CO_2. During this reaction the mitochondrial enzymes in viable cells reduce the tetrazolium salt into formazan crystals. After having removed most of the medium in each well, 200 µL dimethylsulfoxide was added following a 1-h incubation at 37°C. After solubilization of the formazan crystals in dimethylsulfoxide and homogenization of each well, the amount of formazan produced is quantified by measuring the OD of the wells with a V_{max} microtiter plate reader at 540 nm.

To examine Molt-4 cell viability after incubating the cells with increasing HePC standard concentrations, the MTT test was performed as just described, with some adjustment. The cell density of the cell suspension used was generally 4.4×10^5 cells/mL. After 4 d of incubation of the Molt-4 cells with

increasing concentrations of HePC, 20 µL of a 5 mg/mL MTT solution prepared in phosphate-buffered saline (pH 7.4) was added to each well. The plate was sealed with Micropore tape and incubated under the same conditions as already described. Formazan crystals were dissolved in 100 µL acidified isopropyl alcohol (0.04 N HCl in isopropyl alcohol). After homogenization the concentration of the colored crystals in each well, proportional to the number of viable cells, was measured at 490 nm.

Each cell viability assay was performed in duplicate. For both cell lines, the OD of rows 3 to 11 were diminished by the mean OD value of row 2 (blank) and these were expressed as percentages of the mean control OD value, in reporting cell survival as function of increasing drug concentrations.

Uptake and retention. After subculturing the SK-BR-3 cells at different cell densities in 25-cm^2 culture flasks and 1-d growth, the medium was removed and 4.0 mL of the prepared D-19391 solution was added to the cell culture followed by a 36-h incubation at 37°C, 5% CO_2. Therefore, the compound was dissolved in SK-BR-3 medium at a concentration of 18.6 µM (approximately 75% of the compound's ID_{50} value derived from the MTT results, where ID_{50} represents 50% inhibitory concentration). At the end of this incubation period, the medium was removed and retained for analysis. The SK-BR-3 cells were trypsinized during 6 min at 37°C after washing the cell culture with 5 mL 0.1 M phosphate buffer pH 7.4. The cells were then collected in a volumetric flask and brought to a constant volume of 3.0 mL with cell medium. The cell density of this cell suspension was determined using a cell counting chamber by trypan blue exclusion. Because of the similar results obtained for the D-19391 and HePC uptake in the SK-BR-3 cells, the study of the D-19391 cellular retention in this adherent breast cancer cell line was not further investigated. For the uptake and retention measurements of HePC in the Molt-4 cell line, a full-grown cell culture was centrifuged at 360 × *g* for 8 min at 25°C. The supernatant was removed and the cell pellet resuspended in fresh Molt-4 medium, followed by cell density determination. The cell density could eventually be corrected at this point. Of the cell suspension obtained, 1.0 mL was transferred to a 25-cm^2 cell culture flask containing 5.0 mL HePC solution prepared in cell medium at a concentration of 17.9 µM, whereby a compound concentration of 15.0 µM was reached. HePC uptake was determined after 36 h of incubation. The cell suspension culture was first centrifuged at 1250 × *g* for 6 min at 25°C. The medium was then removed and retained for analysis. The cell pellet was resuspended in 3.0 mL fresh Molt-4 medium, and the cell density of this cell suspension was determined, followed by cGC analysis of HePC cellular uptake. For the retention measurements, the cell pellet was resuspended in 6.0 mL fresh sterile Molt-4 medium and transferred to a cell culture flask, followed by another 24-h incubation at 37°C, 5% CO_2. At the end of this incubation, the cell suspension was centrifuged at 1250 × *g* for 6 min at 25°C and further handled as described for the uptake measurements of HePC in the Molt-4 cell line.

For the analysis of the obtained cell suspensions and cell culture media, 1.0 mL of the samples was buffered with 5 mL 0.1 M phosphate buffer (pH 5.2) and sonicated for 6 min to obtain cell membrane disruption. Sample clean-up was performed by solid-phase extraction, followed by derivatization and 1 µL sample injection in the cGC as described in the instrumentation paragraph above. The use of an internal standard made quantitative determination of the compound in cells and cell culture media possible. Data collection was performed with the HP Chemstation, whereby the ratios of the areas of the examined compound vs. the internal standard were calculated to obtain the quantitative amount of compound.

As control, culture flasks without cells (blank) were subjected to the same conditions as described for the cell cultures and subjected to the same analysis as well as data collection.

RESULTS

Cytotoxic effect of D-19391 on the SK-BR-3 and of HePC on the Molt-4 cell line. The effect of D-19391 cytotoxicity on the SK-BR-3 cell viability was examined by the MTT assay, performed in duplicate as described in the Materials and Methods section. The SK-BR-3 cell line was incubated with increasing concentrations of compound ranging from 5 to 100 µg/mL (11.5–230.0 µM) for 4 d in the microtiter plate. At the end of this time a dose-dependent decrease in cell survival was observed for the SK-BR-3 cell line as shown in Figure 1. The mean concentration of D-19391 that is responsible for a 50% decrease in cell viability (ID_{50} value) was 10.8 ± 0.1 µg/mL. This indicates the high sensitivity of the adherent breast cancer cell line for D-19391 cytotoxicity.

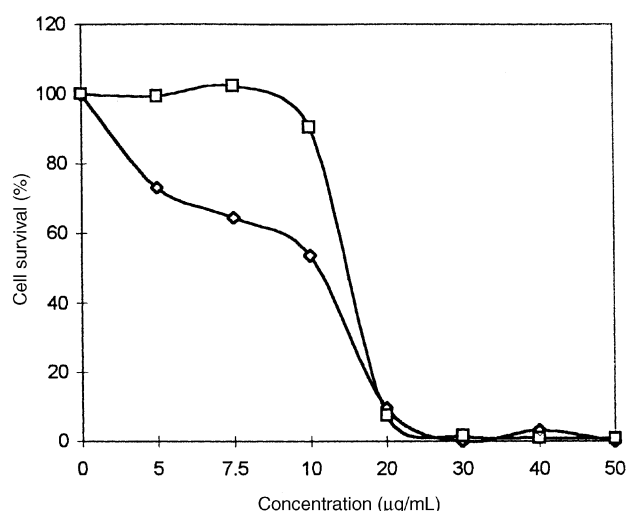

FIG. 1. Cell viability (MTT) test of D-19391 on the SK-BR-3 (◇) and of HePC on the Molt-4 (□) cell line. The test conditions of the MTT assay are given in the Materials and Methods section. The results of one representative assay, with concentrations ranging from 0 to 50 µg/mL alkyllysophospholipid, are shown. Abbreviations: MTT, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-tetrazolium; D-19391, octadecylphosphocholine; HePC, hexadecylphosphocholine.

TABLE 1
Uptake Measurements of D-19391 in SK-BR-3 Cell Cultures and Cell Culture Media[a]

Sample (n = 3)	Cell density (10^3 cells/mL)	D-19391 in the cells (%)	D-19391 in media (%)	D-19391 recovered (%)
1	6.25	10.1 ± 0.5	76.1 ± 8.3	86.2 ± 8.3
2	6.94	11.5 ± 0.2	81.5 ± 2.8	93.0 ± 2.8
3	10.76	30.1 ± 0.04	82.5 ± 5.2	112.6 ± 5.2
4	13.89	19.5 ± 2.4	90.1 ± 3.3	109.6 ± 4.1
5	14.24	18.4 ± 0.3	89.9 ± 11.4	108.4 ± 11.4
6	15.63	17.9 ± 8.1	73.9 ± 5.7	91.8 ± 9.9
7	21.53	19.0 ± 2.5	81.1 ± 10.8	100.1 ± 11.1
8	29.86	17.1 ± 2.8	67.5 ± 1.8	84.6 ± 3.3

[a]The D-19391 amount obtained in the cell cultures and cell culture media is calculated in relation to the recovered drug amount in the blanks. Data represent the mean ± SD. Abbreviation: D-19391, octadecylphosphocholine.

The MTT cell viability assay on the Molt-4 cell line was performed with a HePC concentration range of 5 to 60 µg/mL (12.3–147.2 µM). A dose-dependent decrease in cell survival was observed, resulting in a mean ID_{50} value of 13.2 ± 2.5 µg/mL (Fig. 1). Uptake and retention measurements were performed with compound concentrations below the obtained ID_{50} values.

Uptake of D-19391 in the SK-BR-3 cell line. These antitumor ether lipids possess a high affinity for serum albumin, present in the cell culture medium. Knowing that the cell plasma membrane is the main localization and action site of these agents, one can expect a possible back-extraction of the compound from the plasma membrane into the cell culture medium by serum albumin (17), accompanied by a lowered compound cytotoxicity (18). Therefore, a time-dependent uptake study has been performed for HePC on the SK-BR-3 cell line (14). Based on this previous experiment, a maximal drug uptake in the cells was reached after 36 h of incubation. After incubating the SK-BR-3 cells with D-19391 in a concentration of 18.6 µM, the cells and cell culture media were collected and analyzed as described in the Materials and Methods section. Table 1 shows the results obtained for the D-19391 cellular uptake and the remaining concentration in the cell medium at different cell densities. For the quantitative determination of D-19391 in cells and cell culture media, a standard curve ranging from 2.3 to 11.5 µM with 12.3 µM HePC as internal standard was used. A correlation coefficient of 0.991 was obtained. The average recovered D-19391 percentage in the cells and cell culture media shown in Table 1 is 98.3 ± 11.0% (n = 8) taking into account the values obtained for the recovery in blank experiments. D-19391 cellular uptake was performed at lower cell densities, ranging from 6 to 30 × 10^3 cells/mL of this adherent growing breast cancer cell line, to avoid cell release from a full-grown culture flask during the investigation. The results of a 36-h D-19391 uptake into the SK-BR-3 cells point to a mean cellular uptake of 18.0 ± 6.0% at the tested cell densities.

Uptake and retention of HePC in the Molt-4 cell line. Molt-4 cells were incubated with 15.0 µM HePC during 36 h, eventually followed by a 24-h recovery in drug-free medium. Subsequently the cells and cell culture media were collected and analyzed as described in the Materials and Methods section. In Table 2 the results of the HePC cellular uptake are shown. A standard curve in a concentration range from 1.2 to 12.3 µM HePC in Molt-4 medium with 9.2 µM D-19391 as internal standard was used to determine the compound's cel-

TABLE 2
Uptake Measurements of HePC in Molt-4 Cell Cultures and Cell Culture Media[a]

Sample (n = 3)	Cell density (10^5 cells/mL)	HePC in the cells (%)	HePC in media (%)	HePC recovered (%)
1	1.01	23.0 ± 0.9	67.0 ± 18.2	90.0 ± 18.2
2	2.68	20.2 ± 3.3	69.2 ± 20.0	89.4 ± 20.3
3	2.88	13.9 ± 4.8	99.2 ± 14.4	113.1 ± 15.2
4	3.36	20.7 ± 1.1	54.7 ± 5.6	75.4 ± 5.7
5	4.74	18.1 ± 4.5	91.9 ± 9.4	110.0 ± 10.4
6	5.42	28.1 ± 1.1	97.8 ± 20.9	125.9 ± 20.9
7	5.60	15.9 ± 6.8	78.2 ± 8.9	94.1 ± 11.2
8	6.00	27.8 ± 6.6	97.7 ± 5.3	125.5 ± 8.5
9	9.60	20.3 ± 0.5	81.3 ± 13.6	101.6 ± 13.6
10	10.90	16.6 ± 0.6	71.9 ± 7.9	88.5 ± 8.0
11	11.50	25.0 ± 1.4	74.8 ± 24.0	99.8 ± 24.0
12	11.70	17.3 ± 3.1	76.3 ± 13.3	93.6 ± 13.7

[a]The HePC amount obtained in the cell cultures and cell culture media is calculated in relation to the recovered drug amount in the blanks. Data represent the mean ± SD. Abbreviation: HePC, hexadecylphosphocholine.

lular uptake and retention. A correlation coefficient of 0.998 was obtained. The amount of HePC that was recovered from cell cultures and cell culture media after 36-h incubation was 100.6 ± 15.4% (n = 12) taking into account the values obtained for the recovery in blank experiments. The HePC cellular uptake and retention were performed at different cell densities. The uptake of HePC at varying Molt-4 cell densities amounts to an average of 20.6 ± 4.6% (n = 12). The retention of HePC in Molt-4 cells, after an incubation of 24 h in drug-free medium amounts to 12.8 ± 3.3% (n = 11).

DISCUSSION

By the use of the described cGC method, it was possible after sample clean-up by solid-phase extraction and derivatization to determine the amount of D-19391 and HePC in two cancer cell lines. The cell survival assay proves that the breast cancer cell line SK-BR-3 has a comparable sensitivity to HePC [ID_{50} value of 8.9 ± 2.1 µg/mL (14)] and to D-19391 (ID_{50} value of 10.8 ± 0.1 µg/mL). This is not so uncommon since HePC and D-19391 are structural analogs, differing in alkyl chain length by only two carbon atoms. Also the leukemia cell line Molt-4 is rather sensitive for the HePC action, unlike the human colon cancer cell line Caco-2T [(ID_{50} values of 13.2 ± 2.5 µg/mL and 70.0 ± 3.2 µg/mL, respectively (15)].

Previous experiments performed in our laboratory show that an average of 20% HePC is taken up by the Caco-2T cells after a HePC treatment of 48 h in a concentration of 98.1 µM. After a 48-h recovery period in drug-free medium, 7% is still present in the cells (15). To compensate for the difference in cell counts of each cell culture, the amount of HePC taken up by the cells was expressed per milligram protein. We preferred to determine the cell density to compensate for these cell culture variations. In this way a maximal D-19391 uptake of approximately 18.0 ± 6.0% was reached after a 36-h incubation period in the adherent growing SK-BR-3 cell line. Since the measurements of uptake performed with HePC (14) and D-19391, as structural analogs, in the SK-BR-3 cell line give comparable results we may expect similar retention measurements as well for both agents. HePC still remains present in the breast cancer cells from 6.8 to 9.5% after incubation for 24 h in drug-free medium (14). The uptake of HePC in Molt-4 cells growing in suspension is 20.6 ± 4.6% (n = 12) and the retention after incubation for 24 h in drug-free medium is 12.8 ± 3.3% (n = 11).

Compound cellular uptake is the initial step in a series of events finally leading to cell death. The main target site of these antitumor lipids appears to be the plasma membrane, where they accumulate principally in the phospholipid bilayer. Owing to the lipophilicity of ET-18-OMe (1-octadecyl-2-methyl-*rac*-glycero-3-phosphocholine), a representative of this antitumor class, and its main localization in the cell plasma membrane, Kelley *et al.* (19) propose passive diffusion as the possible uptake mechanism of this ether lipid. Knowing that temperature can influence the cell membrane fluidity, one can adjust this parameter to obtain optimal compound uptake. Bazill and Dexter (20), on the other hand, suggest that endocytotic uptake of antitumor ether lipids is the beginning step in their antitumor activity. Agents interfering with endocytosis inhibit uptake of ether lipids and analogs into cells. The effects of endocytotic as well as metabolic inhibitors, and the use of inhibitors of ATP generation, could give more insight in the mechanism responsible for the results obtained in this study.

We cannot completely compare our results with those obtained for the Caco-2T cell line because of the difference in incubation conditions and the differences in expression of the results. Still, even in a resistant cell line like Caco-2T there is a high HePC uptake that is not very different from the uptake percentage of the compound in the sensitive SK-BR-3 cell line. The difference in cell line sensitivity does not really point to cellular uptake differences. Fleer *et al.* (17) came to the conclusion that both sensitive and resistant cell lines take up HePC in similar amounts, taking the cellular lipid content into account. Other parameters besides uptake must explain the differences in the sensitivity of the cell lines. We are now in the process of investigating apoptosis of these different cell lines under the influence of APC and ALP in order to get more insight in the selective mechanism of action of these antitumor agents.

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Steryl Esters in the Elaioplasts of the Tapetum in Developing *Brassica* Anthers and Their Recovery on the Pollen Surface

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ABSTRACT: The tapetum cells in the developing anthers of *Brassica napus* contained abundant elaioplasts, which had few thylakoid membranes but were packed with globuli of neutral esters. Of the neutral esters, the major ester group possessed mainly 24-methylenecholesterol, 31-norcycloartenol, 24-dehydropollinastanol, and pollinastanol esterified to 18:3 and other unsaturated and saturated fatty-acyl moieties. The minor ester group had a dominant component tentatively identified as 12-dehydrolupeol esterified to mostly 18:0, 16:0, and 20:0 fatty-acyl moieties. The elaioplasts also contained a high proportion (16% w/w of total lipids) of monogalactosyldiacylglycerols (MGDG). This is the first report of plastids having steryl esters as the predominant lipids. We propose that the globuli contain steryl esters and are stabilized by surface MGDG and structural proteins. The tapetosomes, the other abundant lipid-containing organelles in the tapetum, possessed triacylglycerols (TAG) as the predominant lipids. At a late stage of anther development, the minor group of neutral esters and MGDG of the elaioplasts, as well as the TAG of the tapetosomes, were degraded. Steryl esters similar to those of the elaioplasts were recovered from the pollen surface and were the major lipids of the pollen coat. The pollen coat steryl esters and proteins could be extracted with moderately polar or nonpolar solvents. These proteins, which were mostly fragments of oleosins derived from the tapetosomes, had a high proportion of lysine (13 mol %). The possible functions of the steryl esters and the proteins on the pollen surface are discussed.

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Pollen is produced in the locules (pollen sacs) in the anthers of flowers (1,2). The maturing pollen grains interact with the tapetum, which consists of one layer of metabolically active cells enclosing the locule. The tapetum serves several important roles (2). Throughout the maturation process, the tapetum regulates the flow of nutrients and secretes enzymes to the locule for pollen maturation. At a late stage of anther development, the tapetum cells lyse and discharge molecules onto the surface of the pollen, forming the pollen coat. The pollen coat consists of lipids, proteins, and small molecules such as sugars. The pollen coat may play diverse physiological roles, such as protecting the pollen from solar radiation, giving the pollen color, preventing water loss from the pollen, attracting insect pollinators, acting as an adhesive to affix the pollen together and to the insect pollinators and the stigma surface, aiding hydration and thus the initial germination of the pollen, and exerting self-compatibility and incompatibility (2).

The constituents of the pollen coat of most species are largely unknown, but those of the pollen coat of *Brassica* species have been studied (3–6). In *Brassica*, the pollen coat can be extracted from the pollen with an organic solvent such as cyclohexane or diethyl ether without apparent damage to the pollen protoplast. The coat consists of neutral esters as the major lipid constituents, and its predominant proteins are specifically fragmented oleosins. The oleosins belong to the family of proteins described earlier as abundant structural proteins on the storage oil bodies in seeds (7). The neutral esters and oleosin fragments that constitute the pollen coat are produced in the tapetum cells. At a late stage of anther development immediately before lysis of the tapetum, the tapetum cells contain two dominant organelles. Both organelles are spherical and about 3 µm in diameter (8,9). One of the organelles is a novel lipid particle termed tapetosome, which does not have an enclosing membrane and contains patches of triacylglycerols (TAG) situated among densely packed vesicles (6). The other organelle is the elaioplast, which is packed with globuli of neutral esters. Whereas the predominant proteins of the tapetosomes are oleosins, those of the elaioplasts include lipid-associated structural proteins (10), presumably located on the surface of the globuli. Just before or shortly after lysis of the tapetum cells, some of the constituents of the two organelles are degraded, whereas the other constituents remain unchanged or are modified, and are released onto the surface of the maturing pollen (4–6). These latter constituents include fragmented oleosins of the tapetosomes and the neutral esters of the elaioplasts.

We here report that the abundant neutral lipids in the elaioplasts are steryl esters. This is the first report of plastids contain-

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Abbreviations: APCI, atmospheric pressure chemical ionization; EI, electron impact; FAME, fatty acid methyl ester; GC, gas chromatography; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; MGDG, monogalactosyldiacylglycerols; MS, mass spectroscopy; TAG, triacylglycerols.

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ing steryl esters as the predominant lipids. Also, we demonstrate the selective recovery of the lipids and proteins of the tapetum elaioplasts and tapetosomes on the pollen surface.

EXPERIMENTAL PROCEDURES

Plant materials. Brassica napus L. var. Westar seeds were obtained from Calgene Inc. (Davis, CA) and used to produce flowering plants in a greenhouse maintained at 26.5/18.5°C, 14/10 h day/night cycle. The florets were divided into five developmental stages according to the following criteria (10): at stage 1, the florets were 2 mm long or less, and more than half of the microspores (i.e., the maturing pollen) were in a tetrad condition; at stage 2, the florets were 2–3 mm long, and all the microspores were solitary; at stage 3, the florets were 3–4 mm long, and the tapetum cells were unlysed and filled with organelles; at stage 4, the florets were 4–5 mm long, and the tapetum cells had just lysed; and at stage 5, the florets were 5–6 mm long, and the microspores were almost mature as pollen. Mature pollen was collected from flowers that had opened on the same day.

Isolation of tapetosomes and elaioplasts from the florets. All the solutions described in this paragraph contained 0.05 M HEPES-NaOH, pH 7.5. The florets of stage-3 anthers were finely minced with a razor blade in a petri dish containing a grinding medium of 0.8 M sucrose (125 florets of 3.1 g per 8 mL) (6). Light microscopy revealed that chopping broke most of the sporophytic floral tissues, especially the tapetum, but left the gametophytic microspores intact. These microspores were stained positively with the vital dye fluorescein diacetate (observed by light microscopy). The homogenate was filtered through a layer of Nitex cloth (20 × 20 µm pore size), which removed the microspores. The filtrate (4 mL) was placed in a 17-mL centrifuge tube (6). For the preparation of a mixture of the two organelles (tapetosomes and elaioplasts) in a one-step density gradient, a solution of 0.4 M sucrose solution was layered on top of the filtrate. For the separation of the two organelles in a three-step density gradient, successive layers of 4 mL each of 0.4, 0.2, and 0 M sucrose solutions were placed on top of the filtrate. The tube was centrifuged at 9,000 rpm in a Beckman SW 28.1 rotor for 2 h. In the one-step density gradient, the low-density organelle fraction floating on top was collected with a pipet. In the three-step density gradient, the two visible organelle fractions banding at the interfaces between 0.4 and 0.2 M (tapetosomes), and 0.2 and 0 M (elaioplasts) sucrose solutions were collected from the bottom of the centrifuge tube after a hole was punctured through the tube with a needle.

Preparation of surface fractions from the mature pollen. The procedure followed that described earlier (6). Mature pollen was mixed with 2 vol of diethyl ether (or other solvents, to be described in the Results and Discussion section) for 1 min by repeated inversion in a capped test tube. The pollen was separated from the solvent by centrifugation for 10 min at 800 × *g*. The supernatant was retained and evaporated under vacuum.

Analyses of the proteins in the pollen surface fraction. The amino acid composition was analyzed by the Protein Structure Facility at the University of Iowa, Iowa City, IA. The polypeptide composition was analyzed by 12.5% (wt/vol) sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 2.5 h at 100 V (6). After electrophoresis, the gel was stained with Coomassie blue and then destained.

Extraction of lipids from the various samples. Lipids in the various samples were extracted with chloroform/methanol (2:1, vol/vol) using the procedure of Folch *et al.* (11).

Separation of neutral lipids in pollen coat extracted with different solvents. Lipids in chloroform/methanol (2:1, vol/vol) were applied to thin-layer chromatography plates (silica gel 60A, Whatman Inc., Clifton, NJ). The plates were developed in hexane/diethyl ether/acetic acid (80:20:2, by vol), and then charred with sulfuric acid.

Quantitative analyses of the neutral and polar lipids. Nonpolar lipids were quantitatively analyzed using a high-performance liquid chromatography (HPLC) system that consisted of a Hewlett-Packard (Avondale, PA) Model 1050 Quaternary Solvent HPLC and autosampler, and an Alltech-Varex (Deerfield, IL) Mark III Evaporative Light-Scattering Detector. The column was a LiChrosorb Diol column (Chrompack, 3 × 100 mm), and the flow rate was 0.5 mL/min. The solvents were A, hexane/acetic acid, 1000:0.3, vol/vol; and B, hexane/isopropanol, 100:1, vol/vol. (Both were mixed fresh daily to eliminate variability caused by evaporation and/or absorption of moisture). The linear gradient timetable was as follows: at 0 min, 100:0; at 8 min, 100:0; at 10 min, 75:25; at 40 min, 75:25; at 41 min, 100/0 (%A/%B, respectively), with a total run time of 60 min. Polar lipids (glycolipids and phospholipids) were quantitatively analyzed using an HPLC-evaporative light-scattering detection system as previously described (12).

Analysis of alcohols from E1 and fatty acids from E1 and E2. Lipids in HPLC fractions E1 (defined as lipids with a retention time of 2.3 min) and E2 (retention time of 3.4 min) were saponified with 1 N KOH in 80% ethanol (reflux 1 h at 90°C). After cooling, the alcohols were extracted with hexane, the ethanolic phase was acidified with 6 N HCl, and free fatty acids were recovered by a second hexane extraction. The free fatty acids were then derivatized to methyl esters (FAME) with 14% boron trifluoride in methanol (20 min at 70°C sealed under N_2) for gas–liquid chromatography (GLC) analysis. Prior to GLC and gas chromatography–mass spectrometry (GC–MS) analyses, alcohol and FAME fractions were partially purified by hexane/diethyl ether step gradient elution from Pasteur pipet silicic acid columns (13). Free sterols derived from the steryl esters in HPLC fraction E1 were quantified by capillary GLC–flame-ionization detection on a 30 m × 0.25 mm i.d., 0.25-µm SPB-1 film column (Supelco, Bellefonte, PA) as described previously (14). Sterols were identified on the basis of their retention times relative to authentic standards and by GC–MS followed by comparison of their electron impact (EI) mass spectra with those from standards, from the literature, and/or from the NIST (National Institute of Standards and Technology, e-

mail: http://webbook.nist.gov) spectral library. FAME derived from fraction E1 and E2 lipids were quantified by capillary GLC–flame-ionization detaction, with separation on a 15 m × 0.25 mm i.d., 0.20-µm SP-2330 film column (Supelco) as previously described (15). Identification was based on FAME retention times relative to authentic standards, with confirmation by electron impact (EI) GC–MS. Electron ionization spectra (70 eV, source temperature 180°C) of free sterols and FAME were collected from *m/z* 40 to 500 using a Hewlett-Packard 6890 series gas chromatograph and mass-selective detector fitted with a 30 m × 0.25 mm i.d., 0.25-µm SPB-1 film capillary column. For sterols, the column oven was programmed to increase from 200 to 240°C at 10°C per min, followed by an increase from 240 to 260°C at 1°C per min, with a final 12 min at 260°C. For FAME, the column oven program included an initial rise from 100 to 150°C at 10°C per min, followed by a further increase from 150 to 200°C at 2°C per min and a final 10 min at 200°C.

Analysis of E2 and alcohols from E2. The lipids were analyzed by HPLC–MS–atmospheric pressure chemical ionization (APCI). The HPLC system for separation of nonpolar lipids described in an earlier section was connected to a Hewlett-Packard Model 5989A mass spectrometer, with an APCI interface operated in the positive ion mode. The ion source was at 100°C, and the instrument scanned from 50–1000 *m/z* at 2.0 s/scan.

RESULTS AND DISCUSSION

Lipid composition of the florets and the subcellular fractions. Lipids extracted from the whole florets (omitting the maturing pollen) and the various organelle fractions were analyzed *via* one HPLC system for the nonpolar lipids and another HPLC system for the polar lipids (Table 1). The abundant lipids in all the samples were neutral lipids and glycolipids. Those in the whole florets included a major (E1, about 80%) and a minor (E2, about 20%) group of fatty-acyl esters, TAG, and monogalactosyldiacylglycerol (MGDG). E1 and E2 ester fractions were separated in the nonpolar HPLC system, with retention times of 2.3 and 3.4 min, respectively. Whereas the E1 esters co-chromatographed with steryl-fatty acyl ester standards, no known standards co-chromatographed with the E2 ester. The abundant lipids and the minor lipids of the whole florets were also present in roughly the same proportion in the two-organelle fraction, which was a mixture of approximately equal amounts of the elaioplasts and the tapetosomes of the tapetum. The similarity is expected because the tapetum cells contained the two organelles as the predominant constituents and were the most metabolically active cells in the anthers (other than the maturing pollen).

The isolated elaioplast fraction contained E1, E2, and MGDG as the major neutral lipids, whereas the isolated tapetosome fraction contained mostly TAG. Using the neutral esters and TAG as markers of the two organelles (6), we estimate that the elaioplast fraction contained little contaminating tapetosomes, whereas the tapetosome fraction had some (about 15% w/w) contaminating elaioplasts. This pattern of cross contamination is expected because the elaioplasts moved to a lower density region than the tapetosomes during flotation gradient centrifugation, resulting in a relatively pure elaioplast fraction and a less pure tapetosome fraction containing some tailing elaioplasts. The elaioplast fraction possessed a high amount of MGDG (about 16% w/w of total lipids). Since the elaioplasts have minimal envelope and thy-

TABLE 1
Quantitative Analysis of Lipid Classes in Different Extracts of the *Brassica napus* Florets and the Pollen Surface[a]

Lipds	Whole florets	Two-organelle fraction	Elaioplasts	Tapetosomes	Pollen surface
			µg lipid class/mg total lipids[b]		
E1	219	201	347	52	298
E2[c]	68	61	111	26	10
TAG	322	352	20	539	11
FFA	23	9	14	Trace	7
St	35	17	38	18	42
MGDG[d]	96	122	156	24	Trace
DGDG	29	3	3	5	13
PE	15	9	10	48	Trace
Lyso-PE	4	15	15	3	38
PI	5	2	2	11	0
PA	3	2	1	13	3
PC	18	11	32	42	4
Lyso-PC	9	5	27	4	4

[a]The two-organelle fraction was a mixture of approximately equal amounts of the elaioplasts and tapetosomes.
[b]Lipids that were not accounted for in each sample were very nonpolar lipids (e.g., squalene, β-carotene, or other hydrocarbons) that eluted as a single peak in the void volume (1.5 min) by nonpolar high-performance liquid chromatography (HPLC).
[c]For unknown reasons, some preparations of the whole floret, the two-organelle, and the elaioplast fractions contained higher amounts of E2 relative to E1 (about 50–80% rather than the indicated 30–32%)).
[d]The peak fraction of monogalactosyldiacylglycerols (MGDG) resolved by HPLC might contain some steryl glycoside and glucocerebroside. TAG, triacylglycerols; FFA, free fatty acids; ST, sterols, DGDG, digalactosyldiacylglycerols; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; PC, phosphatidylcholine. For explanation of EI and E2 see the Experimental Procedures section.

lakoid membranes and are packed with small (about 0.2 µm in diameter) globuli (6), which presumably are made up of the neutral esters, it is likely that the MGDG is present on the surface of the globuli. The tapetosomes contained abundant internal vesicles of presumably membranous nature (6), and their predominant phospholipids were phosphatidylcholine and phosphatidylethanolamine. The lipids on the pollen surface contained mostly E1 and had little E2 and TAG.

All the fractions (Table 1) contained very nonpolar lipids, which were eluted with the void volume (1.5 min.) in the nonpolar HPLC system. These represented a complex mixture of various lipids, such as squalene, ß-carotene, or other hydrocarbons. No attempts were made to further separate and characterize them.

Characterization of the major neutral esters in the elaioplasts. The major group of neutral esters, E1, and the minor group of neutral esters, E2, were characterized further after saponification. The acyl moieties in E1 and E2 were very different (Table 2). In E1, 50% of the acyl moieties were 18:3, 18:2, and 18:1, and the remaining 50% were the saturated acids 16:0, 14:0, 18:0, and 20:0. In E2, only 6% of the acyl moieties were unsaturated, and the saturated moieties included mostly 18:0, and 16:0.

TABLE 2
Fatty-Acyl Composition of the Elaioplast E1 and E2 Neutral Esters

Acyl moiety	E1	E2
	% (wt/wt) of the fatty-acyl methyl esters	
14:0	11.6	6.7
16:0	17.8	37.8
18:0	10.7	35.9
18:1	3.9	2.3
18:2	11.8	2.6
18:3	34.9	1.7
20:0	8.7	10.0
22:0	0.6	1.3
24:0	0	0.9
26:0	0.9	0.8

The saponified E1 contained a mixture of at least 16 sterols, 14 of which (accounting for about 98% of the total) were identified and quantified by GLC and GC–MS (Table 3). Of these sterols, the common desmethyl sterols typically found in plant cell membranes (i.e., sitosterol, campesterol, stigmasterol, and cholesterol) constituted less than 16%. These desmethyl sterols are likely to represent the constitutive components of the limited envelope and internal membranes (6). The four most abundant components included 24-methylenecholesterol and three unusual 9ß,19-cyclopropane sterols (pollinastanol, 24-dehydropollinastanol, and 31-norcycloartenol) derived from a branch pathway of cycloartenol metabolism. They are likely the major constituents of the globuli in the elaioplasts (6,9). These steryl esters are known to be present in pollen from various species (16).

We have so far been unable to identify definitely the predominant alcohol moiety of E2. For both the intact E2 esters and the major alcohol moiety recovered after saponification, the soft-ionization achieved with APCI–MS in the positive mode yielded an intense peak at *m/z* 407. In assuming that this high-mass fragment represented the molecular ion of the E2 alcohol minus water plus a proton ($M^+ - 18 + 1$), which is often observed in mass spectra of sterols and triterpenols, the alcohol should have a molecular weight of 424. Although the EI GC–MS spectrum of the underivatized E2 alcohol showed only a small number of minor peaks above *m/z* 200, the highest mass ion was *m/z* 424, thus supporting the molecular weight proposed on the basis of the APCI–MS data. The pattern of ions ≤ *m/z* 203 obtained with EI GC–MS was similar to that reported for lupeol, a triterpenol with a molecular

TABLE 3
Sterol Composition of the Elaioplast E1 Steryl Esters Isolated by HPLC

Phytosterol	Retention time[a]	Molecular mass	Formula	Area %
Cholesterol[b]	1.00	386	$C_{27}H_{46}O$	1.1
Lathosterol	1.09	386	$C_{27}H_{46}O$	0.9
Pollinastanol	1.12	400	$C_{28}H_{48}O$	11.5
24-Methylenecholesterol	1.21	398	$C_{28}H_{46}O$	23.2
24-Dehydropollinastanol	1.22	398	$C_{28}H_{46}O$	12.5
Campesterol	1.24	400	$C_{28}H_{48}O$	5.3
31-Norcycloartanol	1.29	414	$C_{29}H_{50}O$	1.2
Stigmasterol	1.33	412	$C_{29}H_{48}O$	0.7
24-Methylenepollinastanol	1.35	412	$C_{29}H_{48}O$	2.1
31-Norcycloartenol	1.38	412	$C_{29}H_{48}O$	20.9
α-Sitosterol	1.47	414	$C_{29}H_{50}O$	8.7
Isofucosterol	1.51	412	$C_{29}H_{48}O$	8.3
Cycloeucalenol	1.53	426	$C_{30}H_{50}O$	0.7
Cycloartenol	1.60	426	$C_{30}H_{50}O$	0.9
Others (unidentified)	—	—	—	2.0

[a]Gas chromatography retention time relative to cholesterol.
[b]Sterol nomenclature: cholesterol, cholest-5-en-3ß-ol; lathosterol, cholest-7-en-3α-ol; pollinastanol, 14α-methyl-9ß,19-cyclo-5α-cholestan-3ß-ol; 24-methylenecholesterol, ergosta-5,24(28)-dien-3ß-ol; 24-dehydropollinastanol, 14α-methyl-9ß,19-cyclo-5α-cholest-24-en-3ß-ol; campesterol, 24[*R*]-ergost-5-en-3ß-ol; 31-norcycloartanol, 4α,14α-dimethyl-9ß,19-cyclo-5α-cholestan-3ß-ol; stigmasterol, stigmasta-5,22-dien-3ß-ol; 24-methylene-pollinastanol, 4α-methyl-9ß,19-cyclo-5α-ergost-24(28)-en-3ß-ol; 31-norcycloartenol, 4α,14α-dimethyl-9ß,19cyclo-5α-cholest-24-en-3α-ol; ß-sitosterol, stigmast-5α-en-3ß-ol; isofucosterol, stigmasta-5,24(28)-dien-3ß-ol; cycloeucalenol, 4α,14α-dimethyl-9ß,19-cyclo-5α-ergost-24(28)-en-3ß-ol; cycloartenol, 4,4,14α-trimethyl-9α,19-cyclo-5α-cholest-24-en-3ß-ol.

weight of 426 (the base ion, however was *m/z* 81 rather than 43 as in lupeol). These limited data are consistent with the E2 alcohol being the triterpenol 12-dehydrolupeol, which has been reported to occur as an acetate ester in *Hemidesmus indicus* (17). Further analysis of this alcohol is required for unequivocal identification.

Green or nongreen plastids containing steryl esters as the predominant lipids have not been previously reported. The best-studied nongreen plastids containing high amounts of specific lipids are the chromoplasts, which possess abundant pigments (18). These pigments, mainly carotenoids, are present in globuli in the stroma or fibril structures associated with the thylakoids. The globuli in chromoplasts of some species also contain a high percentage of TAG. The globuli or the fibrils are covered with special structural proteins that are highly conserved in amino acid sequences among species (19). In comparison with these chromoplasts and other plastids, the *Brassica* tapetum elaioplasts are unique. They contain minimal internal membranes and stroma and are packed with lipid globuli. These globuli contain steryl esters and are presumably covered with MGDG (Table 1) and structural proteins similar to those in chromoplasts (10). Elaioplasts of *in situ* morphology similar to that of *Brassica* elaioplasts have been observed in the tapetum of diverse species (2), indicating that they may also have similar lipid and protein compositions.

Developmental changes of the major neutral lipids in the florets. Changes during floret maturation in the contents of the major neutral lipids in the sporophytic anthers, and ultimately on the pollen surface, were determined (Fig. 1). Elaioplast E1 (steryl esters) accumulated during maturation from stage 1 to stage 5. Electron microscopic observations (8,9) showed that at stage 5, the tapetum cells started to lyse, and the elaioplasts or the released globuli were transferred to the surface of the maturing pollen. Steryl esters similar to those of the elaioplasts were recovered quantitatively from the pollen surface (Fig. 1). The current biochemical analysis and the earlier electron microscopic observations strongly suggest that the steryl esters were transferred from the tapetum elaioplasts to the pollen surface. In contrast with the elaioplast E1 steryl esters, the elaioplast E2 (Fig. 1) and MGDG (Table 1), as well as the tapetosome TAG (Fig. 1), increased to a maximum at developmental stage 3 and then declined to minimal amounts on the pollen surface (Fig. 1). Thus, the pollen surface contained only E1 as the predominant lipid component (Fig. 1 and Table 1). The relatively high percentage of lysophosphatidylethanolamine in the pollen coat (Table 1) is indicative of the presence of phospholipase A2 in the anthers during development.

The physiological significance of the accumulation of E2 in the elaioplasts and TAG in the tapetosomes during the mid-maturation stage of the anthers and of their subsequent degradation rather than transfer to the pollen surface is unknown.

Extraction of lipids and proteins from the surface of the pollen. It was reported that the lipids and proteins on the surface of the pollen could be extracted with cyclohexane or diethyl ether without apparent damage to the pollen protoplasts (3,4,6).

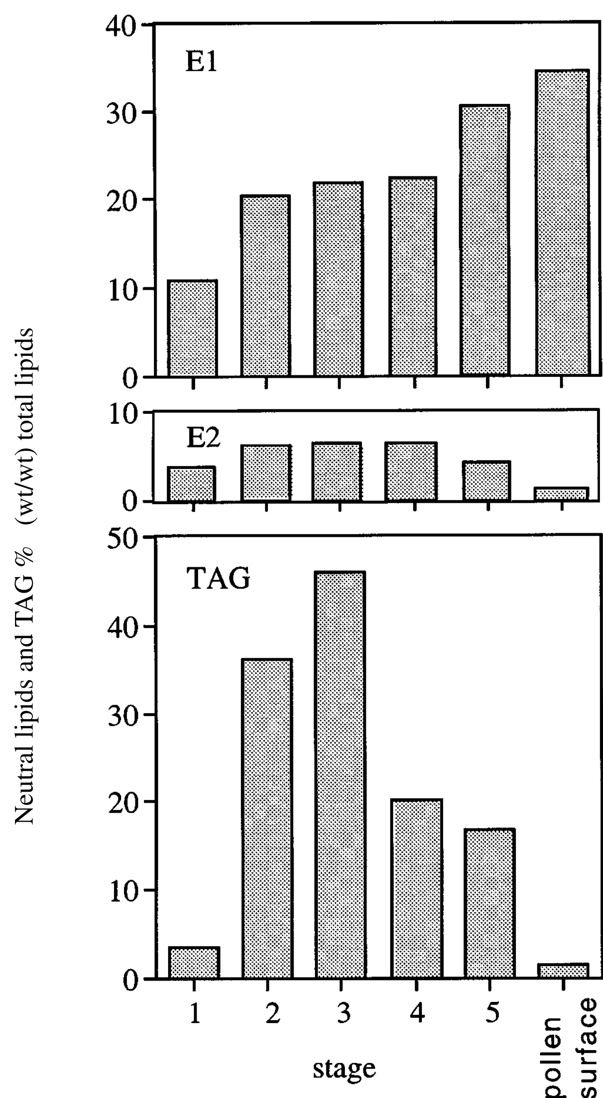

FIG. 1. Proportions of major neutral lipids in *Brassica napus* sporophytic florets at five stages of development and in the pollen coat. Data on the neutral esters E1 and E2 and triacylglycerols (TAG) are expressed as % wt/wt of total lipids. The amounts of total lipids in the florets at stages 1 to 5 and on the pollen surface were 1.01, 2.40, 2.20, 2.22, 2.28, and 2.29 mg per 15 florets, respectively.

In the present study, we found that all the neutral lipids and proteins of the pollen coat could be simultaneously extracted with nonpolar and moderately polar solvents (Fig. 2). They were extracted incompletely with methanol, and not at all with water. The pollen surface lipids shared some similarities with the total pollen lipids (Fig. 2). The total pollen lipids contained the pollen surface E1 and other neutral esters of the pollen interior (6), which comigrated with E1. They also contained TAG, which should be those in the storage lipid bodies in the cytoplasm. The pollen surface proteins were resolved into a pattern by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which differed from that of the total pollen proteins (Fig. 2).

Most of the polypeptides in the pollen coat had sizes in the ranges of 30–37 kDa and <14 kDa (Fig. 2). They largely repre-

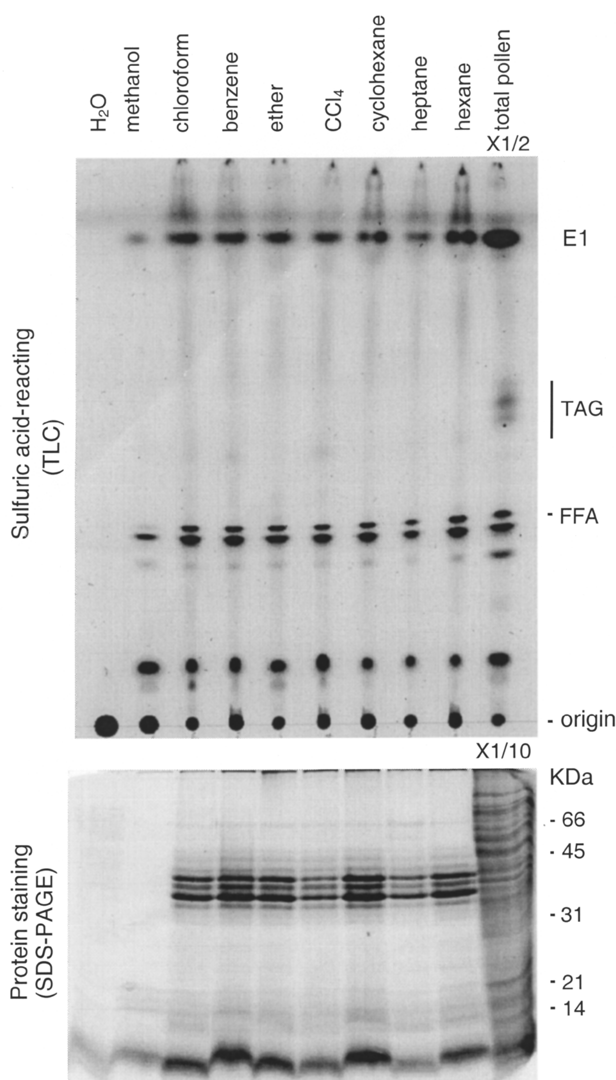

FIG. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins and thin-layer chromatography (TLC) of neutral lipids of pollen coat extracted from the pollen of *Brassica napus* by different solvents. Loading of extracts obtained with various solvents was arranged in the order of decreasing solvent polarity from left to right. Total pollen extract was prepared by grinding pollen in diethyl ether in glass beads with a mortar and pestle. A pollen-coat sample equivalent to an extract from 2 mg (TLC) or 3 mg (SDS-PAGE) of pollen, or total pollen extract from 1/2 of 2 mg (TLC) or 1/10 of 3 mg (SDS-PAGE) of pollen was loaded to each lane. Positions of markers for protein molecular weights and standard lipids are shown on the right. For other abbreviations see Figure 1.

sented specifically fragmented oleosins of the tapetosomes (4, 10). The amino acid sequences of these fragments, as predicted from their gene nucleotide sequences, indicate that they are very hydrophilic. Yet, these very hydrophilic polypeptides and the very hydrophobic steryl esters were extractable by moderately and strongly nonpolar solvents. These findings suggest that on the pollen surface, the neutral lipids of the elaioplasts and the polypeptides of the tapetosomes are mixed and emulsified with amphiphilic molecules, such as phospholipids and galactolipids (Table 1).

TABLE 4
Amino Acid Compositions of the *Brassica* Pollen Coat Proteins and the Two Major Oleosin Fragments Known to Be in the Pollen Coat[a]

	Pollen coat	Fragment from 45-kDa oleosin	Fragment from 48-kDa oleosin
		mol%	
Ala	8.3	2.2	1.9
Cys	3.1	0	0
(Asx) asp	(7.8)	4.9	3.8
(Glx) glu	(8.0)	7.2	7.3
Phe	2.4	0.38	0.32
Gly	13.8	26.0	26.9
His	3.4	7.5	7.6
Ile	4.3	4.9	4.8
Lys	13.3	16.6	14.9
Leu	5.4	0.75	0.63
Met	2.6	2.3	2.9
(Asx) asn	(7.8)	1.9	2.2
Pro	6.1	2.6	3.5
(Glx) gln	(8.0)	0.38	0.63
Arg	3.6	0.38	0.63
Ser	11.5	17.3	18.7
Thr	4.1	3.8	1.9
Val	3.3	0.38	1.3
Try	0	0	0
Tyr	1.5	0.38	0.32

[a]The amino acid compositions of the oleosin fragments were deduced from their known amino acid sequences (4,10).

An analysis of the amino acid composition of the total proteins on the pollen surface revealed a very high proportion (13 mol%) of lysine (Table 4). This is a reflection of the high lysine content in the short repeated sequences of the two predominant oleosin fragments on the pollen surface (4,20).

The high-lysine proteins and the plentiful steryl esters are both essential nutrients of the pollinating bees and other insects. Whether these components make a substantial impact in increasing the population of the pollinating insects is unknown. Some minor constituents of the pollen coat are originated from the pollen gametophyte and involved in self-incompatibility (3). The steryl esters on the pollen surface could play one or more of the roles of the pollen coat described in the Introduction. In addition, they could act as feeding stimulants of insects (21) or hormonal stimulants of pollen germination and tube growth (22).

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COMMUNICATION

Occurrence of γ-Linolenic Acid in Compositae: A Study of *Youngia tenuicaulis* Seed Oil

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ABSTRACT: Seeds of *Youngia tenuicaulis* and other species from the plant family Compositae (Asteraceae) were studied for their oil content and fatty acid composition. The seed oil of *Y. tenuicaulis* growing in Mongolia was found to contain 5.6% γ-linolenic acid (18:3Δ6*cis*,9*cis*,12*cis*) in addition to common fatty acids. The oil was analyzed using chromatographic [capillary gas–liquid chromatography (GLC), thin-layer chromatography] and spectroscopic (infrared, gas chromatography–mass spectrometry) techniques. Seed oil fatty acids of *Saussurea amara* (containing γ-linolenic acid) and of *Arctium minus* (containing 18:3Δ3*trans*,9*cis*,12*cis*), as well as Δ5*cis*- and Δ5*trans*-18:3 were used as GLC reference substances. The evolution in this plant family of a large number of different 18:3 acids as well as the corresponding evolution of unusual desaturases should be investigated. On the other hand, the Δ6*cis*-desaturase required for the biosynthesis of γ-linolenic acid may have evolved independently several times in unrelated families of the plant kingdom.

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Three species of *Youngia* (*Y. tenuicaulis*, *Y. tenuifolia,* and *Y. stenoma*) are known to occur in Mongolia (1). *Youngia* is a genus of the plant family Compositae (Asteraceae). A large number of species of Compositae have been studied, and their seed oils were found to contain a whole range of unusual fatty acids, e.g., *cis*- or *trans*-unsaturated fatty acids with double bonds in positions 3 or 5 (2–4), or conjugated (5), acetylenic (6,7), epoxidized (8–10), or hydroxylated fatty acids (11). A number of these unusual fatty acids are now of interest because they are natural phytochemicals (12–15). The plant family Compositae also contains species that produce edible oils, such as sunflower and safflower. However, the pharmaceutically interesting γ-linolenic acid has never been found in seed oils of Compositae until we recently discovered it in *Saussurea* (16).

γ-Linolenic acid (γ-Ln, 18:3Δ6*c*,9*c*,12*c* or 18:3n-6) is an important essential fatty acid, both as a nutrient and as a therapeutic agent (17). The occurrence of γ-Ln as a seed oil component was reported by previous workers in the plant families Boraginaceae, Onagraceae, Liliaceae, Aceraceae, Moraceae, Scrophulariaceae and Saxifragaceae (18), and by us in Ranunculaceae (19), Primulaceae (20) and in *Saussurea,* a genus of plant family Compositae (16). Seed oils of *Oenothera biennis, Borago officinalis,* and *Ribes nigrum* are currently the most common commercial sources of γ-Ln. However, further screening for the occurrence and distribution of γ-Ln in the plant kingdom is justifiable. This may have practical consequences with respect to genetic engineering or plant breeding for renewable lipid resources. It may also be of academic interest with regard to natural product chemistry, plant chemotaxonomy, and the phylogenetic evolution of fatty acid structures in seed oils. Δ6-Desaturases that take part in the biosynthesis of γ-Ln should also be of interest in future attempts at enzyme design. These enzymes must have evolved several times, independently, during the course of evolution of otherwise unrelated plant families, and so they may contain different primary structures or amino acid sequences.

Capillary gas–liquid chromatography (GLC) on columns of different selectivity is a powerful analytical tool to detect and identify γ-Ln and other unusual positional or geometrical isomers of fatty acids that occur as seed oil components (21–24). However, some care is needed in investigations of Compositae seed oils, because other 18:3 fatty acid isomers may be present. For example, γ-Ln and Δ3*trans*-18:3 peaks may overlap on certain polar GLC columns.

In continuation of our screening project for exploring the oilseed potential of wild species of Compositae, we have analyzed seed oils from many other less well-studied species. These include a large number of species growing in Central Asia, including *Saussurea* spp. (16), *Y. tenuicaulis,* and various *Artemisia* spp. (Tsevegsüren, N., and Aitzetmüller, K., unpublished data). There is no information in the available literature on seed oils from genus *Youngia*. The present communication is one of a series resulting from a project intending to supplement the literature with respect to species not previously studied, as well as to discover new natural seed oil sources for unusual fatty acids in the plant kingdom. γ-Ln was found as a component fatty acid (5.6%) in seed oil from *Y. tenuicaulis*, the second known source of γ-Ln in the Compositae [*Saussurea* (16) was first]. *Youngia tenuicaulis* oil was characterized by capillary GLC separation of fatty acid

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Abbreviations: DMOX, dimethyloxazoline; ECL, equivalent chain length; FAME, fatty acid methyl ester; GLC, gas chromatography; IR, infrared; γ-Ln, γ-linolenic acid; TLC, thin-layer chromatography.

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methyl esters and mass fragmentation of the dimethyloxazoline (DMOX) derivative of γ-Ln. Results on *Youngia*, *Saussurea,* and *Arctium* are compared with other seed oils of the Compositae.

EXPERIMENTAL PROCEDURES

Seed materials. Seeds of *Y. tenuicaulis* were collected in September 1994 in Bayan zag, Bulgan sum region, Umnegobi aimak, Mongolia. Sources of seeds for the other species used were as described (16,19–23,25).

Oil extraction and preparation of fatty acid derivatives. Ground seeds were extracted with *n*-hexane in a Soxhlet extractor. Fatty acid methyl esters (FAME) of the seed oils were prepared by transesterification with 2.0 M sodium methoxide in methanol as described previously (25). The DMOX derivatives of fatty acids were prepared as described by Zhang *et al*. (26).

Thin-layer chromatography (TLC). To obtain additional information on unusual components in oils, we carried out analytical TLC of oils and FAME on 0.25-mm layer TLC plates of Silica gel G (Merck, Darmstadt, Germany) using a solvent system of *n*-hexane/diethyl ether (70:30 and 80:20 vol/vol, respectively). Spots were visualized by spraying with phosphomolybdic acid (5% in ethanol) and heating at 100°C.

Capillary gas–liquid chromatography (GLC). GLC of FAME was performed on either a Hewlett-Packard HP 5890 or a Perkin-Elmer F22 gas chromatograph equipped with a flame-ionization detector and a fused-silica WCOT capillary column (length 50 m, internal diameter 0.25 or 0.22 mm) packed with one or a number of stationary phases of different polarity (Silar 5 CP, CP Sil 88 and BPX 70).

(i) GLC conditions, Silar 5 CP. The temperature was held at 165°C for 1 min, then programmed from 165 to 205°C at 1°C/min, then held at 205°C for *ca*. 60 min before cooling to 165°C. The injector and detector temperatures were 230 and 260°C, respectively.

(ii) BPX 70. The temperature was programmed from 100 to 240°C at 2°C/min and maintained at 240°C for 32 min. The injector temperature was 260°C and the detector temperature was 240°C.

(iii) CP Sil 88. The temperature was programmed from 100 to 220°C at 1.5°C/min and maintained at 220°C for 20 min. The injector temperature was 270°C and the detector temperature was 240°C.

Nitrogen (Silar 5 CP and BPX 70) and hydrogen (CP Sil 88) were used as carrier gases. Flow rates were 1.13 mL N_2/min for the Silar 5 CP, 0.69 mL N_2/min for the BPX 70, and 1.0 mL H_2/min for the CP Sil 88 columns.

Chromatographic data were evaluated with an integrator (Chromato-Integrator D2000, HP 3396 Series II Integrator, or Shimadzu Chromatopac C-R3A). Identification of FAME was confirmed by chromatographic comparison with authentic standards, including seed oils of known composition, and by co-chromatography and calculation of equivalent chain lengths (ECL) (21).

GC–mass spectrometry. GC–mass spectrometric analysis was carried out using the electron impact ionization mode (70 eV) on a Hewlett-Packard instrument Model 5890 Series II/5989A, equipped with a 0.23 μm Permabond OV-1 fused-silica capillary column (Macherey-Nagel, Düren, Germany; 25 m × 0.32 mm i.d.). The column temperature was initially held at 150°C for 5 min, then programmed 150 to 270°C at 4°C/min. The final temperature was held for 5 min. Other operating conditions were split/splitless injector temperature of 300°C, interface temperature of 280°C, and ion source temperature 200°C. The carrier gas was helium at a flow rate of 1.5 mL/min.

Infrared (IR) absorption spectra. IR spectra were determined from oil films deposited on sodium chloride cell windows using a Perkin-Elmer 781 Infrared Spectrophotometer (Beaconsfield, England) with a focusing attachment to check the presence of any unusual (*trans* unsaturated or oxygenated) fatty acid.

RESULTS AND DISCUSSION

The extraction of seeds of *Y. tenuicaulis* yielded 16.2% seed oil. The fatty acid compositions of this oil and some others used for comparison are given in Table 1. Since IR spectra of the *Youngia* oil showed no bands in the 960 cm^{-1} region, *trans*-fatty acids are absent and any unsaturation in the oil has the *cis* configuration only. Analytical TLC and IR spectra of the seed oil and TLC of the mixed FAME confirmed the ab-

TABLE 1
Fatty Acid Compostion of Four Compositae Seed Oils (*Youngia tenuicaulis, Arctium minus, Saussurea amara,* and sunflower)

	GLC (area %)			
Fatty acid	*Youngia tenuicaulis*	*Arctium minus*	*Saussurea amara*	Sunflower oil[a]
12:0	Trace	0.1	Trace	—
14:0	0.2	0.2	0.1	<0.5
15:0	Trace	Trace	Trace	—
16:0	5.8	6.3	4.1	3.0–10
16:1n-9	0.1	0.1	Trace	—
16:1n-7	0.1	2.1	0.2	<1.0
17:0	0.1	—	Trace	—
17:1n-7	Trace	—	Trace	—
18:0	1.8	2.2	2.8	1.0–10
18:1n-9	16.4	13.7	15.5	14–35
18:1n-7	0.6	0.6	0.5	—
18:2n-6	65.1	58.4	62.7	55–75
18:3n-6	5.6	—	10.4	—
18:3Δ3*t*,9*c*,12*c*	—	13.9	—	—
18:3n-3	0.6	0.3	0.5	<0.3
20:0	1.6	0.5	0.7	<1.5
20:1n-9	0.5	0.2	0.2	<0.5
20:2n-6	0.1	—	Trace	—
22:0	0.7	0.3	0.3	<0.5
24:0	0.2	0.2	0.2	<0.5
Others	0.5	0.9	1.8	

[a]Data for sunflower oil are taken from Codex Alimentarius. Abbreviation: GLC, gas–liquid chromatography.

sence of any oxygenated fatty acids. The typical GLC pattern or fingerprint (23) of mixed FAME of *Y. tenuicaulis* seed oil on a Silar 5 CP column is illustrated in Figure 1A. One interesting aspect of the fatty acid analysis was the appearance of an unusual component with ECL 19.09 (5.6% abundance) on the Silar 5 CP column in addition to linoleic (65.1%) and oleic (16.4%) acids as the predominant fatty acids. The ECL suggests that this peak is an isomeric 18:3 acid. Further tentative identification of γ-Ln was carried out by capillary GLC analyses of the methyl esters on capillary columns of BPX 70 and CP Sil 88, which resulted in good separation of the FAME, with ECL values for γ-Ln of 19.66 and 19.95, respectively. These ECL values correspond well to those found with authentic γ-Ln methyl ester (test substance) and to those found with several well-known seed oils containing γ-Ln.

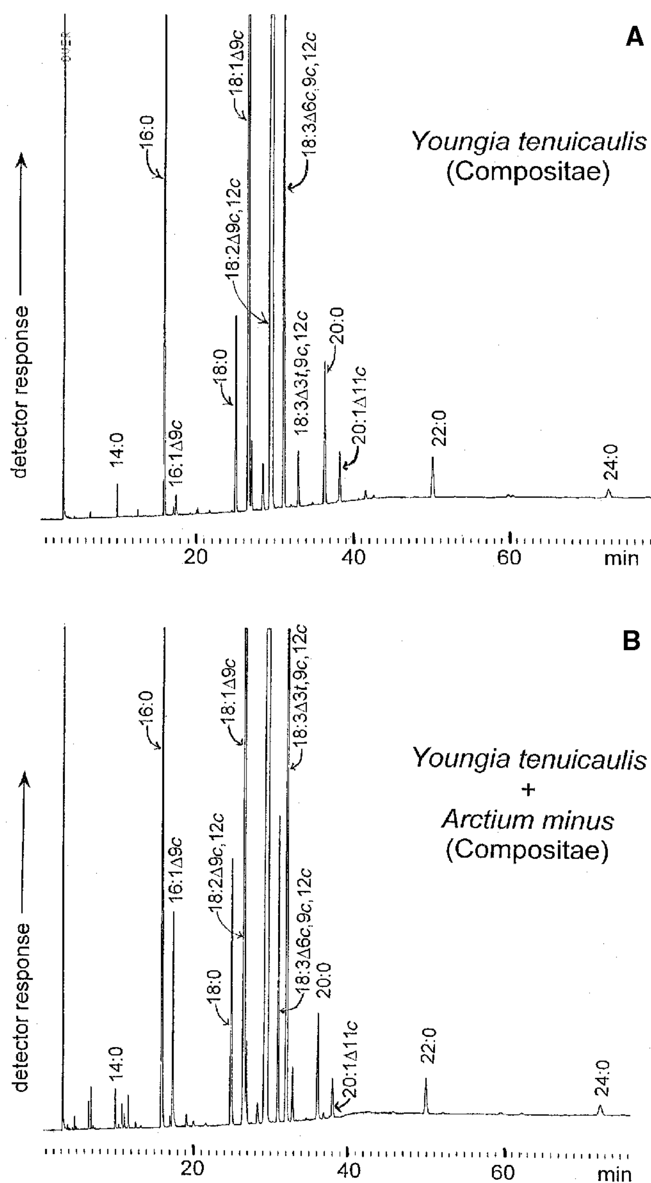

FIG. 1. Gas–liquid chromatograms of the fatty acid methyl esters (FAME) (A) of *Youngia tenuicaulis* (Compositae) seed oil separated on a Silar 5 CP column, and (B) from *Y. tenuicaulis* seed oil mixed with FAME from *Arctium minus* seed oil separated on a Silar 5 CP column. FAME peaks are labeled as usual; 18:3Δ3*t*,9*c*,12*c* is caleic acid and 18:3Δ6*c*,9*c*,12*c* is γ-linolenic acid.

γ-Ln (18:3Δ6*c*,9*c*,12*c*) had never been observed in seed oils of members of the Compositae until it was recently found in several *Saussurea* spp. (16) during the present series of investigations. Another 18:3 isomer (18:3Δ3*t*,9*c*,12*c*, caleic acid) was previously reported to occur more frequently in the Compositae and it is present, for example, in *Arctium minus* seed oil (2). Therefore, seed oils from *Saussurea amara* and *A. minus*, both belonging to the plant family Compositae, were used as reference substances to compare the gas chromatograms and ECL values under our three different gas chromatographic conditions. The absence of 18:3Δ5*c*,9*c*,12*c* and of 18:3Δ5*t*,9*c*,12*c* was also established as described before (21).

We know that caleic acid (18:3Δ3*t*,9*c*,12*c*) is difficult to separate from γ-Ln (18:3Δ6*c*,9*c*,12*c*) on the BPX 70 column under the given conditions. Baseline separation, however, is obtained on Silar 5 CP columns. Co-injection of mixed FAME from *Y. tenuicaulis* seed oil with those from *A. minus* seed oil gave a split peak, with the unknown from *Y. tenuicaulis* eluting just before the peak of 18:3Δ3*t*,9*c*,12*c* from *A. minus* (Fig. 1B). This indicated that the unknown in *Y. tenuicaulis* was not caleic acid. The presence of 18:3Δ6*c*,9*c*,12*c* was established in a similar manner by comparison with *S. amara* which contains this fatty acid in its seed oil. Co-chromatography of *Y. tenuicaulis* seed oil FAME with those from *S. amara* on a Silar 5 CP column resulted in a single peak for the unknown from *Y. tenuicaulis* which coeluted with 18:3Δ6*c*,9*c*,12*c* from *S. amara.* This comparison supported the presence of γ-Ln (18:3Δ6*c*,9*c*,12*c*) in *Y. tenuicaulis.*

The separation of Δ6*cis*-, Δ5*cis*-, and Δ5*trans*-18:3 isomers had been studied in detail before (21), using *Aquilegia* oil as a source of Δ5*trans*-18:3 and *Xeranthemum* as a representative of the Compositae that contains the Δ5*cis* isomer, i.e., pinolenic acid or 18:3Δ5*cis*,9*cis*,12*cis*. Our GLC data in the present case, however, clearly established the absence of both these fatty acids in *Youngia*. No Δ3*trans*- *or* Δ5*trans*-fatty acid or any other peak representing a *trans* fatty acid was found in *Y. tenuicaulis*, thus confirming the earlier IR evidence for the absence of a *trans* peak.

Further evidence for the double bond positions in the unusual 18:3 fatty acid of *Y. tenuicaulis* seed oil is given by the GC–mass spectrometric fragmentation pattern of the DMOX derivatives. The molecular ion at *m/z* 331 indicates the presence of three double bonds. Their position was easily recognized by applying the empirical rule for double bond location (26). The peak indicating the first double bond in mass spectra of polyunsaturated fatty acid DMOX derivatives with a Δ6 double bond (*m/z* 166) was accompanied by a strong odd-mass ion at *m/z* 167, in good agreement with literature data (26–28). The mass intervals of 12 *m/z*, instead of 14 *m/z*, occurred between *m/z* 194 (C_8) and *m/z* 206 (C_9) as well as between *m/z* 234 (C_{11}) and *m/z* 246 (C_{12}). This pattern indicates the presence of a Δ9 and a Δ12 double bond.

In conclusion, this study demonstrates for the first time that a seed oil of the genus *Youngia* contains γ-Ln. γ-Ln was unambiguously identified as a component fatty acid in *Y. tenuicaulis* seed oil which, therefore, besides *Saussurea,* seems to be only the second hitherto known γ-Ln-bearing genus of Compositae. γ-Ln, which is a very common seed oil constituent in the Boraginaceae (25), occurs only sparsely and sporadically in other plant families. The list of the natural sources for this pharmaceutically interesting fatty acid is now extended by another genus of the plant family Compositae, which is presently the only plant family where four different nonconjugated isomers of 18:3 are known to occur: 18:3Δ9*cis*,12*cis*,15*cis* (linolenic acid), 18:3Δ5*cis*,9*cis*,12*cis* (pinolenic acid), 18:3Δ3*trans*,9*cis*,12*cis* (caleic acid), and 18:3Δ6*cis*,9*cis*,12*cis* (γ-linolenic acid). Besides these, conjugated 18:3 acids have also been described in members of the Compositae, for example in *Calendula* and *Osteospermum*. Thus the Compositae are apparently capable of producing a whole range of different 18:3 fatty acids in phylogenetically closely related species or genera. Given the fact that these plant species or genera have evolved from one common ancestor by way of mutations, the biosynthetic pathways and the nature and structure of the enzymes involved in the production of so many different 18:3 fatty acids, including the conjugated ones, should be investigated and may prove useful in any future attempts at desaturase enzyme design. Similar cases had been discussed recently in the Ranunculaceae, where the evolution of desaturases producing Δ5*trans*-, Δ5*cis*- and Δ6*cis*-fatty acids, and changes of substrate preference, appeared to be linked to the phylogenetic evolution of the individual plant genera (29,30).

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ARTICLE

Metabolism of Individual Fatty Acids During Infusion of a Triacylglycerol Emulsion

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ABSTRACT: The triacylglycerol emulsion Intralipid was infused into six normal subjects to investigate the metabolism of individual fatty acids in subcutaneous adipose tissue and forearm muscle, by measurement of arteriovenous differences. The composition of plasma nonesterified fatty acids changed steadily after passage through adipose tissue and became similar to that of the emulsion, reflecting hydrolysis of the Intralipid-triacylglycerol by lipoprotein lipase, since endogenous lipolysis (hormone-sensitive lipase activity plus lipoprotein lipase hydrolysis of very low density lipoprotein triacylglycerol) was decreased. There was no significant net release of total or individual fatty acids from forearm muscle although there was a tendency for the composition of the fatty acids in forearm venous plasma to change during passage through the tissue to reflect the composition of the emulsion. This may reflect hydrolysis of emulsion particles by lipoprotein lipase situated in capillaries which drain into the forearm vein. The behavior of stearic acid in the plasma nonesterified fatty acid pool was consistently aberrant, with arterialized concentrations considerably higher than predicted from adipose tissue release, both before and during Intralipid infusion. We conclude that there are no significant differences in the metabolism of specific fatty acids, with the exception of stearic acid.

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The enzyme lipoprotein lipase (LPL) is attached to the capillary endothelium in a number of extrahepatic tissues and acts on circulating triacylglycerol (TAG) to release fatty acids. These fatty acids may be taken up into the tissue for oxidation or, once reincorporated into TAG by esterification pathways within the cell, they can be stored. Alternatively, they may be released into the plasma as nonesterified fatty acids (NEFA). Both pathways occur in adipose tissue (1), but whether LPL-derived fatty acids may be released as plasma NEFA in other tissues is not known.

The exact mechanism of fatty acid transport into and out of the adipocyte has not been elucidated, although there is evidence to suggest that in mammalian cells, membrane uptake of long-chain fatty acids is mediated by both passive and facilitated processes (2). There is some indirect evidence that the partitioning of LPL-derived fatty acids between tissue uptake and release into the plasma is fatty-acid specific. For instance, after a high-fat meal the composition of plasma NEFA changes to partially reflect the fatty acid composition of the meal (3,4). Unsaturated fatty acids tend to be underrepresented in the change in the plasma NEFA pool (3). Similarly, when a synthetic TAG emulsion is infused, the composition of plasma NEFA changes to reflect that of the emulsion, but again unsaturated fatty acids are underrepresented (5). One possible explanation is preferential tissue uptake of unsaturated fatty acids at the site of LPL action. There is now considerable evidence in both small animals (6,7) and humans (8) for preferential release of certain fatty acids from adipose tissue during fat mobilization. For a given chain length, unsaturated fatty acids are more highly mobilized than saturated, and for a given degree of unsaturation, shorter-chain fatty acids are more highly mobilized than longer-chain. This suggests that the physicochemical properties of fatty acids are important determinants of their transport across the adipocyte cell membrane. Since the composition of adipose tissue TAG broadly reflects that of the diet (9,10) and therefore does not become depleted of specific fatty acids, there must be corresponding replenishment of unsaturated and shorter-chain fatty acids. Perhaps at times of fat deposition there is preferential uptake of the fatty acids that are more readily mobilized. Preferential uptake of certain fatty acids has not been demonstrated directly.

To test this idea directly, we investigated the handling of individual fatty acids in subcutaneous adipose tissue by measurement of their arteriovenous differences during a time of expected fat storage. This technique involves sampling from a cannula inserted into a vein draining the subcutaneous abdominal adipose tissue. At the same time, samples are taken from a cannula inserted in a retrograde fashion into a vein draining a warmed hand, providing arterialized blood (11). Arteriovenous differences across the forearm (mainly skeletal muscle) were measured at the same time to elucidate the fate of LPL-derived fatty acids in skeletal muscle. Since there are problems in interpreting data in the nonsteady state following a fat-rich meal, we infused a synthetic TAG emulsion.

Concentrations of other metabolites including plasma

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Abbreviations: HSL, hormone-sensitive lipase; LPL, lipoprotein lipase; NEFA, nonesterified fatty acids; TAG, triacylglycerol; VLDL, very low density lipoprotein.

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TAG and NEFA in this study have been reported previously (12,13). The findings of that study, in brief, were that plasma TAG and NEFA concentrations increased markedly in response to the lipid infusion. As discussed in Reference 13, the rise in arterialized plasma NEFA was not associated with increased release of fatty acids from subcutaneous adipose tissue as we previously found after the ingestion of a mixed meal. Increased net release of NEFA from adipose tissue during the postprandial period partly represents increased release of fatty acids into the adipose venous plasma by the LPL-mediated hydrolysis of dietary TAG, circulating in the plasma in chylomicron particles (14).

Another unexpected finding was that the calculated adipose tissue hormone-sensitive lipase (HSL) activity was decreased during and after the infusion (13), despite the absence of an increase in plasma insulin concentrations. This finding aids the interpretation of data in the present study by minimizing the dilution of fatty acids leaving adipose tissue with those from intracellular lipolysis. We therefore now report our measurements of specific fatty acids in plasma lipid fractions in order to try to further our understanding of the handling of individual fatty acids in peripheral tissues.

METHODS

Subjects and protocol. The studies were approved by the Central Oxford Research Ethics Committee, and all subjects gave informed consent. Six normal, healthy male subjects aged 21–37 yr, with body mass index 23.0–25.9 $kg \cdot m^{-2}$, ate a low-fat evening meal and were studied the next morning after an overnight fast.

Blood samples from arterialized, forearm venous and adipose venous sites were obtained as previously described in detail (13). Briefly, a cannula was inserted retrogradely into a hand vein and the hand was warmed in a box at 60–70°C in order to obtain arterialized blood samples. Another cannula was inserted retrogradely into an antecubital vein draining deep forearm tissues on the contralateral arm. A 10-cm, 22-gauge catheter was then introduced over a guide wire into one of the superficial veins on the anterior abdominal wall and threaded toward the groin, so that its tip lay just superior to the inguinal ligament. Samples from this cannula represent the venous effluent from the subcutaneous abdominal adipose tissue, uncontaminated by muscle drainage and with only a minor contribution from skin. Blood samples were taken immediately before the start of an intravenous infusion of Intralipid 10% (wt/vol) (Pharmacia Ltd., Milton Keynes, United Kingdom) at a rate of 1.85 $mL \cdot kg^{-1} \cdot h^{-1}$, and at 60, 120, and 240 min during the infusion.

Analytical methods. Blood samples were taken into heparinized syringes and the plasma was separated rapidly at 4°C. A portion (1 mL) was used for preparation of a chylomicron-rich fraction as described previously (2) ("chylomicrons" in this case representing the infused TAG emulsion). Portions of plasma (0.75 mL) and of the "chylomicron" fraction were extracted with chloroform/methanol in tubes containing heptadecanoic acid as an internal standard. The lipid classes were separated by thin-layer chromatography, and specific fatty acids estimated by gas chromatography after methylation, as described previously (8). Samples of Intralipid were analyzed in the same way.

Changes in concentrations with time, and arteriovenous differences across the tissues, were assessed by repeated-measures analysis of variance using time and site (arterialized, forearm venous, and adipose venous) as within-subjects factors. Calculations were done with SPSS for Windows Release 7.1 (SPSS Inc., Chicago, IL). Results are shown as mean value ± SEM.

The proportion of the adipose tissue NEFA veno-arterial difference (AT v-a) that could be attributed to the hydrolysis of endogenous TAG (the total of LPL and HSL-mediated hydrolysis) was calculated from the concentration of palmitoleic acid (16:1) in the adipose venous and arterial plasma. The concentration of palmitoleic acid in Intralipid is less than 0.1%, so it can only arise in the plasma from endogenous sources.

$$\text{endogenous hydrolysis at time } t = \frac{\%\ (\text{mol/mol})\text{ of 16:1 in AT v-a at time } t}{\%\ (\text{mol/mol})\text{ of 16:1 in AT v-a at time 0}} \times 100 \quad [1]$$

At time 0, endogenous hydrolysis is 100%. It is assumed that the proportion of 16:1 to other endogenous fatty acids remained constant during the period of the infusion.

RESULTS

The concentration of TAG in the plasma increased rapidly after the onset of the infusion (Fig. 1) and the concentration of chylomicron-TAG represented over 50% of the total plasma TAG after 120 min. However, despite a high infusion rate, with plasma TAG concentrations increasing threefold within the first 60 min, there was efficient removal of TAG in

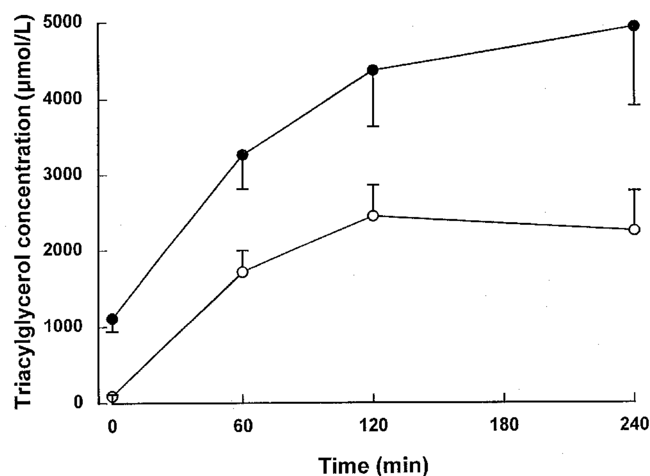

FIG. 1. Plasma triacylglycerol (TAG) (●) and chylomicron-TAG (○) concentrations in arterialized plasma before and during intravenous infusion of Intralipid, as described in the Methods section. The results are plotted as the mean ± SEM for 6 subjects. Error bars have been represented on one side of the mean only for clarity.

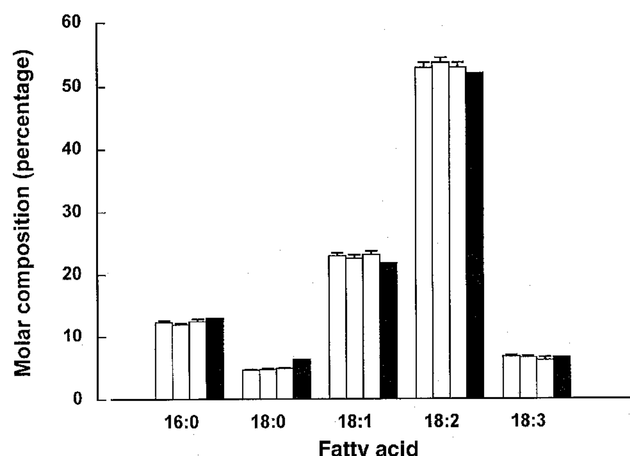

FIG. 2. Fatty acid composition of TAG in the chylomicron fraction taken from arterialized plasma. For each fatty acid the following values are shown (left to right): 60, 120, and 240 min after beginning intravenous infusion of Intralipid. The black bar to the right of each group represents the measured composition of Intralipid. Values are molar percentage composition, shown as mean value ± SEM for 6 subjects. For abbreviation see Figure 1.

the chylomicron-rich fraction, with no further increase in the concentration of chylomicron-TAG after 120 min. The composition of TAG in Intralipid (black bars, Fig. 2), estimated by gas chromatography, was as expected from the manufacturer's literature with a preponderance of 18:2n-6 (linoleic acid). The fatty acid composition of the chylomicron fraction TAG closely resembled Intralipid-TAG throughout (Fig. 2). The concentration and composition were similar in blood samples from all three sites (data not shown).

The concentration of individual fatty acids in the adipose venous plasma (hatched bars) was significantly higher than in arterialized or forearm venous sites ($P < 0.01$ for site effect, for all fatty acids shown on Fig. 3) as found for total plasma NEFA concentrations (13). However, the extent of release of stearic acid into the adipose venous plasma was considerably less than the other fatty acids shown. The relative concentrations of specific plasma NEFA initially (time 0) resembled that usually found after overnight fast (8), with a preponderance of 16:0 (palmitic acid) and 18:1n-9 (oleic acid), a lesser amount of 18:2n-6 (linoleic acid) and very little 18:3n-3 (α-linolenic acid). This changed with time during Intralipid infusion, so that the composition resembled increasingly that of the infused lipid. For example, after 240 min of Intralipid emulsion, 18:2 was the most predominant NEFA in the adipose venous plasma (Fig. 3). There were significant increases ($P < 0.001$) in the proportions of 18:2 and 18:3 and decreases ($P < 0.001$) in the proportions of palmitoleic acid (16:1n-7), 18:1, and 16:0. The composition of the veno-arterial differences for NEFA across adipose tissue initially (time 0) resembled that of plasma NEFA, and again changed with time increasingly to resemble the infused lipid (Fig. 4A). It was noticeable, however, that for stearic acid alone there was no systematic change with time in either its proportion in the veno-arterial difference (Fig. 4A) or its proportion in arterialized plasma NEFA (Fig.4B, black bars). There was no significant release of total NEFA (the sum of individual fatty acids represented in Fig. 3) from forearm muscle into the forearm vein. This was also true for individual fatty acids although there was a tendency for the composition of the fatty acids in forearm venous plasma to change during passage through the tissue to reflect the composition of the emulsion (Fig. 4B). For example, the proportion of 18:2 in the forearm venous plasma was consistently higher than that of the arterialized plasma at each time point, reflecting the high proportion of

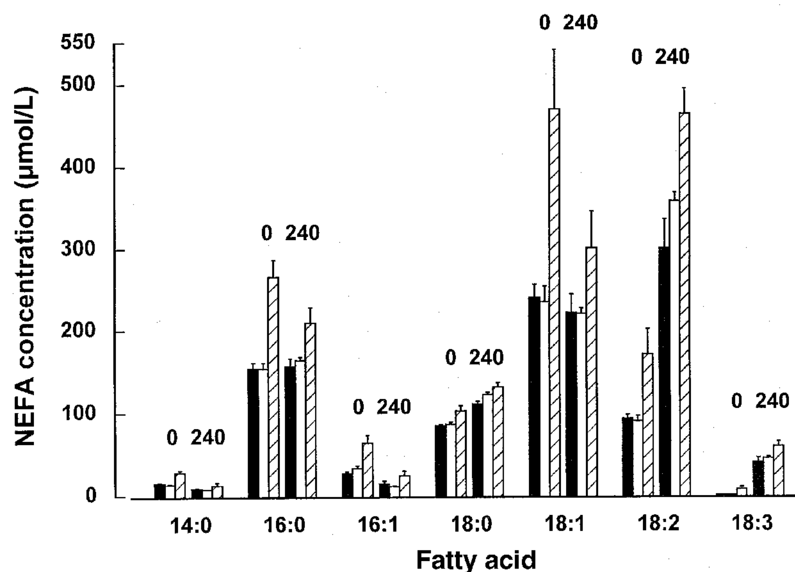

FIG. 3. Concentrations of specific fatty acids in the plasma nonesterified fatty acid (NEFA) fraction. Bars represent mean ± SEM for 6 subjects. For each fatty acid, the following values are shown: arterialized (solid bar), forearm venous (open bar) and adipose venous (hatched bar) plasma NEFA concentrations at time 0 and 240 min.

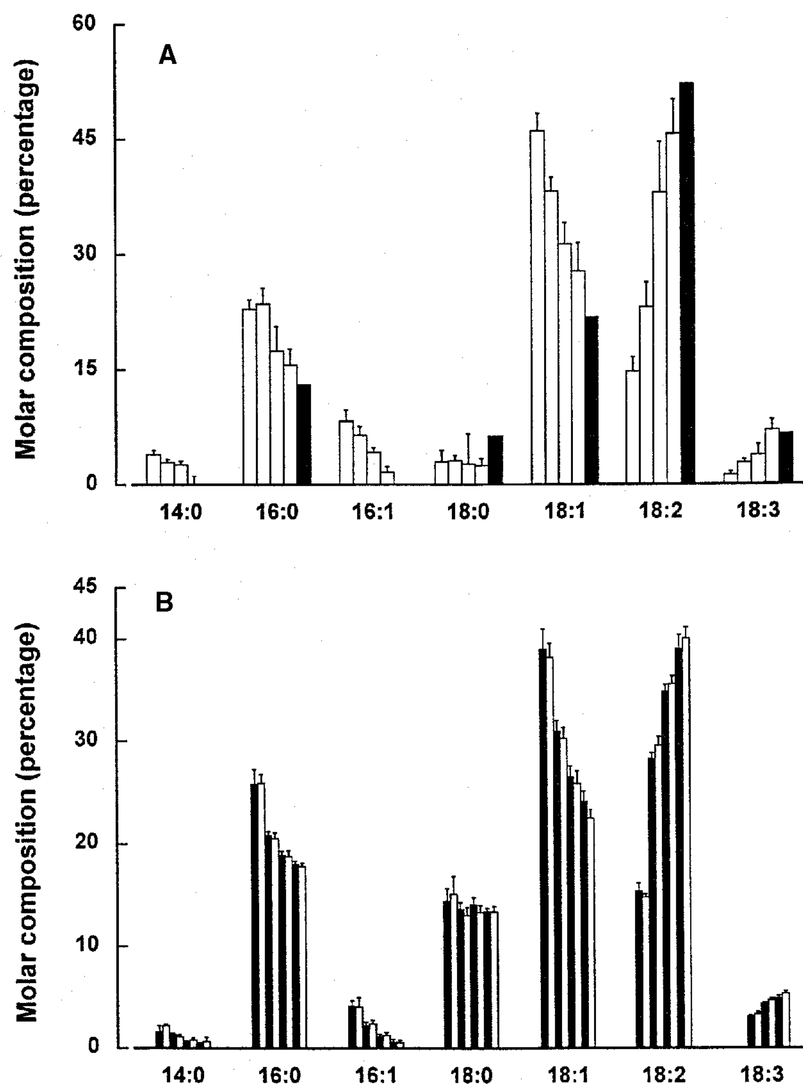

FIG. 4. Fatty acid composition (molar percentages) of the plasma NEFA fraction. Bars represent mean ± SEM for 6 subjects. (A) Composition of the veno-arterial difference for NEFA across adipose tissue (i.e., the composition of the net NEFA release from adipose tissue). For each fatty acid the following values are shown: time 0; 60, 120, and 240 min. The black bar without SEM to the right of each group represents the measured composition of Intralipid triacylglycerol, as in Figure 2. (B) Plasma NEFA composition. For each fatty acid the following values are shown: arterialized (solid bar), forearm venous (open bar) at time 0, 60, 120, and 240 min, respectively. 18:3 was not detected in arterialized and forearm venous samples at time 0. For abbreviation see Figure 3.

18:2 in the lipid emulsion. This may reflect uptake of plasma NEFA by muscle, matched by hydrolysis of emulsion particles by LPL situated in capillaries that drain into the forearm vein.

There is a strong positive relationship between the proportion of each fatty acid in the arterialized plasma NEFA fraction and the composition of the adipose tissue veno-arterial NEFA difference (Fig. 5). That is, the fatty acid composition of the arterialized plasma at any one time can be predicted by the change in fatty acid composition as plasma crosses the adipose tissue depot. The position of individual fatty acids on this relationship changed with time during Intralipid infusion. The proportion of each fatty acid increased or decreased as the composition of the arterialized plasma became similar to the composition of the Intralipid. For example 18:2 (Fig. 5, diamonds) increased from an arterialized value of 15.3 mol% before infusion to a value of 38.9 mol% at the end of infusion, approaching its representation in Intralipid TAG of 52.2 mol%. Despite these changes in arterialized plasma and veno-arterial compositions for individual fatty acids, the relationship over all fatty acids (slope of the line in Fig. 5) did not change. However, it was again noticeable that stearic acid was an outlier in the relationship shown in Figure 5 (black star), and its position remained virtually unchanged during Intralipid infusion (open star). Its concentration in arterial NEFA compared with other fatty acids was therefore higher

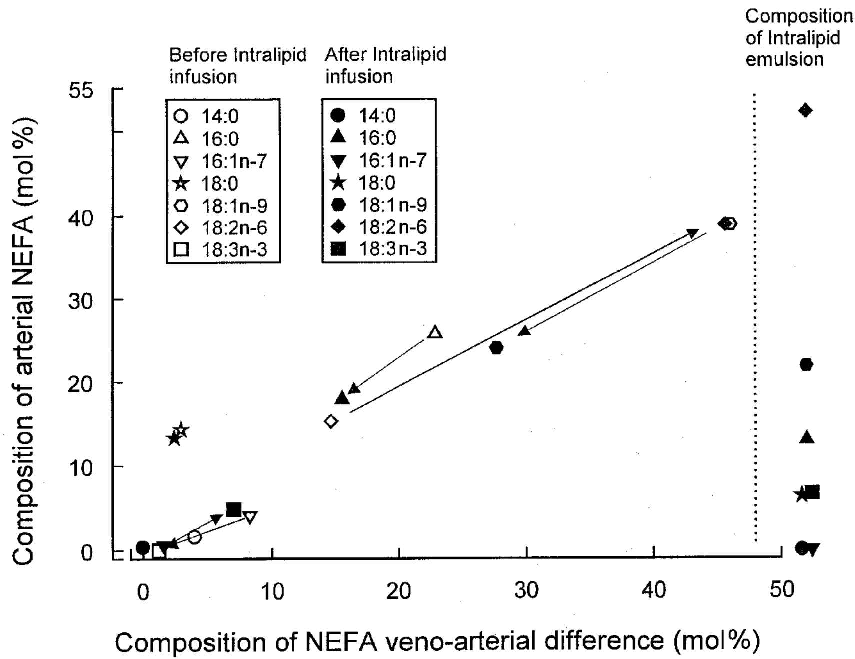

FIG. 5. Relationship between the composition of arterialized plasma NEFA and the composition of NEFA released from adipose tissue before and at the end of Intralipid infusion (comparison of values for 0 and 240 min on Fig. 3). Results are the mean of 6 subjects. Open symbols: 0 min (before Intralipid infusion); solid symbols, 240 min (end of Intralipid infusion). Each symbol represents a different fatty acid (see legend on figure). Arrows illustrate the change in proportions of different fatty acids during the infusion. The column to the right of the dashed line shows the measured composition of Intralipid TAG (mol%). For abbreviations see Figures 1 and 3.

than would be predicted from its composition in Intralipid, despite a relatively low veno-arterial difference.

The difference in concentration of palmitoleic acid (16:1) in the adipose venous and arterial plasma can be assumed to reflect endogenous lipid hydrolysis (LPL-derived fatty acids plus HSL-derived fatty acids) since its concentration in Intralipid was found to be less than 0.1%. We have previously shown that in the fasting state, NEFA leaving the adipose tissue (i.e., the veno-arterial difference) originates mainly from HSL-mediated hydrolysis of adipose tissue stores of TAG (75%) and only 25% from LPL hydrolysis (16). In the fasting state, the source of TAG for both enzymes is endogenous. From Figure 6 in the present study, one can see that the proportion of fatty acids originating from endogenous lipolysis fell steadily during the infusion and, by 240 min, over 80% of the release of NEFA from subcutaneous adipose tissue could be accounted for by exogenous lipid hydrolysis. Since the adipose tissue NEFA veno-arterial difference decreased during Intralipid infusion (13), the decrease in proportion of endogenous fatty acids leaving adipose tissue is likely to be mainly due to suppression of HSL action.

DISCUSSION

The composition of the chylomicron fraction during Intralipid infusion matched closely the measured composition of Intralipid, as expected. However, a surprising finding was that there was no significant change in the concentration of chylomicron-TAG during passage through adipose tissue. This

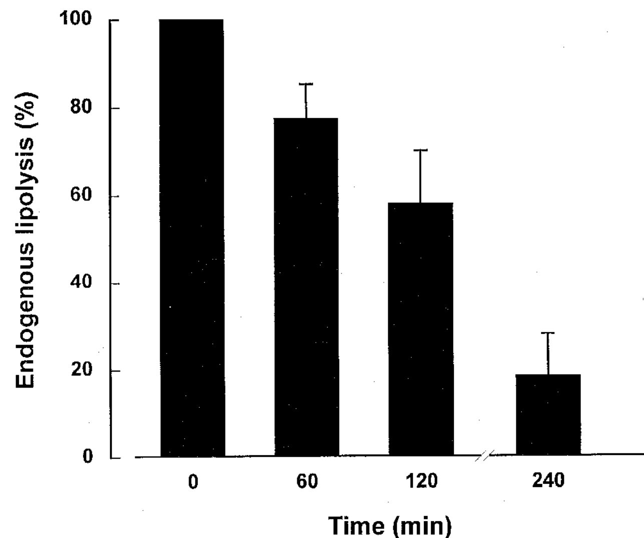

FIG. 6. Calculation of the proportion of plasma NEFA released from adipose tissue (the veno-arterial difference) that is derived from endogenous lipolysis, based on the measurement of 16:1 in the plasma NEFA fraction. Endogenous lipolysis is the net hydrolysis of TAG stored in adipose tissue plus the hydrolysis of very low density lipoprotein-TAG. The calculation and assumptions made are explained in more detail in the Methods section. The results are plotted as the mean ± SEM for 6 subjects. For abbreviations see Figures 1 and 3.

was in accordance with the total plasma TAG measurements in this study, as previously reported (13). Although not measured, the concentrations of very low density lipoprotein (VLDL)-TAG are likely to have increased during the Intralipid infusion owing to increased competition for LPL (17). As discussed in our previous paper, emulsion uptake by extrahepatic tissues may contribute to the clearance of lipid emulsions containing medium- and long-chain TAG (15). In the circumstances of this study, the detection of small changes in the concentration of chylomicron-TAG across the adipose tissue would be difficult to make because of the high concentration of chylomicron-TAG. By ignoring the action of HSL and assuming that reesterification of fatty acids within the adipocytes is virtually zero, one can calculate that the proportion of chylomicron-TAG that would be hydrolyzed to account for the difference in adipose venous and arterial plasma NEFA is 8.4, 5.5, and 3.9% for the time points 60, 120, and 240 min, respectively. However, since HSL was not completely suppressed at those time points, these will be overestimates. Therefore, it is not incompatible that changes in the composition of adipose venous NEFA (reflecting the action of LPL) were detected, and yet LPL-mediated hydrolysis of chylomicron-TAG was not measurable. The composition of the plasma NEFA pool changed steadily during Intralipid infusion, toward that of the Intralipid, as expected from previous work (5). We have shown previously that the composition of arterial NEFA reflects that of NEFA released from adipose tissue (8). This was borne out in the present studies both in the fasting state and as the composition changed during Intralipid infusion, even though arterialized NEFA concentrations did not show the close relationship with the rate of release from subcutaneous adipose tissue that we have shown before (13). NEFA released from adipose tissue arise either from intracellular lipolysis of TAG stores, under the control of HSL, or from the hydrolysis of VLDL- and chylomicron-TAG. In the present study, there was a significant decrease in the calculated action of HSL, derived either from measurements of the transcapillary flux of glycerol (13) or independently from the use of palmitoleic acid as a marker of endogenous lipid. This occurred despite no increase in plasma insulin concentrations (13). This is consistent with the plasma NEFA composition, which rapidly changed to reflect the fatty acid composition of emulsion particles, rather than VLDL- or adipose tissue-TAG. It is also consistent with the finding that the plasma NEFA composition did not appear to have reached equilibrium after 4 h of Intralipid infusion.

The relationship between arterialized NEFA composition and release from adipose tissue appeared consistent for all fatty acids other than stearic. This is somewhat different from the results of Siderova *et al*. (5), who found α-linolenic acid to be almost absent from plasma NEFA during Intralipid infusion. This difference may be explained by the fact that their study used an indirect method for the study of intravascular metabolism of fatty acids. In contrast, the technique of arterio-venous difference is much more direct. The discrepant result for stearic acid is reminiscent of our previous observation that this fatty acid is overrepresented in arterialized plasma compared with its release from adipose tissue (8). This observation could be explained by a rate of clearance of stearic acid which is less than that for other fatty acids. There is evidence of this in the case of splanchnic removal (18). In addition, the proportion of stearic acid among plasma NEFA failed to change during Intralipid infusion, which may result from a low rate of release from adipose tissue, compared with its composition in the emulsion.

In summary, infusion of Intralipid raised chylomicron-TAG and NEFA concentrations as expected. However, clearance of Intralipid-TAG across both subcutaneous adipose tissue and skeletal muscle was considerably less than would have been expected if its metabolism truly reflected chylomicron-TAG metabolism. Intralipid infusion resulted in a decrease of intracellular, HSL-catalyzed lipolysis by mechanisms which are not clear, with the result that the release of fatty acids from adipose tissue changed steadily to reflect hydrolysis of the Intralipid-TAG, presumably brought about by LPL. Our measurements of specific fatty acids again highlight the aberrant behavior of stearic acid among plasma NEFA.

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High-Fat Dairy Product Consumption Increases Δ9*c*,11*t*-18:2 (rumenic acid) and Total Lipid Concentrations of Human Milk[1]

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ABSTRACT: Conjugated octadecadienoic acids (18:2, conjugated linoleic acids) have been shown to be anticarcinogenic and may influence growth and nutrient partitioning. The Δ9*c*,11*t*-18:2 isomer (rumenic acid, RA) is most common in both food sources and human tissues. To determine if maternal diet can influence milk RA concentration, breastfeeding women ($n = 16$) were enrolled in a 3-wk crossover study. Women initially consumed minimal amounts of food containing RA during week 1, then were assigned randomly to consume diets rich in high-fat dairy foods (and thus RA) during week 2 or 3. Milk was collected by complete breast expression twice during each experimental week. Current and chronic RA intakes were estimated by 3-d dietary records and food frequency questionnaires, respectively. Estimated chronic RA intakes ranged from 49 to 659 mg/d. Dietary RA intake was greater during the high compared to the low dairy period (291 ± 75 vs. 15 ± 24 mg/d, respectively; $P < 0.0001$). Milk contained more RA during the high than the low dairy period (13.5 ± 0.1 vs. 8.2 ± 0.4 μmol/g lipid, respectively; $P < 0.0001$). Milk lipid concentration was influenced by diet, such that lipid concentration was greater during the high than the low dairy period (46.6 ± 5.0 vs. 38.3 ± 1.6 mg/g milk, respectively; $P < 0.05$). Additionally, multiple regression analyses suggested that body mass index was the primary predictor of milk RA and lipid concentrations. In summary, these data indicate that both lipid and RA concentrations of human milk can be influenced by diet.

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Conjugated dienoic derivatives of linoleic acid (conjugated linoleic acids; CLA) are a mixture of geometric and positional isomers of linoleic acid (Δ9*c*,12*c*-18:2) that have been identified primarily in ruminant meats and dairy products (1–3). Because of the high CLA content in beef and milk fat, it is estimated that CLA consumption in the United States may be several hundred mg/person/d if meat and dairy products are a significant part of the diet (4). The most prominent isoform of CLA in foods and human tissues is Δ9*c*,11*t*-18:2, which has been recently given the trivial name of rumenic acid (RA; 6).

Industrially produced mixtures of CLA have been shown to inhibit the initiation of mouse skin carcinogenesis by 7,12-dimethylbenz[a]anthracene (6) and mouse forestomach tumorigenesis induced by benzo[a]pyrene (7). Ip *et al.* (8,9) also reported that increased dietary intake of these CLA reduced significantly the number and incidence of mammary tumors in rats. Whereas these studies elucidated the role of CLA in modulating the initiation stage of cancer, the work of Belury *et al.* (10) reported CLA inhibition of mouse skin tumor promotion by the phorbol ester tetradecanoyl-phorbol-13-acetate in a dose-responsive manner. Additionally, Shultz and colleagues (11–13) reported that CLA was cytostatic and cytotoxic to human malignant melanoma, colorectal and MCF-7 breast cancer cells *in vitro*. Other types of dietary lipid such as n-3 fatty acids, when fed to animal models, have also exhibited anticarcinogenic properties (14). Although the amount of fish oil needed to elicit this response usually exceeds 10% in the diet, the effective dose of CLA to produce significant cancer protective effects might be as low as 0.1% (15). Therefore, increasing the dietary intake of CLA may be a possible, nonpharmacologic approach to cancer prevention.

Additionally, consumption of CLA during lactation has been shown to increase the concentration of CLA in milk of rats, as well as the growth and feed efficiency of the suckling pups (16). However, no studies have been conducted to determine the influence of maternal dietary CLA intake on human milk CLA concentration and health of the breastfed human infant. Recently, we reported that human milk but not infant formula contains substantial amounts of the naturally occurring active isomeric form of CLA (i.e., RA) (17); the range of RA concentrations in human milk was 8 to 19.4 μmol/g lipid. Fogerty *et al.* (1) reported values for human milk CLA concentration of 11.1 to 30.3 μmol/g lipid among mothers eating conventional diets, and values of 34.6 to 44.6 μmol/g lipid among Hare Krishna mothers. These data suggest that diet may influence human milk CLA concentration, because members of the Hare Krishna faith consume large amounts of

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Abbreviations: BMI, body mass index; CLA, conjugated linoleic acid; GC, gas chromatography; RA, rumenic acid.

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butter or ghee, as well as cheese. However, dietary intake was not reported, nor was the CLA isoform distribution.

Because of the potential influence of CLA exposure in decreasing risk of cancer in breast tissue and the possible growth-modifying effects of CLA in the neonate, we were interested in determining factors influencing the concentration and variation of CLA, especially RA, in human milk. Therefore, the purpose of this study was to determine whether high-fat dairy product consumption might affect RA concentration in human milk. We tested the following main hypotheses: (i) Short-term consumption of high-fat dairy foods by lactating women increases the concentration of RA in human milk. (ii) Chronic total RA consumption (as contributed mostly from beef and dairy products) by lactating women is correlated positively with the concentration of RA in human milk.

SUBJECTS, MATERIALS AND METHODS

Subjects. Healthy breastfeeding women ($n = 16$) between 1 and 26 mon postpartum (mean ± SEM: 9.1 ± 2.0 mon) were recruited from the Moscow, Idaho–Pullman, Washington, area. The general purpose and hypotheses of the study were explained to each subject, and written informed consent was obtained. Average age of subjects was 32 ± 2 yr (range: 21 to 38 yr). All procedures were approved by the University of Idaho Human Assurances Committee and the Washington State University Institutional Review Board.

Study design and milk collection. This study was conducted as a crossover experiment lasting 3 wk and beginning on a Sunday. Upon enrollment, women were blocked by number of months postpartum (<12 vs. ≥12 mon) and randomized to be in either Group A (high-fat dairy period during week 2; $n = 8$) or Group B (high-fat dairy period during week 3; $n = 8$). Women were asked to eat diets low in RA throughout the experiment except during their assigned week of eating diets high in dairy foods. In an attempt to standardize initial milk RA concentration, subjects were asked to consume foods containing minimal amounts of RA during week 1 (depletion period). All women were provided with a supply of food items containing an abundance of RA (i.e., cheese, yogurt, whole milk, and ice cream) during the high-fat dairy period. Because of a concern that energy intake would be decreased during the baseline and low dairy periods, subjects were advised to consume sufficient nondairy, high-energy foods during these periods.

Milk was collected by complete breast expression using an electric breast pump (Model SMR-B-R; Ameda-Egnell, Inc., Cary, IL) on days 3 and 7 of each period between 1500 and 2200 h. Subjects were asked either (i) to pump all milk from both breasts or (ii) to pump all milk from one breast while nursing on the other breast until milk flow ceased. Milk was frozen immediately in the homes of the subjects and then transported to the laboratory and stored at −80°C until later analyses.

Dietary assessments. Subjects were requested to keep complete records of their dietary intakes during the last 3 d of each period for estimation of current dietary RA intake. Chronic intake of RA was estimated by a semiquantitative food frequency questionnaire developed by the investigators which estimated mean daily dietary RA consumption. This food frequency questionnaire was designed to obtain information concerning the chronic intake of several ($n = 24$) commonly consumed food items, mostly of beef and dairy origin.

Data from the 3-d dietary records and food frequency questionnaire were analyzed using a nutrient database (18) modified by us to contain previously published values for the RA contents of foods (1–3,19–22). Results obtained from the 3-d dietary records were averaged for each period, and this value is referred to herein as "current" RA intake. Each dietary record was also analyzed for intakes of energy, protein, carbohydrate, fiber, lipid, 18:2 (linoleic acid), 18:3 (linolenic acid), 18:1 (oleic acid), and saturated, monounsaturated and polyunsaturated fatty acids as well as cholesterol. Results obtained from the food frequency questionnaire are referred to herein as "chronic" RA intake and are thought to reflect typical intakes during the previous year.

Anthropometric assessments. Self-reported height and weight of all subjects were obtained at the beginning of the study. For 11 subjects, body weight was measured weekly using an electronic scale (Model 770; Seca Alpha, Germany). Body mass index (BMI) was calculated as measured weight (kg) divided by height squared (m^2) for each subject. For the five subjects for whom weights were only self-reported, BMI was calculated from these self-reported values.

Lipid extraction and analysis of fatty acids. Lipid was extracted from milk in duplicate using standard procedures (23). Hydrolysis of lipid and methylation procedures were performed as described previously (2,24). Briefly, the lipid extract (45 mg) was hydrolyzed with 1 mL 1 N NaOH in methanol, and the free fatty acids were methylated with 1 mL 14% boron trifluoride in methanol at room temperature for 30 min. Hexane (2 mL) and water (1 mL) were added; samples were vortexed thoroughly. Following phase separation, the organic layer was dried with sodium sulfate and quantified by gas chromatography (GC). A lipid extract from a human milk pool was analyzed to determine interassay variability.

Fatty acid methyl esters were analyzed on a Hewlett-Packard gas chromatograph (model 5890; Hewlett-Packard, Avondale, PA) with an on-column injector port and flame-ionization detector. A Supelcowax 10 capillary column (60 m × 0.75 mm i.d.; phase thickness, 1.0 µm; Supelco Inc., Bellefonte, PA) was used for separation of the CLA fatty acid isomers. Conditions of GC analysis were set according to the method of Werner *et al.* (24). CLA peaks (including that of RA) were identified by comparison with the retention time of a CLA standard mixture obtained from Dr. Michael W. Pariza (Food Research Institute, University of Wisconsin, Madison, WI). Fatty acid concentrations were quantified based on a linoleic acid methyl ester standard curve. The coefficient of variation of duplicate extracts was <6.5%, while interassay variabilities of RA and linoleic acid were 6.7 and 7.2%, respectively ($n = 9$).

Statistical analysis. Data were analyzed using the General Linear Models procedure of the Statistical Analysis System

(25). The effects of low and high dairy consumption on milk lipid, RA, and fatty acid concentrations were analyzed using an analysis of variance model for a three-period, crossover design with two treatments. The model parameters included two groups (8 subjects nested within each group), three measurement periods, two dietary treatments (high and low dairy products consumption), and carry-over of treatment into the subsequent measurement period (26). Paired *t*-tests were performed to compare the milk lipid and fatty acid concentrations between sampling days within a dietary period. In all instances these were found to be statistically nonsignificant ($P > 0.05$). Subsequently, the two measurements for milk lipid, RA, and fatty acid concentrations within a dietary period were averaged, and the analysis of variance model was used to assess differences in milk lipid, RA, and fatty acid concentrations for the group, period, treatment, and carry-over means. Differences between means were considered statistically significant at the $P < 0.05$ level.

The relationships among intakes of several nutrients (as assessed by 3-d dietary records) and day 7 milk lipid and RA concentrations were tested by partial Pearson correlation analyses. Multiple regression analyses were conducted to investigate the possible relationships among milk RA and lipid concentrations, BMI, and chronic and current intake of RA. Independent effects were considered statistically significant at the $P < 0.05$ level, and interactions were considered significant at the $P < 0.10$ level.

RESULTS

Maternal anthropometrics. Self-reported height and weight of subjects at enrollment were 1.63 ± 0.01 m and 63.7 ± 3.1 kg (mean ± SEM), respectively. For the 11 subjects for whom weights were measured weekly, there was no difference between weight after the high- and low-fat dairy periods (65.3 ± 4.1 and 64.7 ± 4.1 kg, respectively). BMI was 23.9 ± 1.0, 22.0 ± 1.3, and 22.1 ± 1.3 during the baseline, high and low dairy periods, respectively.

Dietary intake. Dietary intake data are presented in Table 1. Consumption of energy, protein, lipid, RA, oleic, saturated, and monounsaturated fatty acids and cholesterol was greater during the high than the low dairy period. However, dietary intakes of carbohydrate, fiber, linoleic, linolenic, and polyunsaturated fatty acids did not differ. Chronic intake of RA as estimated from the food frequency questionnaire ranged from 49 to 659 mg/d (mean ± SEM: 227 ± 180 mg/d).

Milk fatty acid and lipid concentrations. Concentrations of RA, myristic (14:0), palmitic (16:0), stearic (18:0), and oleic acids increased ($P < 0.05$), while linoleic acid concentration tended to decrease ($P < 0.06$) during the high dairy period as compared to the low dairy period (Table 2). In addition, linolenic acid expressed as μmol/g lipid was lower, whereas palmitoleic acid (16:1), expressed as μmol/g milk, was higher during the high than the low dairy period. Milk RA concentrations of all subjects increased during the high dairy period (Fig. 1). Overall, increased high-fat dairy product consumption resulted in a significant ($P < 0.0001$) increase in RA concentration of milk [8.2 ± 0.4 (0.3 ± 0.1) vs. 13.5 ± 1.1 (0.6 ± 0.1) μmol/g lipid (μmol/g milk) during low and high dairy periods, respectively].

TABLE 1
Mean Daily Nutrient Intakes During Depletion, High and Low Dairy Periods as Estimated from 3-d Dietary Records[a]

	Dietary periods		
Variables	Depletion	High dairy	Low dairy
Energy (kJ)	10,200 ± 628	12,500 ± 1,560[b]	9,180 ± 599
Protein (g)	84 ± 6	137 ± 21[b]	82 ± 7
Carbohydrate (g)	370 ± 29	378 ± 59	327 ± 19
Fiber (g)	25 ± 2	47 ± 18	29 ± 6
Lipid (g)	71 ± 6	117 ± 17[b]	64 ± 5
Δ9*c*,11*t*-18:2 (RA)[c] (mg)	34 ± 8	291 ± 75[b]	15 ± 24
18:2 (g)	9 ± 2	8 ± 3	6 ± 1
18:3 (mg)	176 ± 26	274 ± 237	118 ± 76
18:1 (g)	13 ± 1	19 ± 5[b]	11 ± 2
Saturated fatty acids (g)	20 ± 2	49 ± 10[b]	16 ± 3
Monounsaturated fatty acids (g)	23 ± 2	38 ± 8[b]	21 ± 2
Polyunsaturated fatty acids (g)	15 ± 2	11 ± 4	13 ± 1
Cholesterol (mg)	232 ± 34	569 ± 103[b]	269 ± 33

[a]Mean ± SEM; *n* = 16.
[b]Values differ ($P < 0.05$) between the high and low dairy periods.
[c]Rumenic acid.

TABLE 2
Concentrations of Various Fatty Acids in Human Milk During Depletion, High and Low Dairy Periods[a]

	Dietary periods		
Fatty acids[b]	Depletion	High dairy	Low dairy
		μmol/g lipid (μmol/g milk)	
Δ9*c*,11*t*-18:2 (RA)	7.8 ± 0.8	13.5 ± 1.1[c]	8.2 ± 0.4
	(0.3 ± 0.1)	(0.6 ± 0.1)[c]	(0.3 ± 0.1)
14:0	190.3 ± 22.5	264.7 ± 34.3[c]	195.2 ± 11.0
	(7.4 ± 1.2)	(11.3 ± 2.1)[c]	(6.9 ± 0.7)
16:0	500.0 ± 39.6	707.0 ± 51.5[c]	511.3 ± 16.4
	(19.4 ± 4.2)	(27.5 ± 5.4)[c]	(18.5 ± 1.7)
16:1	65.1 ± 7.0	81.7 ± 10.2	65.8 ± 3.1
	(2.5 ± 0.4)	(3.7 ± 0.6)[c]	(2.6 ± 0.2)
18:0	143.9 ± 13.4	219.0 ± 23.1[c]	154.3 ± 7.4
	(6.1 ± 1.3)	(9.5 ± 1.8)[c]	(5.9 ± 0.6)
18:1	831.5 ± 73.1	1055.0 ± 103.4[c]	874.3 ± 33.3
	(34.0 ± 6.6)	(48.5 ± 6.7)[c]	(34.3 ± 2.1)
18:2	415.4 ± 34.1	328.2 ± 56.5	419.2 ± 18.1
	(17.6 ± 3.5)	(17.5 ± 2.3)	(17.4 ± 0.9)
18:3	17.2 ± 1.5	10.7 ± 3.6[c]	17.6 ± 1.2
	(1.4 ± 0.3)	(1.6 ± 0.5)	(1.5 ± 0.1)

[a]Mean ± SEM; *n* = 16.
[b]These fatty acids accounted for approximately 92, 89, and 91% of the total fatty acid composition identified in milk samples during the baseline, high and low dairy periods, respectively.
[c]Values differ ($P < 0.05$) between the high and low dairy periods. For abbreviation see Table 1.

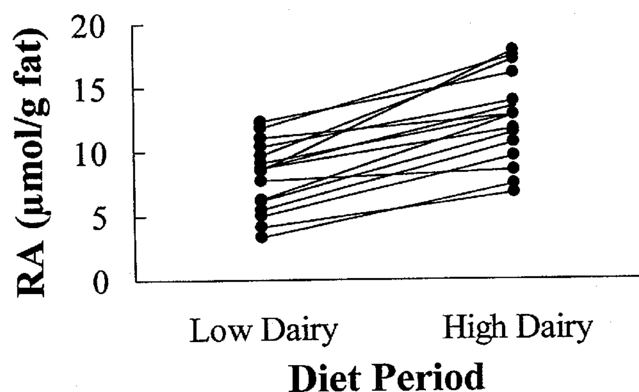

FIG. 1. Concentration of rumenic acid (RA; 9*c*,11*t*-18:2) in milk from individual subjects during the low and high dairy periods. Mean RA concentration was lower during the low than the high dairy periods (8.2 ± 0.4 vs. 13.5 ± 1.1 μmol/g lipid, respectively; $P < 0.0001$).

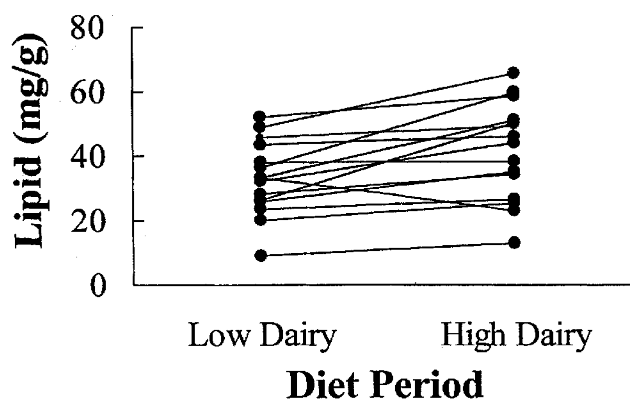

FIG. 2. Lipid concentrations in milk from individual subjects during the low and high dairy periods. Mean lipid concentration was lower during the low than the high dairy periods (38.3 ± 1.6 vs. 45.6 ± 5.0 mg/g milk, respectively; $P < 0.05$). It should be noted that data from one subject (26 mon postpartum; 108.2 and 114.7 mg lipid/g milk during the low and high dairy periods, respectively) were omitted from the figure, because they were substantially greater than all others, and thus were not suitable for this illustration.

Milk lipid concentrations for each subject are illustrated in Figure 2. Consumption of high-fat dairy products resulted in an overall increase in milk lipid, as compared to that found in the low dairy period (45.6 ± 5.0 vs. 38.3 ± 1.6 mg/g milk, respectively; $P < 0.05$).

Factors related to milk composition. After controlling for dietary intakes of energy, protein, lipid, linoleic acid, saturated fatty acids, and cholesterol, analyses using partial Pearson correlation coefficients suggested that current dietary intakes of RA ($r = 0.32$), oleic ($r = -0.41$), and monounsaturated fatty acids ($r = 0.38$) were correlated with milk RA concentration (μmol/g lipid). However, when milk RA was expressed as μmol/g milk, there were no statistical relationships between these dietary components and milk RA. Further, there were no significant relationships between milk lipid concentrations and any of the dietary nutrients investigated.

Although milk RA and lipid concentrations of most subjects increased during the high dairy period, there was considerable between-subject variation in all periods. Thus, multiple regression analyses were conducted to explore the possible relationships among milk lipid and RA concentrations, BMI, and chronic and current RA intakes; the interaction between BMI and chronic RA intake was also included in the model, because the effect of chronic RA intake on milk RA concentration may be influenced by BMI of subjects (Table 3). BMI was related positively to milk lipid concentra-

TABLE 3
Results (β ± SEM) of Multiple Regression Analyses Investigating the Relationships Among BMI, Chronic and Current RA Intakes, and Milk Lipid and RA Concentrations During Depletion, High and Low Dairy Periods[a]

Dietary period	Independent variables	Dependent variables: Lipid (mg/g milk)	CLA (μmol/g lipid)
Depletion	Constant	−87.2 ± 42.1	−9.31 ± 5.38
	BMI	5.68 ± 1.78[b]	0.60 ± 0.23[b]
	Chronic RA intake	0.23 ± 0.15	0.04 ± 0.02
	Current RA intake	−0.23 ± 0.14	0.02 ± 0.02
	BMI × chronic RA intake	−0.01 ± 0.006	−0.001 ± 0.0007
High dairy	Constant	−86.1 ± 53.9	−7.88 ± 5.56
	BMI	5.55 ± 2.30[b]	0.69 ± 0.24[b]
	Chronic RA intake	0.27 ± 0.18	0.07 ± 0.02[b]
	Current RA intake	−0.004 ± 0.04	0.003 ± 0.004
	BMI × chronic RA intake	−0.01 ± 0.007	−0.002 ± 0.00072[b]
Low dairy	Constant	−91.4 ± 48.4	−6.16 ± 6.26
	BMI	5.31 ± 2.02[b]	0.51 ± 0.26
	Chronic RA intake	0.21 ± 0.17	0.03 ± 0.02
	Current RA intake	0.08 ± 0.25	0.02 ± 0.03
	BMI × chronic RA intake	−0.008 ± 0.007	−0.001 ± 0.0009

[a]$n = 16$; BMI, body mass index (kg/m^2); CLA, conjugated linoleic acid; for other abbreviation see Table 1.
[b]Significant relationship ($P \leq 0.05$ for individual effects and $P < 0.10$ for interactions).

tion during all periods and to RA concentration during the depletion and high dairy periods. After controlling for BMI, chronic RA intake was correlated positively with milk RA concentration during the high dairy period. Interestingly, after controlling for BMI and chronic RA intake, current RA intake was not a significant predictor of milk lipid or RA concentration in any period.

The interaction between BMI and chronic RA intake was related to milk RA concentration during the high dairy period, such that the positive effect of chronic RA intake on milk RA concentration was greater in subjects with lower BMI. In addition, we observed similar relationships for milk RA concentration when expressed as μmol/g milk (data not shown).

DISCUSSION

Results from this study indicate that the RA concentration in human milk increases when the consumption of high-fat dairy products increases. Previously, we reported that human milk contains predominantly the RA isomer of CLA (17), and we suspected that variation among milk samples was primarily due to maternal intake of RA. The presence of RA in human milk has been confirmed by Jensen and colleagues (27) using GC/mass spectrometry. Data from the only other study reporting CLA concentration in human milk also suggested that diet may also influence the CLA concentration of human milk, such that women of the Hare Krishna faith produced milk with higher CLA concentration than did women consuming conventional diets (1). It is noteworthy that the CLA concentration of milk produced by Hare Krishna women was much higher and of greater range than concentrations reported in this study, and that this difference may be due to their greater use of butter or ghee in food preparation (28).

Data from the present study also suggest that dietary RA, especially chronic intake, is related positively to milk RA concentration. Although the dietary intakes of energy, protein, lipid, and cholesterol increased during the high dairy period, the aforementioned dietary nutrients were not correlated with milk RA concentration. The dietary intakes of oleic and monounsaturated fatty acids were correlated with milk RA concentration, although their correlation coefficients were not as strong as that of current dietary RA with milk RA concentration. However, when both current and chronic intake of RA were included in the multiple regression analyses, only chronic RA intake remained significant, suggesting that current intake of RA is not as strong of a predictor of milk RA concentration, at least not in the time frame used for this study. This may mean that small changes in milk RA concentration can be made in a short period of high-fat dairy product consumption. However, to observe large changes in milk RA concentration a high RA diet must be consumed chronically.

In addition, multiple regression analyses showed that BMI was a primary factor in predicting milk RA concentration. Analyses also suggest that BMI modifies the effect of chronic RA on milk RA concentrations, such that chronic RA intake was a more powerful predictor of milk RA concentration in women with lesser body fat content. These observations suggest that the leaner subjects experience substantial mobilization of adipose stores even when consumption of high-fat dairy foods is encouraged. The interactions between current RA intake and BMI, and between energy intake and BMI on milk RA concentration were not significant.

To our surprise, these data also show that milk lipid concentration increased during the high dairy period. One explanation may be that RA enhanced *de novo* lipid production in the mammary gland or lipid partitioning to the mammary gland. However, results of the partial Pearson correlation and multiple regression analyses showed that neither chronic nor current dietary RA intakes were related to milk lipid concentration. Additionally, the intakes of energy, protein, lipid, and carbohydrate were not correlated with milk lipid concentration. Other investigators have shown previously that milk fat concentration in humans is not influenced by energy intake (29–31). Thus, our findings raise an important question as to what dietary factor(s) resulted in milk lipid depression observed during the low dairy period.

Ruel *et al*. (31) reported that variability in milk lipid concentration could be explained partly by body fatness. Multiple regression analyses of our data also suggested that BMI was a significant factor in predicting milk lipid concentration. Further, Lovelady *et al*. (32) found a significant association between maternal percent body fat and milk lipid. These investigators suggested that an interaction between body fatness and dietary lipid existed for milk lipid concentration. Secondary analysis of our data, however, shows no interaction between dietary lipid intake and BMI on milk lipid concentration.

Recent reports indicate that lactating cows (33–36) and mice (37) are susceptible to milk lipid depression when they are fed partially hydrogenated vegetable oils containing *trans* fatty acids. Koletzko (38) found that *trans* fatty acid concentrations in plasma of premature infants were inversely related to long-chain polyunsaturated fatty acid biosynthesis pathways. Therefore, it is possible that substitution of foods containing industrially produced *trans* fatty acids for dairy lipid (e.g., substitution of margarine for butter) during the low dairy period may have decreased milk lipid content by inhibition of lipid partitioning to or *de novo* fatty acid synthesis in the mammary gland. Unfortunately, we have no information on the *trans* fatty acid intakes of our study participants.

Noteworthy are two limitations in this study. First, the food frequency questionnaire used to estimate chronic RA intake is semiquantitative in nature and is not expected to provide an accurate estimate of chronic RA intake (39). Nonetheless, it is clear that, although information collected using this instrument may not be quantitatively accurate, it does provide useful qualitative information concerning relative chronic RA intakes. Second, the relative homogeneity of subjects in regard to body weight and adiposity resulted in limitations of power when conducting multiple regression analyses using these variables. It is suggested that further studies designed

to investigate the influence of body fatness on milk fat and fatty acid concentrations take this factor into account.

The significance of milk RA concentration to maternal and infant health is not known. However, the potential for RA to prevent cancerous growth in mammary tissue of breast-feeding women is of public health importance. During both pregnancy and lactation, the mammary gland may be sensitive to the effects of dietary lipid, such as carcinogenic (e.g., n-6 polyunsaturated) and anticarcinogenic (e.g., RA) fatty acids. In fact, Hilakivi-Clarke *et al*. (40) recently reported that a diet high in n-6 polyunsaturated fatty acids, mainly linoleic acid, consumed by rats during pregnancy increases their susceptibility to mammary tumorigenesis. Epidemiologic data suggest independent protective effects of lactation (41–43) and bovine milk consumption (44) on breast cancer incidence in humans; to our knowledge, the importance of the interaction between lactation and dairy consumption has not been tested. Thus, we suggest further research be done to test the hypothesis that a portion of the inhibitory nature of lactation on the incidence of breast cancer might be due to the increased exposure of mammary tissue to dietary anticarcinogens (such as RA) due to lactation. Further, we hypothesize that an increase in RA intake during lactation might be related to an increased magnitude of this exposure, thus resulting in decreased risk of breast cancer.

CLA consumption may also have health benefits to the infant, since RA has been shown to enhance growth of the suckling rat pup (16) and to increase lean body mass in mice (45). However, the relevance of these findings in animal models to the human infant is unknown.

In conclusion, our data show that increased maternal intake of high-fat dairy foods can increase the concentrations of both lipid and RA in human milk. Further, chronic RA intake is correlated positively with milk RA concentration. Thus, these data support our initial hypotheses. Further research is necessary to determine the public health significance of these findings.

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Elevated Plasma Levels of $F_{2\alpha}$ Isoprostane in Cystic Fibrosis

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ABSTRACT: Cystic fibrosis (CF) is associated with chronic lung infection, inflammation, and elevated indices of oxidative stress. Recently, isoprostanes were shown to be a reliable *in vivo* marker of oxidant injury with 8-*iso*-$PGF_{2\alpha}$, shown to cause airflow obstruction and plasma exudation in guinea pig lung. The present study was designed to examine the relationship between 8-iso-$PGF_{2\alpha}$ levels, plasma antioxidants, and clinical status in CF. We hypothesized that plasma 8-iso-$PGF_{2\alpha}$ levels would be higher in subjects with CF compared to healthy controls. Plasma 8-iso-$PGF_{2\alpha}$ levels were prospectively measured in 22 subjects with CF and nine healthy controls using an 8-isoprostane enzyme immunoassay kit along with plasma vitamins A, E, and β-carotene. Plasma 8-*iso*-$PGF_{2\alpha}$ levels were shown to be significantly elevated in the CF subjects compared to controls (319.6 ± 52.6 vs. 145.0 ± 21.0 pg/mL, $P = 0.005$). Plasma levels of antioxidants were significantly lower for the CF subjects compared to the controls (vitamin A, $P < 0.003$; vitamin E, $P < 0.001$; and β-carotene, $P < 0.01$). This study confirms significantly elevated lipid peroxidation in CF using 8-*iso*-$PGF_{2\alpha}$ levels.

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Cystic fibrosis (CF) is associated with chronic lung infection and inflammation leading to progressive lung destruction. While mediators are important to resolution of inflammation, they do have some negative effects, which include free radical generation and release of hydrogen peroxide and proteinases by activated immunocytes, which contribute to cellular damage and reduced lung function (1–3). Peroxidation of lipids is the main feature of oxidant injury (4), and many authors reported increased lipid peroxidation in CF (2,5–9). In a comprehensive review, van der Vliet *et al.* (10) reported potential contributors to oxidative stress in CF, such as factors which increase oxidative reactions and metabolic rate, chronic infection and inflammation with increased immune responses, cytokine-mediated reactions, malabsorption of antioxidants, and disordered iron metabolism. Much of the morbidity and most of the mortality in CF are due to progressive lung disease (11), so strategies that have the potential to impact on the rate of progression seem worthy of further investigation. Antioxidant supplementation may attenuate lipid peroxidation (5), and Winklhofer-Roob *et al.* (1) showed that supplementation with β-carotene (a potent lipid-soluble antioxidant) can attenuate both lung inflammation and lipid peroxidation. Reduced levels of plasma antioxidants in CF were also associated with increased lipid peroxidation compared to controls.

Many authors (10,12–14) questioned the reliability of some commonly used methods of assessing lipid peroxidation. In a review of methodology used to study free radical reactions in humans, Halliwell and Grootveld (13) point out that lack of adequate methodology hindered examination of the contribution of free radical production to tissue injury vs. production secondary to the injury. The assessment of breath pentane (14) and thiobarbituric acid-reactive substances were reported to be unreliable measures of lipid peroxidation in human body fluids (12). While these methods appear to be accurate for measurement of lipid peroxidation *in vitro*, they have limitations when used to measure oxidative stress *in vivo*.

Morrow *et al.* (4) reported (4) the discovery of a unique series of prostaglandin-like compounds, termed isoprostanes, produced in humans *in vivo*, with 8-*iso*-$PGF_{2\alpha}$ being the most abundant. Isoprostanes are produced by free-radical catalyzed peroxidation of arachidonic acid, independently of the cyclooxygenase enzyme (4). They are a reliable measure of *in vivo* oxidant injury while being produced in quantities an order of magnitude higher than cyclooxygenase-derived prostaglandins (4). In a recent review of the isoprostanes, Morrow and Roberts (4) reported that 8-*iso*-$PGF_{2\alpha}$ is an extremely potent renal vasoconstrictor. In the pulmonary bed in rats and rabbits, it acts primarily as an antagonist of platelet thromboxane receptors but interacts with a receptor on vascular smooth muscle that is distinct from the thromboxane receptor (4). Recently, 8-*iso*-$PGF_{2\alpha}$ was shown to cause airflow obstruction and plasma exudation in guinea pig lung *in vivo* (15). However, the role of the isoprostanes in the progression of lung disease in CF was not investigated.

The objectives of this study were to measure 8-*iso*-$PGF_{2\alpha}$ levels, plasma levels of β-carotene, retinol and α-tocopherol

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Abbreviations: BSA, body surface area; CF, cystic fibrosis; ELISA, enzyme-linked immunosorbent assay; $\%FEV_1$, forced expiratory volume in 1 s as a percentage of the predicted; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$; RBC, red blood cell.

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and red blood cell (RBC) membrane fatty acids in patients with CF, to compare these levels to those in normal controls and to examine the relationship of these variables with measures of clinical status in the CF patients. The hypothesis to be tested was that 8-*iso*-$PGF_{2\alpha}$ levels were elevated in CF.

METHODS

This study was a cross-sectional measurement of plasma 8-*iso*-$PGF_{2\alpha}$ levels, plasma antioxidant (retinol, α-tocopherol, and β-carotene), RBC membrane fatty acids, clinical status in 22 patients with CF, mean age 17.4 yr (range 5.8 to 37.8 yr) attending the John Hunter Children's Hospital Cystic Fibrosis clinic and 9 healthy controls, mean age 29.8 yr (range 22.8 to 46.1 yr). Ethics approval was obtained from the Hunter Area Health Service and the University of Newcastle Ethics Committees. Written consent was obtained from the subjects, parent, or both. Subjects were enrolled continuously through the CF outpatient clinic, and a 2-mL blood sample was obtained for isoprostane and fatty acid analysis. An exclusion criterion was aged less than 4 yr. Healthy controls were recruited from advertisements placed on hospital and university notice boards. For control subjects, exclusion criteria were relevant gastrointestinal, medical, or surgical history. Clinical status was measured by the Shwachman score as part of routine clinical care (16) which evaluates subjects in the four categories of nutritional status, physical findings, X-ray findings, and general activity. Ten of the CF subjects were receiving antibiotics for acute pulmonary exacerbation with 10 not experiencing acute exacerbation. The methods of Cameron (17) were used for all the anthropometric measures. Heights were measured with a Holtain, Crymych, Dyfed, stadiometer. Weights were recorded to the nearest 0.001 g on GEC/Avery digital scales, model number 824/890. FEV1, the forced expiratory volume in 1 s (18), was expressed as the percentage predicted based on weight and height.

Blood collection. Whole blood was collected into EDTA-coated tubes containing 2 mg of reduced glutathione acting as an antioxidant (Sigma Chemical Company, St. Louis, MO). The samples were centrifuged at 3000 rpm for 10 min at 4°C to separate the RBC from the plasma. Aliquots 1-mL of each of the lower cell layer and the upper plasma fraction were removed and stored separately at −80°C until commencement of analysis with 200 μg butylated hydroxytoluene (Sigma Chemical Company) added to the plasma fraction and vortexed prior to storage.

Total (free and esterified) isoprostane analysis. To 1 mL of sample, tritiated thromboxane B_2 (Amersham, Buckinghamshire, United Kingdom) of a known amount of counts per minute (cpm) was added to enable later determination of recovery. Thromboxane behaves similarly to isoprostane during subsequent purification, allowing recovery to be determined. An equal volume of 15% wt/vol KOH was added, and the sample was incubated at 40°C for 60 min to cleave esterified isoprostane. The sample was purified by passing through a Sep-Pak C-18 reverse-phase cartridge (Waters, Milford MA), and the solvent was evaporated with N_2 to permit reconstitution in assay kit buffer. Using a liquid scintillation counter, 500 μL of the purified sample was counted with 15 mL of aqueous biodegradable counting scintillant (Amersham, Arlington Heights, IL). A quantity of the remaining portion was analyzed with an 8-isoprostane enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI). Absorbance values were measured using a plate reader (Molecular Devices, V_{max}, Kinetic microplate reader; Beckman) set to record at a wavelength of 405 nm, and the raw data corrected for recovery. The immunoassay was validated to obtain a high correlation (0.99) between added known amounts of 8-*iso*-$PGF_{2\alpha}$ and the concentration measured by the immunoassay kit.

Sep-Pak extraction procedure. The Sep-Pak C-18 reverse-phase cartridges (Waters) were rinsed with 5 mL of methanol followed by 5 mL of ultrapure water. The samples were passed through the cartridges then rinsed with 5 mL of ultrapure water and allowed to dry. They were then rinsed with 5 mL of hexane. The 8-*iso*-$PGF_{2\alpha}$ was eluted with 5 mL ethyl acetate (containing 1% methanol).

Preparation of erythrocyte membranes. RBC previously stored at −80°C were thawed to room temperature. The erythrocytes were lysed and their membranes solubilized and purified using a method described by Tomoda *et al.* (19), and modified by Brown *et al.* (20). Cells were washed with 12 mL of hypotonic Tris buffer (10 mM Tris hydroxymethylamino methane/5 mM ascorbate buffer, pH 7.4) and vortexed. After standing on ice for 5 min, 12 mL of 0.25 M glucose solution was added. The sample was vortexed, allowed to stand on ice for a further 5 min, then centrifuged at 10,000 rpm for 10 min, 4°C. This procedure was repeated twice more (resuspending the pellet by vortexing), using the same quantities of Tris and glucose solutions as described above, but centrifuged at 12,000 rpm for 10 min, 4°C, and then 15,000 rpm for 20 min, 4°C, respectively. The pellet was then resuspended in approximately 1 mL of the remaining final supernatant and stored at −20°C prior to analysis for fatty acid composition.

Total (free and esterified) fatty acid analysis. Total fatty acids were determined using the method established by Lepage and Roy (1986) (21). Two mL of a methanol/toluene (4:1, vol/vol) mixture containing 13:0 (0.02 mg/mL) and 21:0 (0.02 mg/mL) as internal standards (Nu-Chek-Prep, Elysian MN) was added to the RBC membrane preparation. Methylation of fatty acids was carried out by addition of 200 μL of acetyl chloride while vortexing, then subsequent heating for 60 min at 100°C. After cooling, the reaction was then stopped by the addition of 5 mL 6% K_2CO_3. The sample was centrifuged at 3000 rpm for 10 min at 5°C, to facilitate separation of layers. An aliquot of the upper toluene layer was used for gas chromatographic analysis of the fatty acid methyl esters, using a 30 m × 0.25 mm (DB-225) fused carbon-silica column, coated with cyanopropylphenyl (J&W Scientific, Folsom, CA) and a method modified from Garg and Blake (22). Both injector and detector port temperatures were set at 250°C. The oven temperature was 170°C for 2 min, increased 10°C/min to 190°C

and held for 1 min, then increased 3°C/min up to 220°C and maintained for 15 min to give a total run time of 30 min. The chromatograph was equipped with a flame-ionization detector. Sample fatty acid methyl ester peaks were identified by comparing their retention times with those of a standard mixture.

Vitamin intake. Subjects' self-reported current intake of vitamin and mineral supplements and the quantities of vitamins A, E, β-carotene, C, zinc, and selenium were determined from these.

Data analysis. Statistical analysis was performed using Minitab 10Xtra for Windows. The mean ± standard errors are reported. Statistical comparisons were performed using *t*-tests for normally distributed data with a significance level of $P < 0.05$. Correlation studies were undertaken to assess how closely the variables studied were related within both subject groups (CF and controls), and regression analysis was used to explore how much of the variation in isoprostane levels could be explained by the other variables.

RESULTS

Twenty-two subjects with CF and nine controls were studied. Clinical data are provided in Table 1. Clinical characteristics of the subjects with CF and the controls are summarized in Table 1. There was a significant difference in age between the subject groups, and mean height z-score was significantly lower in CF. Although mean weight for height was lower in the CF group, this did not reach significance. All plasma antioxidant levels were significantly lower in the CF subjects despite 13 subjects taking vitamin supplements routinely. These self-reported vitamin intakes are reported in Table 2. Nine subjects with CF were not taking multivitamin supplements. One subject with CF was taking a 50 mg/d selenium supplement (with a corresponding total 8-*iso*-$PGF_{2\alpha}$ level = 133.3 pg/mL), and one person was taking 3 mg/d β-carotene (with a total 8-*iso*-$PGF_{2\alpha}$ level = 155.0 pg/mL).

Isoprostane results. Figure 1 compares the 8-*iso*-$PGF_{2\alpha}$ values in CF subjects and controls. Significantly higher levels of 8-*iso*-$PGF_{2\alpha}$ were found in the CF group compared to the controls (319.6 ± 52.6 pg/mL vs. 145.0 ± 21.0 pg/mL, $P = 0.005$). While there was a trend toward increased 8-*iso*-$PGF_{2\alpha}$ levels in the CF subjects, less than 14 yr compared to controls (Table 1), $P = 0.059$ the 8-*iso*-$PGF_{2\alpha}$ levels were greater for the CF subjects greater than 14 yr compared, $P = 0.038$. While higher levels of 8-*iso*-$PGF_{2\alpha}$ were found in the group experiencing an acute exacerbation of pulmonary disease at the time of blood collection for the study, the difference was not statistically significant [nonacute ($n = 10$) 275 ± 184 pg/mL vs. acute ($n = 12$) 374 ± 308 pg/mL, $P > 0.05$]. For CF subjects taking any type of vitamin supplement, there was no difference between mean total 8-*iso*-$PGF_{2\alpha}$ level when compared to those not taking supplements (334 ± 73 pg/mL, $n = 13$ vs. 299 ± 78 pg/mL, $n = 9$, $P = 0.75$). When the 8-*iso*-$PGF_{2\alpha}$ levels for the 12 subjects with CF ≥ 14 yr of age were compared with the controls, the difference still remained significant [$P = 0.038$, 95% confidence interval (13,373 pg/mL)]. Mean 8-*iso*-$PGF_{2\alpha}$ levels (± standard error) for subjects with CF < 14 yr ($n = 10$) were 297.9 ± 68.4 pg/mL, for those ≥ 14 yr ($n = 12$), 337.9 ± 80.1 pg/mL and for controls 145 ± 21.0 pg/mL.

Within each subject group, there were no significant correlations between 8-*iso*-$PGF_{2\alpha}$ and age (Fig. 1), growth parameters, or plasma levels of vitamins A and E. For the CF group alone, there were no significant correlations between 8-*iso*-$PGF_{2\alpha}$ and FEV_1 (Fig. 2) function or erythrocyte membrane fatty acids. There was, however, a significant correlation be-

TABLE 2
Self-Reported Vitamin Intake from Supplements for Subjects with CF Who Regularly Consume Them[a]

Supplement	*n*	Median (interquartile range)
Retinol (i.u.)	13	1250 (650, 7000)
α-Tocopherol (i.u.)	13	70 (5, 126)
Ascorbic acid (mg)	13	86 (0, 86.3)
β-Carotene (mg)	1	3 mg
Zinc (mg)	13	1.7 (0, 1.7)
Selenium (mg)	1	50 mg

[a]Data are median and interquartile range to highlight the intersubject variability of the CF subjects. See Table 1 for abbreviation.

TABLE 1
Clinical Characteristics of Subjects with CF <14 yr, ≥14 yr, and Healthy Controls Highlighting Shorter Stature and Lower Plasma Antioxidant Concentrations[a]

Clinical variable	CF < 14 yr (*n* = 10)	CF ≥ 14 yr (*n* = 12)	Control (*n* = 9)
Age (yr)	11.2 ± 0.8*	22.5 ± 1.8	29.8 ± 2.7
z-score for height	–0.67 ± 0.43*	–1.0 ± 0.24*	0.12 ± 0.29
%Weight for height	105.5 ± 5.1	105.6 ± 4.4	114.1 ± 6.8
FEV_1 (% predicted)	84.2 ± 4.8	67.1 ± 7.9	n/a
8-*iso*-$PGF_{2\alpha}$ (pg/mL)	297.6 ± 68.4	337.9 ± 80.1	145.0 ± 21.0
Retinol (μmol/L)	1.28 ± 0.15**	1.2 ± 0.19**	2.18 ± 0.19
α-Tocopherol (μmol/L)	14.1 ± 1.9**	11.0 ± 1.6**	27.1 ± 2.55
β-Carotene (μmol/L)	0.09 ± 0.03**	0.04 ± 0.01**	0.48 ± 0.11

[a]Data are mean ± standard error. *Difference from controls = $P < 0.05$. **Difference from controls = $P < 0.01$. CF, cystic fibrosis; FEV, forced expiratory volume in 1 s as a percentage of the predicted; n/a, not applicable; $PGF_{2\alpha}$, prostaglandin $F_{2\alpha}$.

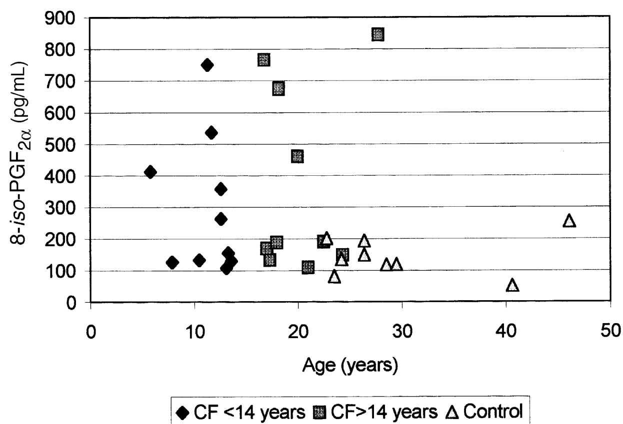

FIG. 1. Plot of 8-*iso*-prostaglandin $F_{2\alpha}$ (8-*iso*-$PGF_{2\alpha}$) concentrations in subjects with cystic fibrosis (CF) <14 yr ($n = 10$), ≥ 14 yr ($n = 12$), and controls ($n = 9$).

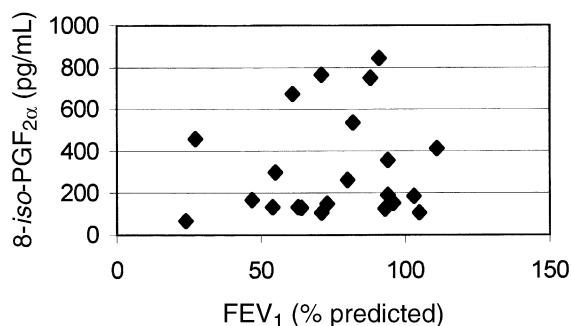

FIG. 2. Plot of 8-*iso*-$PGF_{2\alpha}$ concentrations vs. pulmonary function [forced expiratory volume in 1 s as a percentage of the predicted (FEV_1), % predicted].

tween β-carotene and FEV_1% predicted, r = 0.58, P = 0.009 (Spearman's rank correlation), which remained significant when the one outlier with a high plasma β-carotene was removed, r = 0.57, P = 0.013.

Fatty acid results. Table 3 summarizes the erythrocyte membrane fatty acid results for CF and controls. Subjects with CF had significantly more 18:1 with less 24:0 than normal controls. Females with CF had significantly less 22:5 compared to the males (2.74 ± 0.08% vs. 3.13 ± 0.11%, P = 0.011) with no other significant differences in fatty acid content. Subjects taking vitamin supplements had significantly less 22:5 compared to those not taking supplements (3.2 ± 0.11% vs. 2.78 ± 0.09%, P = 0.0078).

DISCUSSION

We demonstrated that the level of 8-*iso*-$PGF_{2\alpha}$, a reliable marker of *in vivo* oxidative stress in CF, is higher in relatively healthy, young people with CF. This is despite mean plasma levels of vitamins A and E within the laboratory normal range and a clinic policy of multivitamin supplementation, with vitamins A and E supplements for patients known to have low-plasma vitamin A and/or E. This finding is consistent with other reports (2,5–9) and confirms that lipid peroxidation is increased in CF using a reliable *in vivo* marker of oxidative stress. The 8-*iso*-$PGF_{2\alpha}$ levels for the 12 subjects with CF ≥ 14 yr of age were elevated when compared with the controls (P = 0.038).

No correlation exists between 8-*iso*-$PGF_{2\alpha}$ levels and the degree of pulmonary dysfunction. However, the group with acute exacerbation of pulmonary disease did show higher levels of 8-*iso*-$PGF_{2\alpha}$ compared to those who were clinically stable, although this difference was not statistically significant, $P > 0.05$. The numbers in each CF group (10 nonacute, 12 acute) were small, and baseline data were not available for the acute CF group. Therefore the potential bias toward increased 8-*iso*-$PGF_{2\alpha}$ levels could not be assessed and the results should be interpreted with caution. Further studies are currently underway to examine 8-*iso*-$PGF_{2\alpha}$ levels during pulmonary exacerbation as the results will be important in determining whether a rationale for reducing 8-*iso*-$PGF_{2\alpha}$ levels is important in CF lung disease.

Subjects with CF were relatively healthy, as indicated by their high percentage predicted FEV_1, high percentage weights for height, and, as we have previously shown, high energy intakes (23). There was, however, a degree of height stunting, as suggested by the lower mean z-score for height. There was no significant linear relationship between 8-*iso*-$PGF_{2\alpha}$ levels and clinical status or plasma antioxidants (A, E, and β-carotene) although antioxidant levels were all significantly lower in the CF subjects, despite supplementation with vitamins A and E in 13 of the 22 subjects. Plasma β-carotene for the CF group was lower than the lower limit of the laboratory normal range, with eight patients having no β-carotene detectable in their plasma (Table 2). There was also a significant correlation with pulmonary function although this should be interpreted with caution due to the large number of patients whose plasma β-carotene was less than this lower limit of detection. Low β-carotene in CF was also found by other groups (1,2,5,24) and was recently the subject of intervention trials (1,5,25). Supplementation with β-carotene reduced lipid peroxidation (5) and also lung inflammation (1). Winklhofer-

TABLE 3
Comparison of Red Blood Cell Membrane Fatty Acids in Subjects with CF and Controls and Between Subjects with CF Taking Vitamin Supplements Compared to Thkose Not Taking Vitamin Supplements[a]

Fatty acid (%)	CF (n = 22)	Control (n = 9)	P-value, CF vs. controls	CF, no supplements (n = 13)	CF, supplements (n = 9)	P-value, unsupplemented vs. supplements
16:0	20.37 ± 0.32	19.73 ± 0.16	0.081	20.10 ± 0.61	20.56 ± 0.34	0.53
18:0	16.31 ± 0.16	16.32 ± 0.16	0.98	16.33 ± 0.18	16.30 ± 0.24	0.92
18:1	13.55 ± 0.26	12.64 ± 0.28	0.026	13.74 ± 0.36	13.42 ± 0.37	0.54
18:2	8.57 ± 0.34	9.37 ± 0.38	0.14	8.29 ± 0.63	8.76 ± 0.40	0.54
20:3	2.32 ± 0.08	2.20 ± 0.16	0.53	2.18 ± 0.10	2.41 ± 0.10	0.13
20:4	15.59 ± 0.22	15.55 ± 0.39	0.94	15.83 ± 0.37	15.42 ± 0.27	0.38
22:0	2.13 ± 0.07	2.20 ± 0.08	0.51	2.13 ± 0.10	2.13 ± 0.09	0.99
22:4	3.71 ± 0.14	3.46 ± 0.12	0.21	3.62 ± 0.10	3.77 ± 0.224	0.58
22:5	2.95 ± 0.08	2.82 ± 0.09	0.29	3.20 ± 0.11	2.78 ± 0.09	0.0078
22:6	3.99 ± 0.17	4.80 ± 0.44	0.12	4.12 ± 0.26	3.91 ± 0.22	0.54
24:0	5.06 ± 0.13	5.77 ± 0.19	0.007	5.11 ± 0.24	5.03 ± 0.15	0.79
24:1	5.45 ± 0.14	5.14 ± 0.20	0.22	5.34 ± 0.16	5.53 ± 0.21	0.49

[a]Data are mean ± standard error of the mean. See Table 1 for abbreviation.

Roob *et al.* (25) found that the β-carotene dose required to normalize β-carotene status was 0.5 mg/kg/d. While plasma β-carotene correlated significantly with pulmonary function in the present study, the total antioxidant intake may be more important in limiting lipid peroxidation. Because antioxidants inhibit 8-*iso*-$PGF_{2\alpha}$ production by scavenging free radicals, any potential association with clinical status may have been confounded by other factors we did not measure such as plasma vitamin C, iron, copper, and selenium. Relative efficacies of antioxidant vitamins to reduce lipid peroxidation are not known and deserve further investigation. Lepage *et al.* (5) found that lipid peroxidation in CF is inversely related to plasma β-carotene, and our findings of low plasma β-carotene and increased oxidative stress are consistent with this.

In order to adequately assess the effects of antioxidant supplementation on lipid peroxidation, it is important to choose a reliable *in vivo* marker. The limitations of previous measures of oxidative stress in CF (12) and current knowledge of the isoprostanes (4) suggest that measurement of 8-*iso*-PGF_2 levels could be a useful outcome measure in clinical trials of antioxidant treatment. While problems with the use of the enzyme-linked immunosorbent assay (ELISA) kit to measure 8-*iso*-$PGF_{2\alpha}$ are acknowledged, we subsequently completed an age- and gender-matched comparison of 8-*iso*-$PGF_{2\alpha}$ levels in CF patients and healthy controls. In this follow-up intervention study, we confirmed significantly higher 8-*iso*-$PGF_{2\alpha}$ levels in the CF patients ($P << 0.01$) with the levels the same order of magnitude as the results in the present study (Wood, L., Collins, C.E., and Garg, M.L., unpublished observations).

As Morrow *et al.* repeatedly acknowledged, the gas chromatography, mass spectrometric method of assay is a procedure that, while highly accurate, is expensive and labor-intensive, with the technology not available to all laboratories. While the ELISA kit was used in a number of recent studies (28–31), further development of specific immunoassays should expand research in this area.

Erythrocyte membrane fatty acids were measured to determine if 8-*iso*-$PGF_{2\alpha}$ production was circuitous to reduced levels of arachidonic acid and if this contributed to essential fatty acid deficiency, commonly found in CF (32). However, these subjects did not exhibit reduced levels of arachidonic acid or essential fatty acid deficiency compared to the controls, which is likely to be due to the relative good health and high energy intakes of the CF subjects, a finding which was suggested previously by dietary intervention studies (33,34). Fatty acid composition, particularly essential fatty acids, depends in part on the availability of fatty acids (32). While normal erythrocyte fatty acids were reported previously for patients who were pancreatic-sufficient (35), all of these subjects were pancreatic-insufficient.

Increased lipid peroxidation in CF could plausibly exacerbate essential fatty acid deficiency in CF by increasing peroxidation of arachidonic acid and upregulating conversion of 18:2 to 20:4. Lower linoleic acid levels (18:2) are associated with higher $PGF_{2\alpha}$ levels in CF (32), and reduced levels of 18:2 in association with increased 16:1 or monoenes in RBC membranes were previously described in CF (32,36). Differences in eicosanoid levels between subjects with CF and controls were found (32), and it was hypothesized that eicosanoid production contributes to the progression of disease (37). A higher percentage of 22:5 was found in males with CF compared to the females which potentially impacts on differences in eicosanoid production. The gender differences in erythrocyte fatty acids in the CF subjects suggest that future studies, particularly in subjects with worsening clinical status, should examine gender differences in both erythrocyte fatty acid composition and eicosanoid production in light of gender differences in survival.

In conclusion, this study found significantly increased lipid peroxidation in CF using 8-*iso*-$PGF_{2\alpha}$ levels as a marker of *in vivo* oxidative stress. It appears that current level and/or type of antioxidant supplements in CF may not be sufficient to reduce lipid peroxidation to comparable levels in healthy controls. The optimal antioxidant supplementation regime for people with CF, whether achieved through megadosing a single antioxidant or through a combination of supplements, is yet to be determined.

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Cholesterol-Derived Hydroperoxides in Alcoholic Liver Disease

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ABSTRACT: Human liver samples from 33 patients were collected at autopsy (controls, $n = 9$; fatty liver, $n = 12$; liver cirrhosis, $n = 12$), and samples homogenized. Lipids extracted with chloroform and methanol were injected into the octyl column of a high-performance liquid chromatograph with post-column chemiluminescence. Liquid chromatography–mass spectrometry was developed to identify 7-hydroperoxycholest-5-en-3β-ol (7-OOH). We found that two cholesterol-derived hydroperoxides, 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH), are present in significantly elevated amounts (12.4 and 25.0 nmol/g tissue, respectively) in lipid extracts from alcoholic fatty liver, but not in extracts from alcoholic cirrhotic liver. 7α-OOH and 7β-OOH are early intermediates produced during free radical-mediated cholesterol oxidation and can serve as molecular indicators of chain peroxidative damage in cell membranes. This is the first demonstration of 7α-OOH and 7β-OOH accumulations in human liver, and it is presumed to reflect greater oxidative stress pathology in alcoholic fatty liver.

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Results of animal studies show that lipid peroxidation modulates liver injury induced by alcohol (1,2). Thus far, lipid peroxidation was monitored in various ways; e.g., by the thiobarbituric acid assay as malondialdehyde equivalents in plasma and erythrocytes of rats chronically treated with ethanol (3) and in the liver (4), by conjugated diene formation (5), and by the 4-hydroxynonenal concentration in microsomes of rats fed ethanol intragastrically (4,6). These methods lack specificity, in that lipid peroxide itself was not measured. The recent development of high-performance liquid chromatography with chemiluminescence (HPLC-CL) detection made direct measurement of lipid peroxides with high sensitivity and specificity possible.

Previously, we identified cholesta-3, 5-dien-7-one, a metabolite of 7α-hydroperoxycholest-5-en-3β-ol (7α-OOH) and 7β-hydroperoxycholest-5-en-3β-ol (7β-OOH), in erythrocyte membranes of alcoholic patients using gas chromatography–mass spectrometry (7).

The purpose of this investigation was to clarify the relationship between lipid peroxidation and alcoholic liver disease. We used liquid chromatography–mass spectrometry (LC–MS) with an atmospheric-pressure chemical ionization interface to identify cholesterol-derived hydroperoxide in human liver sample, and quantified cholesterol hydroperoxides in alcoholic fatty liver (FL), liver cirrhosis (LC), and control liver using HPLC-CL.

MATERIALS AND METHODS

Materials. Cholesterol was obtained from Sigma (St. Louis, MO). 3,5-Di-*tert*-butyl-4-hydroxytoluene, luminol (3-aminophthaloylhydrazine), and cytochrome c (from horse heart, type VI) were purchased from Wako Pure Chemical Co. (Osaka, Japan). 5α-Hydroperoxycholest-6-en-3β-ol (5α-OOH), 7α-OOH, and 7β-OOH were synthesized as described previously (8,9). β-Sitosterol 5α-hydroperoxide as internal standard was prepared by irradiating a solution of β-sitosterol and hematoporphyrin.

Liver samples. Human liver samples were obtained at autopsy. Historical information on alcohol intake and previous illness was reviewed in medicolegal autopsy files. Twenty-four male, heavy drinkers who had drunk ethanol equivalent to more than 160 g per day for almost uninterrupted periods of longer than 10 yr were included in this study. The control liver group ($n = 9$) consisted of nonalcoholics, who had been teetotalers or, in some cases, drank less than 20 g of ethanol per day. Those who had viral hepatitis, neoplasms, or metabolic disorders were excluded from the study.

The liver samples were cut immediately into small sections and immersed in a 10% volume of formol saline for fixation, after which hematoxiline- and eosin-stained sections were prepared for microscopic examination. The remaining tissue was used for the chemical examination. The diagnosis of alcoholic liver disease was established by microscopic examination. In the FL group ($n = 12$), the histological criterion was fat infiltration with fat vacuoles in more than 30% of the hepatocytes. In the LC group ($n = 12$), the criterion was micronodular- to macronodular-type cirrhosis with necrosis, fi-

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Abbreviations: 7-OH, cholest-5-ene-3β, 7-diol; 7-OOH, 7-hydroperoxycholest-5-en-3β-ol; 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol; CYP2E1, cytochrome P4502E1; FL, fatty liver; HPLC-CL, high-performance liquid chromatography with chemiluminescence; LC, liver cirrhosis; LC–MS, liquid chromatography–mass spectrometry.

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brosis, and varying severity of fatty change. The control subjects had histologically normal livers.

Extraction. In the analysis of lipid oxidation products, total lipid was extracted as follows: 5 mL of ice-cold chloroform/methanol (2:1, vol/vol) containing 0.005% butylated hydroxytoluene as the antioxidant and 1,000 pmol β-sitosterol 5α-hydroperoxide as the internal standard was added to *ca.* 0.2 g of liver, and the mixture homogenized under the ice-cold condition. The homogenate was combined with 5 mL of chloroform/methanol (2:1, vol/vol) and 1 mL of distilled water, and the whole was mixed vigorously for 1 min in a vortex mixer. The mixture then was centrifuged at 800 × *g* for 20 min, after which the chloroform layer was collected and concentrated in a rotary evaporator then dried under a nitrogen stream, leaving the residue total lipid.

The cholesterol-rich fraction was subsequently isolated from the total lipid by solid-phase extraction: A silica column (Sep-Pak®; Waters Co., Milford, MA) of 3-mL capacity packed with aminopropyl-derivatized silica ($-NH_2$) and conditioned by washing it with 5 mL of acetone and 10 mL of *n*-hexane was used. The total lipids dissolved in a small amount of chloroform were applied to the column, which then was flushed with a mixture of 2 mL chloroform and 1 mL isopropanol, giving an eluate which mainly consisted of cholesterol. This was concentrated in a rotary evaporator and dried under a nitrogen stream. The cholesterol-rich fraction obtained was dissolved in 1 mL methanol, and a 10 µL portion injected to the HPLC column and LC–MS column.

LC–MS. A Hitachi L-7000 series LC system fitted with Spherisorb ODS-2-5 (250 × 4.6 mm i.d.) and a model M-1200AP LC–MS system which incorporated an atmospheric chemical ionization system (Hitachi, Tokyo, Japan) were used to identify 7-hydroperoxycholest-5-en-3β-ol (7-OOH). The mobile phase, methanol containing 10 mM ammonium acetate, was delivered at the flow rate of 0.7 mL/min. Application parameters for the mass spectrometer were positive-ion measurement mode, a nebulizer temperature of 170°C, a desolvator temperature of 400°C, and a needle-electrode voltage of 3000 V.

HPLC–CL. Cholesterol hydroperoxides were determined by HPLC–CL comprised of two LC-10AD vp pumps (Shimadzu, Kyoto, Japan), a CLD-10A chemiluminescence detector (Shimadzu), and a Chromatopac C-R4A integrator (Shimadzu). A TSK gel Octyl-80Ts column (Tosoh, Tokyo, Japan) was used (150 × 4.6 mm i.d.). As the mobile phase methanol/water/acetonitrile (89:9:2, by vol) was delivered by one pump at the flow rate of 0.7 mL/min, the chemiluminescent reagent was delivered by the other pump at the flow rate of 0.5 mL/min. The reagent, prepared by the method of Miyazawa *et al.* (10), consisted of cytochrome c and luminol (10 and 2 µg/mL, respectively) in alkaline borate buffer (pH 10). After the column eluant passed through an ultraviolet detector set at 210 nm to determine cholesterol appearing at retention time of 12.8 min, it was mixed with the luminescent reagent in the post-column mixing joint of the chemiluminescence detector. Standard curves were prepared from the analyses of 1, 2, 4, 10, and 20 ng of 5α-OOH and 7β-OOH and from 0.5, 1, 2, 5, and 10 ng of 7α-OOH using 2.5 ng of the internal standard. Regression lines of the ratios of hydroperoxides to the internal standard vs. the standard concentrations (pmol) were linear ($r > 0.998$). The recoveries of 7α-OOH and the internal standard varied from 55 to 65%. The coefficient of variation within-day was 4.5 (%) and between-day 7.8 (%).

Statistical analysis. Comparisons of the experimental data for all the groups were made using a one-way analysis of variance or the Kruskal-Wallis test followed by Scheffe's test. The difference in the mean values of the two cholesterol hydroperoxides (7α-OOH and 7β-OOH) in each group was assessed by the Student's *t*-test. $P < 0.05$ was deemed significant.

RESULTS

Demographic data on the subjects studied are presented in Table 1. There were no differences among the three groups with regard to age, body weight, height, or postmortem period.

Analyses of lipid extracts from human liver in alcoholic FL and the control by LC–MS gave four peaks in the total ion chromatograms. Amounts of oxysterols in FL were greater than control. The peak in alcoholic FL, appearing at a retention time of 7.0 min, was subjected to MS. The mass spectra of standard 7-OOH, cholest-5-ene-3β 7-diol (7-OH), and the peak from FL are shown in Figure 1. Standard 7-OOH had an ion $[M + H - H_2O]^+$ at *m/z* 401 and fragment ions at *m/z* 385 and 367, whereas standard 7-OH had an ion $[M + H - H_2O]^+$ at *m/z* 385 and a fragment ion $[M + H - 2H_2O]^+$ at *m/z* 367 but not at *m/z* 401. The retention times of standard 7-OOH, and 7-OH, and the peak were all about 7.0 min, almost identical. Accordingly, the peak from FL which had a larger ion at *m/z* 385 than at *m/z* 401 was assumed to be a mixture of 7-OOH and 7-OH.

Cholesterol hydroperoxide measurements were made by HPLC–CL. Figure 2 shows the chromatograms of a mixture of standard 7β-OOH, 7α-OOH, 5α-OOH, and the internal standard, when a TSKgel Octyl-80Ts column with methanol/water/acetonitrile as the mobile phase was used. The retention times of standard 7β-OOH, 7α-OOH, 5α-OOH, and the internal standard, respectively, on the chromatograms were 6.8, 7.3, 7.8, and 9.5 min, and they were separable. Figure 2

TABLE 1
Demographic Data for the Samples Studied[a]

	Control	FL	LC
n	9	12	12
Age (yr)	54.0 ± 12.6	61.3 ± 15.0	55.6 ± 7.9
Body weight (kg)	67.7 ± 11.3	60.8 ± 15.5	57.9 ± 10.3
Height (cm)	165.6 ± 5.5	164.9 ± 6.4	162.1 ± 5.6
Postmortem time (h)	13.4 ± 4.7	12.4 ± 4.5	14.6 ± 5.1

[a]Values are means ± SD. FL, fatty liver; LC, liver cirrhosis.

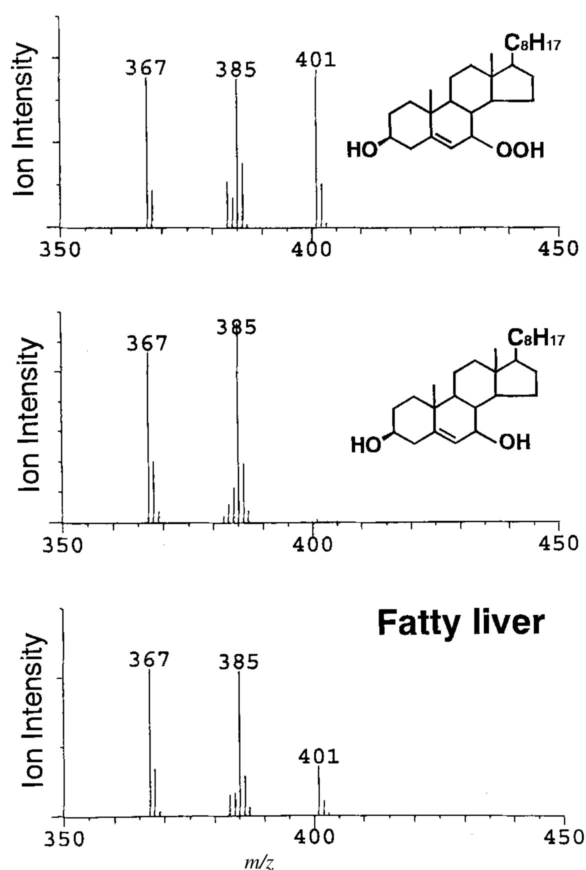

FIG. 1. Mass spectra of standard 7-OOH, 7-OH, and the peak from alcoholic fatty liver sample obtained by liquid chromatography–mass spectrometry with an atmospheric-pressure chemical ionization interface. 7-OOH, 7-hydroperoxycholest-5-en-3β-ol; 7-OH, cholest-5-ene-3β, 7-diol.

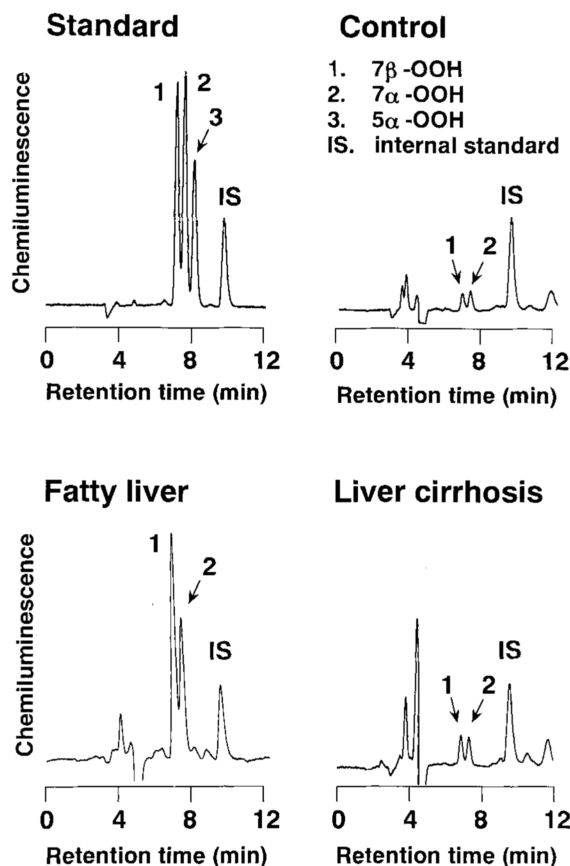

FIG. 2. High-performance liquid chromatograms for standard hydroperoxycholesterols, and the control, fatty, and cirrhotic liver samples. 5α-OOH, 5α-hydroperoxycholest-6-en-3β-ol; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol.

also shows HPLC–CL chromatograms for liver samples of the control, FL, and LC groups. Peaks 1 and 2 of the liver samples, at the respective retention times of 6.8 and 7.3 min, corresponded with those of the respective standards 7β-OOH and 7α-OOH. Not only lipid extracts from FL but also those from LC and the control liver had 7β-OOH and 7α-OOH, but no 5α-OOH was detected in these samples.

The concentrations of 7α-OOH and 7β-OOH in human liver samples are shown in Table 2. In FL both 7-OOH (7α-OOH = 12.4 nmol/g, 7β-OOH = 25.0 nmol/g) were significantly higher than in the control and LC, whereas both 7-OOH in LC (7α-OOH = 2.3 nmol/g, 7β-OOH = 3.2 nmol/g) were as low as in the control. The reason why the standard deviations in Table 2 were high was that we used human liver samples. The postmortem period varied from 4 to 24 h although there was no significant difference among the three groups. The concentration of cholesterol in human liver samples as determined by HPLC at 210 nm also is shown in Table 2. The percentages of 7α-OOH and 7β-OOH compared to cholesterol in liver samples are also shown in Table 2. The

TABLE 2
7-Hydroperoxycholest-5-en-3β-ol Concentrations[a]

	n	7α-OOH (nmol/g)	7β-OOH (nmol/g)	Cholesterol (μmol/g)	7α-OOH/cholesterol (%)	7β-OOH/cholesterol (%)
Control	9	1.2 ± 1.7	2.0 ± 3.3	3.8 ± 1.3	0.027 ± 0.029	0.045 ± 0.054
FL	12	12.4 ± 7.1[b]	25.0 ± 14.0[b,c]	4.1 ± 1.6	0.33 ± 0.20[b]	0.71 ± 0.43[b,d]
LC	12	2.3 ± 3.5	3.2 ± 5.8	3.9 ± 1.5	0.079 ± 0.13	0.11 ± 0.22

[a]Values are means ± SD. FL, fatty liver; LC, liver cirrhosis; 7α-OOH, 7α-hydroperoxycholest-5-en-3β-ol; 7β-OOH, 7β-hydroperoxycholest-5-en-3β-ol.
[b]7α-OOH, 7β-OOH, 7α-OOH/cholesterol and 7β-OOH/cholesterol in FL are significantly higher than in the control and LC groups ($P < 0.05$).
[c]7β-OOH is significantly higher than 7α-OOH in FL ($P < 0.05$).
[d]7β-OOH/cholesterol is significantly higher than 7α-OOH/cholesterol in FL ($P < 0.05$).

data expressed by percentage in liver samples were similar to those by concentration. Moreover, 7β-OOH in FL was significantly higher than 7α-OOH. The ratio of 7β-OOH to 7α-OOH was 2.09 ± 0.38 in FL. There were no significant differences between 7α-OOH and 7β-OOH in the LC and control groups.

DISCUSSION

Many researchers reported that lipid peroxides contributed to the pathogenesis of alcoholic liver disease. To assess the effect of lipid peroxidation on alcoholic liver disease, we tried to analyze directly cholesterol-derived hydroperoxide. Phospholipid and cholesterol esters consist of many kinds of fatty acids. When active oxygen species and free radicals attack them, several fatty acid-derived hydroperoxides of them should be present. We must have many standard compounds of fatty acid hydroperoxides, phospholipid hydroperoxides or cholesterol ester hydroperoxides when we analyze such lipid peroxidation products. The reason why we chose cholesterol-derived hydroperoxide as the indicator of lipid peroxidation was that we could synthesize standard compounds of cholesterol hydroperoxides.

Ozawa *et al*. (11) reduced 7-OOH to 7-OH by $NaBH_4$ and then, using gas chromatography–mass spectrometry, identified the 7-OOH in rat skin as the *O*-trimethylsilyl esters of 7-OH. We are the first to succeed in identifying 7-OOH directly in human FL samples using LC–MS. We could distinguish 7-OOH with its base peak at *m/z* 401 from 7-OH without a peak at *m/z* 401, by using LC–MS with methanol containing ammonium acetate as the mobile phase.

Previously, we examined the extraction procedure and some antioxidants to avoid artifact formation of cholesterol hydroperoxides from cholesterol itself. We proceeded to analyze 7α-OOH and 7β-OOH in erythrocyte membranes of seven healthy volunteers. The respective mean concentrations of 7α-OOH and 7β-OOH were 2.5 ± 1.6 and 5.4 ± 3.5 pmol/mL blood (12). Thus, we confirmed that in the previous measurement, artifact formation was minimal. As we developed the method to determine 7-OOH-preventing artifact formation, artifact formation was not included in the present study even if the concentration of the 7-OOH in FL was high enough.

Ours are the first measurements of 7α-OOH and 7β-OOH in human liver samples. We found that the amounts of 7α-OOH and 7β-OOH in FL, respectively, were 12.4 ± 7.1 and 25.0 ± 14.0 nmol/g tissue (mean ± standard deviation). The 7-OOH concentrations in rat skin reported by Ozawa *et al.* (11) were higher than in the liver of the present study, partly because lipid peroxides accumulate readily in rat skin. The concentration of 7β-OOH in human FL was significantly higher than that of 7α-OOH and was similar to the findings for rat skin (11). The reason for the higher level of 7β-OOH may be that 7α-OOH is easily epimerized to 7β-OOH. In contrast, both the 7-OOH in LC were as low as in the control. As the cholesterol per liver tissue in LC might decrease due to fibrosis, we calculated the percentage of 7-OOH to cholesterol. As shown in Table 2, significantly elevated percentage was observed in FL, but not in LC.

It is a well-known fact that cytochrome P4502E1 (CYP2E1) contributes to generation of free radicals. Chronic ethanol administration resulted in CYP2E1 induction (13,14). Albano *et al.* (15) showed the evidence of links between the induction of CYP2E1 by ethanol and the formation of hydroxyethyl radicals and suggested the stimulation of lipid peroxidation. Several investigators reported an association between the induction of CYP2E1 by ethanol and an increase in lipid peroxidation. (16,17). Moreover, animal studies by Takahashi *et al*. (18) and Koivisto *et al*. (19) provided evidence that ethanol-induced CYP2E1 was associated with the pathogenesis of alcoholic liver disease. Kato *et al*. (20) showed that pretreatment with xanthine oxidase inhibitor decreased xanthine metabolism and reduced ethanol-induced lipid peroxidation. They suggested that xanthine oxidase has a role in ethanol-induced lipid peroxidation in rats. We recognized the possibility of enhanced generation of lipid peroxides owing to increased production of active oxygen species (superoxide and hydroxyl radicals) in alcoholic FL.

In view of a decrease in antioxidants, the acute administration of ethanol resulted in a substantial decrease in the reduced glutathione concentration in the human liver, indicative that the ability to eliminate oxygen radicals was diminished (21). This difficulty in eliminating oxygen radicals may permit enhanced lipid peroxidation which, in turn, may cause the accumulation of cholesterol hydroperoxides. Glutathione peroxidase could play a major role in protecting mammals from the toxicity of ingested lipid peroxides. Plasma glutathione peroxidase as well as classical cellular glutathione peroxidase is present in liver (22). Plasma glutathione peroxidase can reduce phospholipid hydroperoxides but cannot reduce cholesterol hydroperoxides (23). In addition, plasma glutathione peroxidase has a low level of reducing activity toward cholesterol 7α-OOH (24). In contrast, it was unknown if cellular glutathione peroxidase can reduce cholesterol hydroperoxide. The present result showing the accumulation of 7-OOH in FL suggests that cellular glutathione peroxidase does not have enough reducing activity against oxidative stress. We therefore assume that long-term ethanol ingestion leads to excessive oxidative stress and to the accumulation of cholesterol hydroperoxides in alcoholic FL.

Finally, mention must be made of the 7-OOH concentration in alcoholic LC being as low as that in the controls. Tsukamoto *et al.* (4) succeeded in producing an animal model of LC and reported that malondialdehyde and 4-hydroxynonenal, indicators of lipid peroxidation, increased in their model. Our findings, which show that 7-OOH values in human LC are not significantly higher than control values, contradict the findings of previous investigators. Although as yet we have no satisfactory data to explain this contradiction, differences of the substance used to measure the index of lipid peroxidation may partly account for the conflicting results. Generation of free radicals by enzymatic reaction might occur

in alcoholic FL, whereas it could scarcely occur in LC due to decrease of normal liver cells.

In conclusion, the markedly high accumulation of 7-OOH in FL and the low level in LC suggest elevated lipid peroxidation in the early stage of alcoholic liver disorder.

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Lymphatic Absorption of Phytosterol Oxides in Rats

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ABSTRACT: Two of the main classes of oxyphytosterols (7-keto and epoxides) were synthesized from sitosterol and campesterol and given to mesenteric duct-cannulated adult male rats. Lymph was collected during 24 h and was analyzed for oxysterols. The results showed that the lymphatic recovery of the phytosterol oxides was low: 4.7% of the given dose for epoxy derivatives and 1.5% for 7-keto compounds. The campesterol oxides presented a better absorption than the sitosterol oxides. During the process of absorption, the epoxyphytostanols were also partly transformed in campestanetriol and stigmastanetriol.

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Cholesterol oxides are compounds containing additional oxygenated functional groups such as ketone, epoxide, hydroperoxide, or hydroxyl on the cholesterol molecule (1). These oxycholesterols (often called oxysterols) are described to elicit potent biological effects including cytotoxicity, atherogenicity, mutagenicity, and carcinogenicity. They are also involved in the biosynthesis of bile acids, steroid hormones, and cholesterol (2–5). Some of these compounds are formed *in vivo* (6,7), but they can also originate from foods (8–13). Several studies on laboratory animals showed that these dietary oxycholesterols can be absorbed by the intestine (14–18). Direct evidences of the absorption of such compounds in man were also brought out by Emanuel *et al.* (19) using an egg powder meal and by Linseisen and Wolfram (20) on men fed with meals containing salami and Parmesan cheese.

In foods, several sterols other than cholesterol occurred. In particular, the plant sterols (phytosterols) are often found in similar amounts as cholesterol in our diet (21) and may be ingested in large quantities by vegetarians (22). Their chemical structure is very close to that of cholesterol: campesterol contains only an additional methyl group at C24; sitosterol is characterized by an ethyl group at C24; stigmasterol has an ethyl group at C24 and an ethylenic bond at C22. These phytosterols can be oxidized in the same way as cholesterol (23–25). The occurrence of oxyphytosterols in foods was much less studied than that of oxycholesterols. Some studies pointed out little amounts of these compounds in french fries and chips (26–28), in wheat flour (29), and in coffee (30). Data on biological effects of these compounds are very scarce: abortifacient effects were described in mice (31); more recently Meyer and Spiteller (32) described an increase in oxyphytosterols during aging of cell cultures of *Chenopodium rubrum*, as well as cytotoxicity of these compounds on mealworms *Tenebrio molitor* (33).

However, there was a lack of data about the intestinal absorption of these oxyphytosterols, and we cannot presume their possible biological effects in humans. It is well-known that the nonoxidized phytosterols are less absorbed by the human intestine than cholesterol: 5% for sitosterol and 10–16% for campesterol as compared to about 30–43% for cholesterol (34–37). It is sometimes assumed that the reduced absorption of phytosterols could lead to a low absorption of the oxyphytosterols. The present study was undertaken to investigate the intestinal absorption of such compounds.

A blend containing mainly sitosterol oxides and a small amount of campesterol oxides was chosen, in order to better look like the processed foods (27,28). Epoxides and 7-keto derivatives were used, because they are usually present among the sterol oxides (27,28).

EXPERIMENTAL PROCEDURES

Reagents. The compounds used for chemical syntheses (*t*-butyl hydroperoxide, chromium hexacarbonyl, *m*-chloroperoxybenzoic acid) as well as pyridine, cholestanetriol, and 5α-cholestane were purchased from Sigma-Aldrich (L'Isle d'Abeau Chesnes, France). Pyridine was dehydrated and maintained on 4Å molecular sieves. The phytosterol blend, containing 76.2% sitosterol, 8.6% campesterol, 1.4% stigmasterol, and 13.8% Δ5-avenasterol, was provided by Merck (Darmstadt, Germany). Triolein and acetic anhydride were

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Abbreviations a.m.u., atomic mass unit; campestanetriol, 5α-campestane-3β,5α,6β-triol; cholestanetriol, 5α-cholestane-3β,5α,6β-triol; α-epoxycampestanol, 5α,6α-epoxycampestan-3β-ol; β-epoxycampestanol, 5β,6β-epoxycampestan-3β-ol; α-epoxycholestanol, 5α,6α-epoxycholestan-3β-ol; β-epoxycholestanol, 5β,6β-epoxycholestan-3β-ol; α-epoxysitostanol, 5α,6α-epoxystigmastan-3β-ol; β-epoxysitostanol, 5β,6β-epoxystigmastan-3β-ol; GC, gas chromatography; 7-ketocampesterol, 3β-hydroxycampest-5-en-7-one; 7-ketocholesterol, 3β-hydroxycholest-5-en-7-one; 7-ketositosterol, 3β-hydroxystigmast-5-en-7-one; MS, mass spectrometry; stigmastanetriol, 5α-stigmastane-3β,5α,6β-triol; TMS, trimethylsilyl.

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purchased from Prolabo (Fontenay sous bois, France). The silylation reagent bis-trimethylsilyl-trifluoroacetamide with 1% trimethylchlorosilane was obtained from Pierce (Rockford, IL). The other solvents were obtained from SDS (Peypin, France) and were distilled before use. All chemicals and reagents were of the highest grade available.

Chemical syntheses. The method used by Chicoye *et al.* (38) to prepare 7-ketocholesterol was adapted to 7-ketositosterol and 7-ketocampesterol. Briefly, the phytosterol blend was acetylated using acetic anhydride. After purification, the phytosterol acetates were oxidized by *t*-butylhydroperoxide in acetonitrile, in the presence of chromium hexacarbonyl. The oxidized compounds were purified by silica column chromatography, using hexane/ethyl acetate (8:2, vol/vol) as eluent. The acetyl radical was then removed using a sodium hydrogen carbonate solution. After purification on a silica column, using hexane/ethyl acetate (1:1, vol/vol), a blend containing 90.3% of 7-ketositosterol and 9.7% of 7-ketocampesterol was obtained. The structure of these compounds was checked by gas chromatography–mass spectrometry (GC–MS) after formation of trimethylsilyl (TMS) derivatives as described later.

The phytosterol epoxides were synthesized as described by Nourooz-Zadeh and Appelqvist (29). The obtained mixture contained 2.1% of 5β,6β-epoxycampestanol, 8.2% of 5α,6α-epoxycampestanol, 13.6% of 5β,6β-epoxysitostanol, and 76.1% of 5α,6α-epoxysitostanol. Their chemical structures were also checked by GC–MS of their TMS derivatives.

The oxidized derivatives of Δ5-avenasterol and stigmasterol were discarded during the purification steps of the synthesis processes, and only sitosterol- and campesterol-oxides were retained for experiment.

Animals and diets. Specific pathogen-free Wistar male rats, weighing about 250 g, were obtained from Iffa-Credo (L'Arbresle, France). They were separated into three groups of seven animals. Under ether anesthesia, the main mesenteric lymphatic duct was cannulated using a heparinized polyethylene catheter (i.d. 0.3 mm, o.d. 0.7 mm, Guerbet Medical, Louvres, France). Postoperatively, the 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). The morning after surgery and immediately before lipid administration, basal lymph of fasting rats was collected for 1 h in tubes placed inside a polystyrene insulating box containing ice. They received then intragastrically 1 mL of triolein heated at 37°C containing the experimental lipids (Table 1). Triolein was chosen as carrier oil to avoid feeding other sterols. The rats of the control group received 5 mg of the nonaltered phytosterols. Group K rats were given 5 mg of the synthesized 7-ketophytosterols and group E rats 5 mg of the synthesized epoxyphytostanols fraction. The lymph was then collected during the first 6 h and for the next 18 h from the conscious animals. The two samples were pooled, and the entirety of lymph (about 15–20 mL) was used for oxyphytosterols analysis.

Oxyphytosterols and phytosterols analysis. Lipids of the lymph were obtained by extraction with chloroform/methanol (2:1, vol/vol) (39). They were saponified at room temperature during 18 h (40). Unsaponifiable matter was extracted by diethyl ether, redissolved in hexane, and purified by solid-phase extraction on silica cartridges (LC-Si, 3 mL, 500 mg; Supelco, L'Isle d'Abeau, France) according to Morgan and Armstrong (41). As recommended by Lai *et al.* (42), a vacuum manifold (Supelco) was used to ensure a regular solvent flow rate of 0.6 mL/min through the cartridge. A known amount of 5α-cholestane was added as internal standard to the acetone fraction containing the oxyphytosterols. The blend was evaporated to dryness under nitrogen in a conic glass vial. The compounds were redissolved in 100 µL of anhydrous pyridine, and 100 µL of bis-trimethylsilyl-trifluoroacetamide containing 1% of trimethylchlorosilane was added. After vortex mixing, the TMS ether derivatives were obtained by heating 15 min at 60°C. The reagents were evaporated under nitrogen and the residue dissolved in hexane for gas chromatographic analysis as described later.

TABLE 1
Composition of the Sterols Given to Rats by Gastric Intubation (in mg/rat)

	Control group: phytosterols	Group K: 7-ketosterols	Group E: epoxystanols
Campesterol	0.43		
Stigmasterol	0.07		
Sitosterol	3.81		
Δ5-Avenasterol	0.69		
7-Ketocampesterol		0.48	
7-Ketositosterol		4.52	
β-Epoxycampestanol			0.11
α-Epoxycampestanol			0.41
β-Epoxysitostanol			0.68
α-Epoxysitostanol			3.81
Total sterols	5	5	5

The eventual loss of oxysterols during analysis was checked by adding 50 µg of each oxyphytosterol blend to aliquots of control lymphs. The obtained recovery was between 85 and 105%.

The purification of the phytosterols in the lymph samples of the control group was effected using the method of Pie *et al.* (11) originally described for cholesterol. The TMS derivatives were obtained as described above and analyzed by GC.

GC and GC coupled with MS. The TMS ether derivatives were analyzed on a 5890 Series 2 Hewlett-Packard (Palo Alto, CA) gas chromatograph with a flame-ionization detector. A 20 m × 0.32 mm i.d. DB-5 (0.25 µm film thickness) capillary column (J&W Scientific, Folsom, CA) with helium as carrier gas was used. The injection was made in splitless mode. After 1 min at 50°C, the oven temperature was raised at 20°C/min until 270°C, and then reached 290°C at 1°C/min. The completion of analysis was effected at 290°C. The injector and detector temperatures were, respectively, 290 and 320°C. Quantitative analyses were performed using a Chromjet SP 4490 integrator and Winner software (Thermo Separation Products, Les Ulis, France).

The structure of the compounds was checked by GC cou-

pled with MS using a Hewlett-Packard 5890 gas chromatograph equipped with a 5970 mass selective detector. A 30 m × 0.25 mm i.d. HP-5 column (Hewlett-Packard) was used. The chromatographic conditions were the same as for flame-ionization detection GC. The mass spectrometer was operated at an ionization energy of 70 eV. Mass spectra were recorded between 100 and 700 a.m.u.

RESULTS AND DISCUSSION

The mass spectrometric fragmentation of the synthesized oxyphytosterols as TMS derivatives is summarized in Table 2. The *m/z* values were similar to those of the corresponding cholesterol derivatives (43–45), with increments of 14 or 28 for the fragments including the lateral chain. The spectra of the synthesized 7-ketophytosterols were very close to those recently published (27), with some minor differences: in particular in our conditions, the major fragment was not 174, but the molecular ions 500 and 486 for 7-ketositosterol and 7-ketocampesterol, respectively. Moreover, the (M^+ − TMS − CH_3) fragments 395 and 381 were bigger than the (M^+ − TMS) ions (410 and 396). Some little differences with the results of Dutta and Appelqvist (27) could also be noted for α-epoxysitostanol, β-epoxysitostanol, and α-epoxycampestanol concerning base ions or relative intensities. In a recent review including data on MS of cholesterol oxides, Addis *et al.* (45) found that there was little agreement in terms of relative intensities of characteristic ions in the mass spectra literature concerning the epoxycholestanol epimers. The same is probably true for the epoxyphytostanols. Concerning the β-epoxycampestanol, the difference with the spectrum published by Dutta and Appelqvist is rather important: the ion 470 is the base ion in their study and is only 4.5% of the base ion in our study. The different instrumental conditions may explain these little discrepancies.

The recovery of phytosterols in the lymph of the control animals was reported in Table 3. We observed that the intestinal absorption of these compounds was low (about 1.6%), but with important differences between phytosterols: the amount of campesterol in the lymph is about six times higher than that of sitosterol. These results agree with the data of the literature (46,47). It was assumed (36,37) that increasing the length of the side chain of cholesterol decreases the absorbability of the sterol. However, the absorption results obtained in this study are lower than some published data. For example, Vahouny *et al*. (48) obtained 3 to 4% absorption for sitosterol and stigmasterol. However, they used a higher dose of phytosterols (50 vs. 5 mg in this study), and they added a high level of sodium taurocholate in their lipid sample to improve the absorption. The absorption of stigmasterol seemed very low. However, the ingested level was low, and it was difficult to quantify accurately its recovery in the lymph. Concerning the Δ5-avenasterol, we do not know any data on its intestinal absorption. This study indicated that the absorption of this compound is low.

To evaluate the quantity of phytosterols eventually transformed into oxyphytosterols during the analytical process, the oxyphytosterols were checked in the lymph of control animals, which were given 5 mg of the nonoxidized phytosterol blend. It is well-known indeed that the sterols are rather fragile and that artifactual formation of oxysterols could occur during analysis (1,9,49–51). In our experimental conditions, only trace components were observed by GC. The MS of these compounds revealed some characteristic ions of oxyphytosterols. However, the total amount of these compounds was less than 0.5 µg, corresponding to 10^{-4} of the given phytosterols.

The results concerning the lymph of the K group are summarized in Table 4. The 7-ketophytosterols were recovered in the lymph of the rats, but in very low quantities, similar to those of nonoxidized phytosterols. In our experimental conditions, the mean absorption value was only 1.5% of the in-

TABLE 2
Mass Spectrometric Data (*m/z*) for Synthesized Oxyphytosterols (as trimethylsilyl ethers)[a]

	M	Major significant ions								
7-Ketositosterol	500 (100)	485 (12.9)	483 (11.7)	444 (9.0)	431 (5.0)	410 (23.8)	395 (69.9)	187 (45.6)	161 (61.6)	129 (71.2)
7-Ketocampesterol	486 (100)	471 (14.6)	469 (12.2)	430 (11.3)	417 (4.9)	396 (27.0)	381 (72.7)	187 (51.0)	161 (65.8)	129 (80.1)
α-Epoxysitostanol	502 (60.0)	487 (15.8)	474 (6.0)	473 (5.6)	412 (71.7)	397 (23.3)	394 (55.7)	384 (22.0)	379 (21.0)	73 (100)
β-Epoxysitostanol	502 (77.3)	487 (15.4)	474 (11.5)	473 (15.6)	412 (85.3)	397 (30.8)	394 (30.6)	384 (36.3)	379 (19.0)	73 (100)
α-Epoxycampestanol	488 (57.9)	473 (15.8)	460 (7.8)	459 (8.7)	398 (84.8)	383 (28.2)	380 (63.7)	370 (26.5)	365 (21.8)	73 (100)
β-Epoxycampestanol	488 (69.1)	473 (22.1)	460 (8.4)	459 (17.5)	398 (69.4)	383 (29.8)	380 (18.6)	370 (41.7)	365 (6.0)	95 (100)

[a]Relative abundances are reported in parentheses.

TABLE 3
Phytosterols in Lymph of Rats of the Control Group

	Campesterol	Stigmasterol	Sitosterol	Δ5-Avenasterol	Total phytosterols
Ingested amount (μg)	430	70	3810	690	5000
Amount found in lymph[a] (μg)	30.1 ± 10.3	0.6 ± 0.1	45.1 ± 13.8	5.1 ± 2.0	80.9 ± 26.1
Mean percentage of absorption (%)	7.0	0.8	1.2	0.7	1.6
Minimal absorption (%)	5.1	0.7	0.7	0.5	1.0
Maximal absorption (%)	10.4	1.0	1.6	1.1	2.3

[a]Results expressed as mean ± standard deviation.

TABLE 4
Oxyphytosterols in Lymph of Rats Given 7-Keto-phytosterols

	7-Keto-campesterol	7-Keto-sitosterol	Total 7-keto-phytosterols
Ingested amount (μg)	485	4515	5000
Amount found in lymph[a] (μg)	12.2 ± 4.8	61.9 ± 21.5	74.1 ± 26.3
Mean percentage of absorption (%)	2.5	1.4	1.5
Minimal absorption (%)	1.3	0.8	0.9
Maximal absorption (%)	4.5	2.2	2.5

[a]Results expressed as mean ± standard deviation.

gested oxyphytosterols. A large variability between rats was observed: the standard deviation was rather important and the ratio between maximal and minimal values of absorption was about 3. This variability was in the range of that observed by other authors investigating lipid absorption in similar experimental conditions (16,52,53). The 7-ketocampesterol appeared to be more absorbed than 7-ketositosterol (2.5% vs. 1.4%). As for nonoxidized phytosterols, the length of the side chain seemed to have an effect on the absorption of the 7-ketophytosterols.

In the E group rats, the four epoxyphytostanols intubated to the animals were recovered in different proportions in the lymph, but an additional peak appeared on all the chromatograms (Fig. 1). The mass spectrum of this compound (Fig. 2) showed some characteristic ions at *m/z* 431, 129, 484, 349, 574, 395, 469, 545, and 559. This fragmentation is very close to that obtained by Dutta and Appelqvist (27) for dihydroxysitosterol (stigmastanetriol) as TMS derivative. The only standard available for this kind of compounds is the cholestane-3β,5α,6β-triol. Using our conditions of derivatization, the cholestane-3β,5α,6β-triol was found to form the bis-TMS with the hydroxyl groups at the 3- and 6-positions being derivatized, as described by Park and Addis (54). If we compare the mass spectra of cholestane-3β,5α,6β-triol and that of our unidentified peak as TMS derivatives, we observed many similar ions with an increment of 28, corresponding to an ethyl group (Fig. 2). The major ions corresponding to the fragmentation of cholestane-3β,5α,6β-triol as TMS are fragments including the lateral chain (55). Then it appears that the unknown compound has an additional ethyl group on its lateral chain and is supposed to be a triol issued from the epoxysitostanols. To confirm this assumption, we prepared stigmastanetriol and campestanetriol from the blend containing the epoxysitostanols and the epoxycampestanols, using periodic acid (56). Thus we verified that the stigmastanetriol and the unknown compound had the same retention time by GC and the same mass spectrum, after transformation in TMS derivatives. The unknown compound is then a stigmastanetriol, probably the stigmastane-3β,5α,6β-triol.

We can wonder why no campestanetriol peak was detected on the chromatogram (Fig. 1). So, we made a research of the ions 417, 470, and 560, considered as characteristic of the TMS derivative of campestanetriol (27) and detected them as very minor components in the beginning of the peak of 5α,6α-epoxysitostanol. Unfortunately, our chromatographic

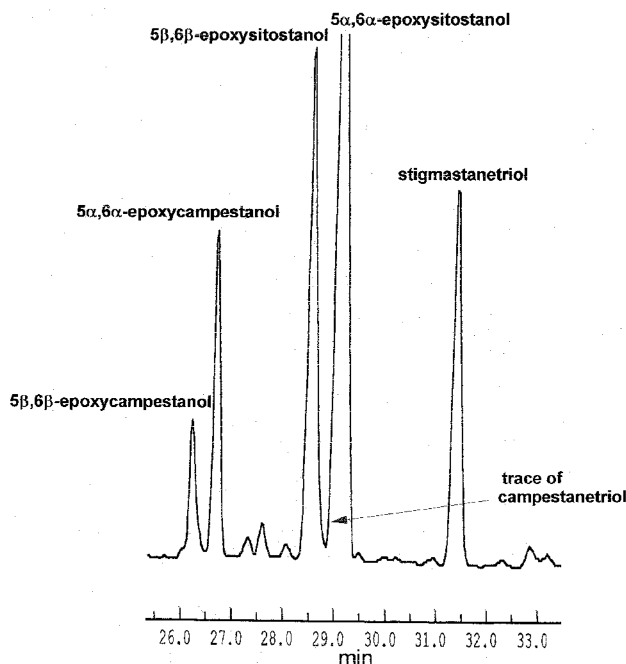

FIG. 1. Part of a gas–liquid chromatogram of oxyphytosterols as trimethylsilyl (TMS) ethers, originating from a sample of lymph of a rat of group E (given the epoxyphytosterols blend). Gas chromatographic conditions are described in the Experimental Procedures section.

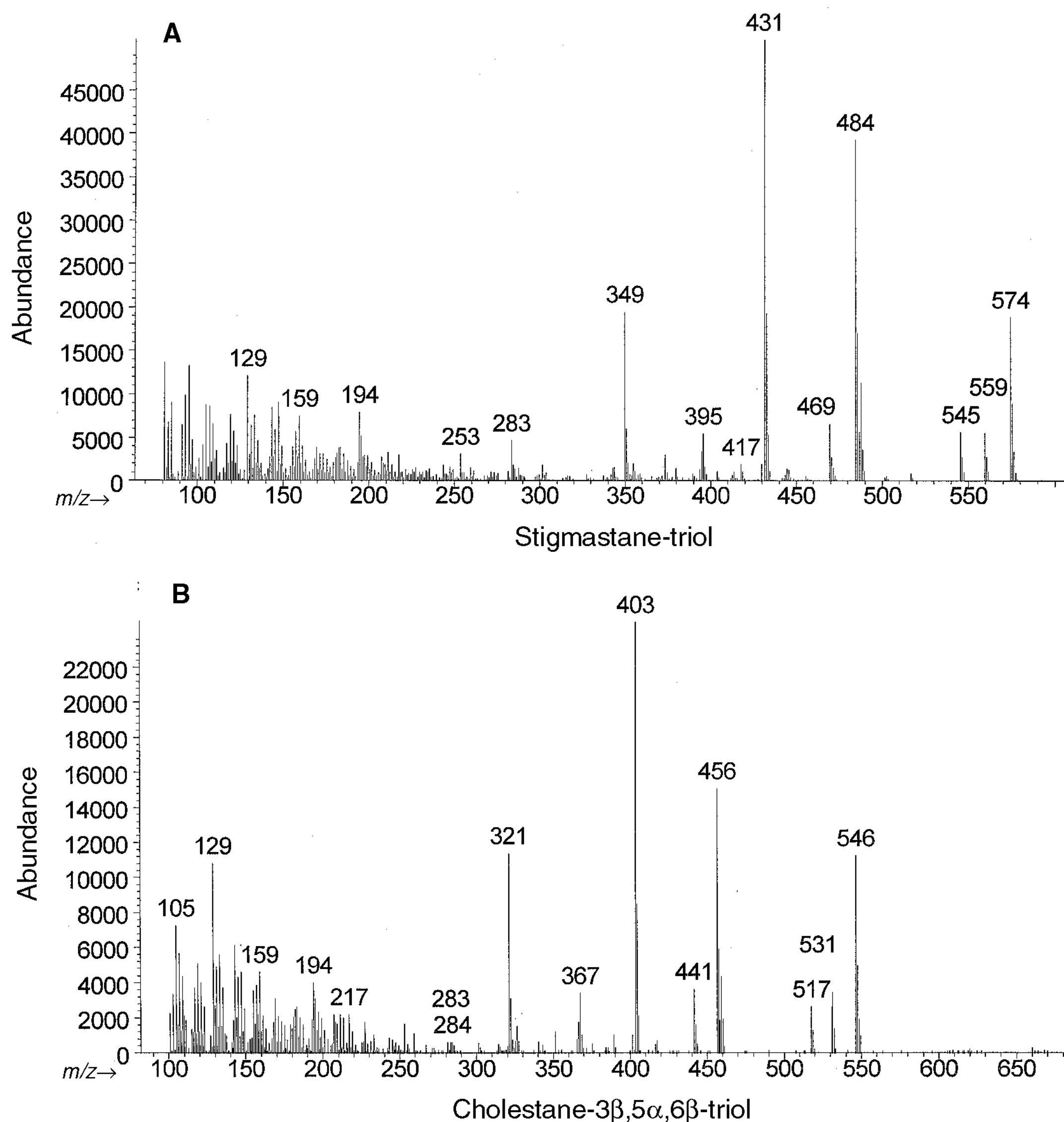

FIG. 2. Mass spectra of (A) an unknown compound (presumed to be stigmastane-3β,5α,6β-triol) and (B) cholestane-3β,5α,6β-triol as TMS ethers. Mass spectrometric conditions in the Experimental Procedures section. See Figure 1 for abbreviation.

conditions failed to separate 5α,6α-epoxysitostanol from campestanetriol.

Only few reports were concerned with the metabolism of oxyphytosterols. In some of them, there were data evidencing the possibility of conversion of epoxyphytostanols as well as epoxycholestanols (57). It is then interesting to look at all the data concerning the transformation of epoxystanols in triols. About 30 yr ago, the transformation of cholesterol epoxides in cholestanetriol in the gastrointestinal tract of the rat was already described (58). Maerker *et al.* (59) studied this event, using simulated gastric juice and demonstrated that both epoxides produced α- and β-chlorohydrins which were then partly hydrolyzed in triols. In less acidic conditions, such as those prevailing in the small intestine, the residual chlorohydrins could revert to epoxycholestanols (59). Another possibility is that the epoxides could be modified by the intestinal flora: an epoxide hydrase activity was indeed described in human intestinal microflora (60). But, we can also hypothesize that the epoxides were hydrolyzed inside the intestinal cells: a microsomal hydrolase able to convert epoxysitostanols and epoxycholestanols in triols was indeed described in rat liver (57). However, there were also descriptions of artifact formation of cholestanetriol during analysis, by simple hydration (61), or due to exposure to light (62). We determined if the triols could be formed during lymph analysis, using blank samples containing similar epoxide quantities as our lymph samples, but deprived of triols. The results of this test showed that a very little transformation of epoxides in triol cannot be excluded, but that the compounds originating from this reaction did not exceed 1% of the initially added epoxides.

The lymph recovery of oxyphytostanols from rats given

TABLE 5
Oxyphytosterols in Lymph of Rats Given the Epoxyphytosterols

	β-Epoxy-campestanol	α-Epoxy-campestanol	β-Epoxy-sitostanol	α-Epoxy-sitostanol[a]	Stigmastane-triol	Total oxyphytosterols
Ingested amount (μg)	105	410	680	3805		5000
Amount found in lymph[b] (μg)	8.3 ± 3.5	21.0 ± 9.0	39.3 ± 17.1	102.2 ± 51.7	62.9 ± 23.5	233.8 ± 92.5
Mean percentage of absorption (%)	7.9	5.1	5.8	2.7		4.7
Mean percentage of absorption for all epoxycampestanols (epoxysitostanols)	5.7		4.6			
Minimal absorption (%)	2.8		2.3			2.5
Maximal absorption (%)	8.5		6.9			7.0

[a]The α-epoxy-sitostanol peak also contains a little amount of campestanetriol.
[b]Results expressed as mean ± standard deviation.

the epoxyphytostanols is reported in Table 5. It appeared that these compounds were better absorbed than the 7-ketophytosterols (4.7 vs. 1.5%). The β-epoxy compounds were recovered in larger proportions in lymph than the α-epoxyphytostanols. This could be due either to a better absorption, or to a lower metabolic transformation into triol, as compared with the conversion process noted in rat liver microsomes (57). The epoxycampestanols were slightly better absorbed than the epoxysitostanols (5.7 vs. 4.6%). However, the peak of α-epoxysitostanol contains an unknown quantity of campestanetriol. On the assumption that the epoxycampestanols were transformed into triol in the same proportion as the epoxysitostanols, their absorption would reach 6.9 and 4.4%, respectively. The campesterol oxides, as well as the campesterol, seemed to be always better absorbed than sitosterol oxides and sitosterol.

The results of this study provide evidence that some phytosterol oxides, eventually present in food, can pass the intestinal barrier. Nevertheless, their lymphatic recovery is rather low and depends on the kind of administered oxysterols. The epoxides are recovered in larger quantities in lymph than the 7-keto compounds. The campesterol derivatives are better absorbed than those of sitosterol. Phytostanol epoxides are partly transformed in corresponding triols which appeared in lymph lipids. Further studies are necessary to elucidate if this conversion occurs in stomach, or if intestinal cells' hydrolases are able to modify the epoxides. Despite their low absorption, it would also be of great interest to study the metabolic fate and the physiological effects of these compounds.

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The Incorporation of Fatty Acids of Different Chain Length into Liver and Biliary Lipids in the Perfused Rat Liver

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ABSTRACT: In an attempt to correlate the incorporation of fatty acids (FA) of different chain length into liver and biliary lipids, isolated rat livers were perfused for 2 h with Krebs-Ringer bicarbonate containing 1% albumin and 10 μmol of [1-^{14}C]-labeled FA: C_2, C_8, C_{10}, C_{12}, C_{16}, and $C_{18:1}$. One to 1.36 μmol of medium-chain fatty acids (MCFA, C_8, C_{10}, and C_{12}) and 6.6 μmol of long-chain FA (LCFA) were incorporated into liver lipids, 40% of the latter into phosphatidylcholine (PC). ^{14}C-acetate (13 nmol) was incorporated into biliary cholesterol; ^{14}C-MCFA contributed only 3.2–5 nmol; LCFA did not lead to newly synthesized cholesterol. Newly synthesized liver PC (2.75 to 3.25%) and newly synthesized liver cholesterol (6.5 to 10%) were secreted into bile. The specific radioactivity of biliary PC after infusion of all-saturated FA was 3.8–6.8 times higher than that of liver PC; for $C_{18:1}$ it was only 1.7-fold. The specific radioactivity of biliary cholesterol, as compared to liver cholesterol, was 12 times higher for C_2 and five times higher for MCFA. This indicates that a considerable proportion of the newly synthesized lipids was secreted into bile prior to significant mixing with preexisting liver PC and cholesterol pools. Liver PC contained 8% of unchanged ^{14}C-C_{12}; while ^{14}C-C_{10} was not detected. Biliary PC, in contrast, contained 18% of unchanged ^{14}C-C_{12} and 3% ^{14}C-C_{10}. These results suggest that after prolonged infusion of medium-chain triacylglycerols/long-chain triacylglycerols to patients, biliary PC may become enriched with MCFA. In addition, the oxidation of these FA may provide C-2 units which increase cholesterol synthesis.

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The correlation between cholesterol solubility and the concentration of biliary bile salts, phospholipids (PL), and cholesterol were formulated by Admirand and Small (1). Subsequent research on factors affecting cholesterol gallstone formation concentrated mainly on the role of cholesterol and bile salts (1,2). The importance of PL in cholesterol solubilization was appreciated only more recently (3–5).

Biliary PL are generally composed of 95% phosphatidylcholine (PC) and 4% phosphatidylethanolamine (PE) (6). Biliary PC is a complex mixture of different molecular species. In all animal and human studies, PC was found to contain saturated fatty acids (FA) (mainly 16:0 and 18:0) in the *sn*-1 position and unsaturated FA (mainly 18:2, 18:1, and 20:4) in the *sn*-2 position. In man, 1-palmitoyl-2-linoleyl-glyceryl-phosphoryl choline amounted to over 40% of the PC (7,8). Addition of different PC to human bile samples *in vitro* (5,9) caused a marked prolongation of the nucleation time. Since *in vitro* addition of bile acids did not affect the cholesterol nucleation time, it was suggested that increasing PL concentrations in bile might be more effective for prevention of cholesterol crystallization (9).

Experiments performed in animals and humans attempted to induce changes in the composition or concentration of PL in native bile by dietary manipulations. Nervi *et al.* (10) correlated the high incidence of gallstones among young Chilean men with a selective decrease in biliary PL, possibly due to a large intake of legumes. A similar abnormality was induced in rats by a high legume diet (11). Dietary supplementation of fish oil in contrast to more saturated triacylglycerols (TAG) markedly increased the percentage of 20:5 and 22:6 in biliary PC, reducing the lithogenicity and incidence of cholesterol precipitates in rats (12), African green monkeys (13), and hamsters (14–16). Likewise, addition of fish oil to the diet of gallstone patients induced an increase in 20:5 and 22:6 in biliary PC with a concomitant reduction in 18:2 concentration (17). Robins and Patton (8) pointed out that in order to understand the changes occurring in biliary PC, analysis of the molecular species is required. When rats were fed pure TAG-containing FA which were more hydrophilic than the FA normally present in bile PC, bile became highly enriched in new molecular species containing the particular FA fed. In contrast when the fat contained less hydrophilic FA, the composition of biliary PC did not change. To further characterize these changes, rat livers were perfused with FA of different chain lengths and degree of unsaturation (18). Results indicated that whereas 18:1, 20:4, and 20:5 were enriched in the *sn*-2 position of biliary PC, 16:1, 17:1, and 18:2 were found

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Abbreviations: C_2, acetic acid; C_8, octanoic acid; C_{10}, decanoic acid; C_{12}, dodecanoic acid (lauric acid); C_{16}, hexadecanoic acid (palmitic acid); $C_{18:1}$, oleic acid; FA, fatty acid; FFA, free fatty acid; GLC, gas–liquid chromatography; KRB, Krebs-Ringer bicarbonate; LCFA, long-chain fatty acids; LCT, long-chain triacylglycerols; MCFA, medium-chain fatty acids; MCT, medium-chain triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; TAG, triacylglycerols; TLC, thin-layer chromatography.

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in both positions. The above findings clearly indicate that the FA composition of biliary PC can be selectively changed. Only relatively few experiments were carried out attempting to change the head-group composition. Addition of ethanolamine and linoleic acid to the diet of rats and hamsters caused a slight increase in biliary PE; cholesterol concentration and the cholesterol saturation index were significantly lower (19). In humans addition of PE (rich in 18:2) to the diet was found to increase the percentage of 18:2 in biliary PC without changing PE concentration (20).

Total parenteral nutrition may result in the development of gallstones (21,22). In our clinical studies (23) we observed marked differences in the concentration of lipids in blood and bile following short-term infusion (24 h) of TAG containing a mixture of long-chain triacylglycerols (LCT) and medium-chain triacylglycerols (MCT). To try to understand the effect of MCT, we carried out a systematic *in vitro* study (24) on the metabolism of FA of various chain lengths by HepG-2 cells. It was found that C_8 was incorporated only after β oxidation. C_{10}, C_{12}, and C_{16} were largely incorporated unchanged into cellular TAG and PL. In the HepG-2 model, the secretion of biliary lipids could not be studied. In contrast, in the perfused rat liver model, both lipid synthesis and secretion into bile could be studied simultaneously. In the present manuscript, we report the effect of FA of different chain lengths on liver and biliary lipid composition.

MATERIALS AND METHODS

Materials. [1-^{14}C]Sodium acetate (52.1 mCi/mmol), [1-^{14}C]-lauric acid (52.8 mCi/mmol) and [1-^{14}C]palmitic acid (57.0 mCi/mmol) were obtained from Amersham (United Kingdom); [1-^{14}C]sodium octanoate (55.0 mCi/mmol) and [1-^{14}C]oleic acid (50.0 mCi/mmol) from NEN (Boston, MA); and [1-^{14}C]sodium decanoate (10.6 mCi/mmol) from Sigma (St. Louis, MO). All ^{14}C-labeled FA were diluted with nonlabeled FA to yield a final specific radioactivity of 2 mCi/mmol; ^{14}C-acetate was diluted to a specific radioactivity of 4 mCi/mmol. All FA were obtained from Sigma. Thin-layer Silica gel 60 plates (0.25 mm) were obtained from Merck (Darmstadt, Germany), KC 18 Silica gel plates (0.2 mm) from Whatman (Maidstone, England). All solvents were of analytical grade (Merck). Albumin, bovine-fat poor, was obtained from Sigma. Scintillation fluid-Ultima-Gold was obtained from Packard (Meriden, CT).

Isolated perfused rat liver studies. Male Wistar rats weighing 300–360 g were used. They were fed Purina Rodent Chow *ad libitum* and maintained under a constant light cycle of 12 h. The surgical procedures were performed as described by Corasanti *et al.* (25). Rats were anesthetized with pentobarbitone (50 mg/kg body wt i.p.) (Ceva, Paris, France). The bile duct was cannulated with PE-10 tubing (Clay Adams, Parsippany, NJ). The portal vein was cannulated with a 16-gauge Teflon intravenous catheter (BOC Ohmeda AB, Helsingborg, Sweden). The liver was then perfused at a constant flow of 20 mL/min with oxygenated Krebs-Ringer bicarbonate (KRB) buffer containing (in mM) 120 NaCl, 4.7 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 1.8 $CaCl_2$, and 25 $NaHCO_3$. Added to the KRB buffer were 5.5 mM glucose and 200 U heparin/100 mL.

After cannulation of the inferior vena cava, the livers were removed from the rat, transferred into a heated perfusion chamber, and perfused in a recirculating closed system (volume: 200 mL) at a constant flow rate of 40 mL/min with KRB buffer containing 5.5 mM glucose and 1% bovine serum albumin. The KRB buffer was gased continuously with a humidified mixture of 95% O_2–5% CO_2 and maintained at 37 ± 0.5°C. After 10 min of equilibration, a bolus of the following lipids was added: 10 μmol of [1-^{14}C]-labeled FA: C_2, C_8, C_{10}, C_{12}, C_{16}, and $C_{18:1}$. Taurocholic acid (30 μM) was infused continuously to stimulate bile secretion. The livers were perfused for 2 h. At the end of the experiment, the livers were perfused for 15 min with fresh KRB. Perfusion pressure was monitored continuously. Bile and perfusate samples were collected every 15 min. Bile was collected into pretared tubes which were changed every 15 min. The tubes were then weighed, and bile flow was calculated as μg bile/min/g liver. At the end of the experiments, bile samples were pooled. Livers, bile, and perfusate samples were frozen at −70°C for lipid analysis. Each perfusion experiment was done twice, and livers and biles were analyzed separately. Approximately 2 mL of bile was collected. The mean weight of the livers was 10.4 ± 1.0 g. Two 1-g samples of each liver were used for lipid extraction and analysis. The results presented in the tables are the mean of these four determinations. A sample of the perfusate was removed at the end of the experiment, and the distribution of the radioactivity among the different lipid classes was determined.

The viability of the perfused liver was assessed throughout the perfusion by monitoring perfusion pressure (7–12 cm H_2O), determining baseline bile flow, and measuring the release of lactate dehydrogenase as well as observing the general appearance of the liver.

Liver and bile lipid extraction and analysis. Liver samples of 1 g were homogenized with 5 mL of saline using an Ultra-Turax (Janke and Kunkel Ika-Werk, Staufen, Germany). The homogenates were then extracted with chloroform/methanol according to Bligh and Dyer (26). Pooled bile (1 mL) from each experiment and 5 mL of the perfusate collected at the end of the experiment were similarly extracted (26).

The amount of the radioactivity in the extracts was determined using a Kontron-Betamatic counter (Switzerland). Aliquots of the lipid extracts were used for the separation of neutral lipids and PL by thin-layer chromatography (TLC) as previously described (24). Neutral lipids were separated on Silica gel G plates employing hexane/diethylether/methanol/acetic acid (90:20:3:2, by vol) as solvent. PL were separated using chloroform/methanol/acetic acid/water (100:20:12:5, by vol) as solvent. For determination of radioactivity, lipid spots were scraped into scintillation vials and suspended in 3.5 mL scintillation fluid. The overlapping spot of diglycerides and cholesterol on the TLC plates was collected and

saponified with 0.5 M methanolic KOH for 30 min at 50°C. Samples were than acidified and extracted with hexane. Aliquots were placed on TLC plates as described above.

To determine the chain length of the FA which were incorporated into PL and TAG, the corresponding spots were collected and saponified with 0.5 M methanolic KOH. FA were recovered and separated according to their chain length by reversed-phase TLC on KC18 plates utilizing acetonitrile/methanol/water (6:3:1, by vol) as solvent (24,27). Under our experimental conditions, 18:0 and 18:1 co-migrated with 16:0. Spots were scraped directly into scintillation vials, and the radioactivity was determined immediately after addition of scintillation fluid.

To determine the FA composition of PL and TAG, the FA recovered after alkaline hydrolysis were methylated with diazomethane (28). The methyl esters were separated by gas–liquid chromatography (GLC) on a 30 m PAG (poly alkylene glycol) column (0.25 μm film thickness; Supelco, Bellefonte, PA) at a temperature range of 185–220°C, employing a Hewlett-Packard 5790A gas chromatograph equipped with a flame-ionization detector. The relative composition of FA mixtures was calculated employing a Hewlett-Packard 3396 integrator. For quantitative analysis, heptadecanoic acid was added as an internal standard, assuming that the response of the detector for all methyl fatty esters was identical. Saturated and unsaturated methyl FA were separated on $AgNO_3$-impregnated plates (29), employing hexane/diethyl ether (80:20, vol/vol) as solvent.

Free cholesterol was determined directly from the chloroform/methanol extracts by an enzymatic colorimetric method using a cholesterol oxidase kit from Boehringer Mannheim GmbH (Ingelheim, Germany).

RESULTS

Incorporation of ^{14}C-labeled FA into liver lipids. Addition of 10 μmol of [1-^{14}C]-labeled FA (C_2, C_8, C_{10}, C_{12}, C_{16}, and $C_{18:1}$) to the perfusion buffer resulted in the incorporation of 0.66 to 6.61 μmol (6.6 to 66.1% of the radioactivity added to the perfusate) of ^{14}C-FA into liver lipids (Table 1). More long-chain FA (LCFA) (C_{16} and $C_{18:1}$) were incorporated than medium-chain FA (MCFA) (C_8, C_{10}, and C_{12}). Only acetate was extensively incorporated into cholesterol (25% of the amount incorporated into liver lipids). The other FA were incorporated mainly into PC (*ca.* 40%) and to a lesser extent into TAG and PE. Diacylglycerols, FA, cholesterol ester, and phosphatidylserine contained only relatively small amounts of radioactivity (not shown). LCFA did not lead to newly synthesized cholesterol.

Incorporation of ^{14}C-labeled FA into biliary lipids. Bile was collected throughout the perfusion period. Figure 1 shows that bile secretion became stable after 30 min at an average rate of 1.8 μg/min/g liver and remained constant for the rest of the perfusion period. As can be seen in Figure 2 secretion of ^{14}C-labeled metabolites into bile was dependent on the ^{14}C-FA added. In most cases, maximal values were obtained 40 to 50 min after addition of the ^{14}C-substrate. ^{14}C-Metabolites include ^{14}C-lipids and ^{14}C-water-soluble compounds. For C_2, C_8, C_{10}, and C_{12} the ^{14}C-water-soluble compounds amounted to 65–75% of the total radioactivity secreted into bile. In the presence of C_{16} and $C_{18:1}$, only 15% of the radioactivity was water-soluble. ^{14}C-Free fatty acid (FFA) were not secreted into bile. ^{14}C was not detected in bile acids (30). Attempts to identify the water-soluble compounds indicated that these were not volatile and could not be extracted into ether (after acidification). ^{14}C-Acetoacetate was not detected after incubation with β-hydroxybutyrate dehydrogenase (31).

Table 2 shows the incorporation of ^{14}C-FA into biliary lipids. When ^{14}C-acetate was perfused, 17 nmol were recorded in biliary lipids—75.5% of which were incorporated into cholesterol. In the presence of MCFA, 19 to 22 nmol were incorporated into biliary lipids—71–78% into PC; in the presence of C_{16} and $C_{18:1}$, 90 and 70 nmol were incorporated into biliary lipids; over 95% of the radioactivity was detected

TABLE 1
Incorporation of Different ^{14}C-FA into Liver Lipids[a]

FA	^{14}C-FA-incorporated (μmol)	Radioactivity incorporated (%)			
		TAG	Cholesterol	PC	PE
C_2	0.66 ± 0.01	18.18 ± 1.46	25.03 ± 4.2	31.42 ± 3.77	10.79 ± 0.77
C_8	1.36 ± 0.41	29.83 ± 7.12	2.49 ± 1.47	39.84 ± 6.01	13.58 ± 1.42
C_{10}	0.99 ± 0.19	21.41 ± 1.37	7.59 ± 1.73	38.66 ± 3.97	14.78 ± 1.97
C_{12}	1.21 ± 0.27	25.49 ± 3.19	3.82 ± 0.61	37.7 ± 2.59	13.19 ± 0.73
C_{16}	6.61 ± 1.88	22.77 ± 1.87	Trace	46.51 ± 1.15	13.91 ± 1.65
$C_{18:1}$	6.61 ± 1.78	35.01 ± 4.58	Trace	33.38 ± 5.03	12.60 ± 0.59

[a]Lipids were extracted from liver samples and separated by thin-layer chromatography (TLC) as described in the Materials and Methods section. Hexane/diethylether/methanol/acetic acid (90:20:3:2, by vol) was used for the separation of neutral lipids, chloroform/methanol/acetic acid/water (100:20:12:4, by vol) for the separation of phospholipids (PL). Lipid spots were scraped directly into scintillation vials and radioactivity determined after addition of scintillation fluid. The incorporation of ^{14}C-fatty acid (FA) is expressed as μmol per total liver, assuming incorporation of intact FA. Diacylglycerols, FA, cholesterol esters, and phosphatidylserine contained 1–3% each. The results given are the mean (±SD) of data obtained from two different experiments. Liver weight was in the range of 9 to 11 g. TAG, triacylglycerols; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

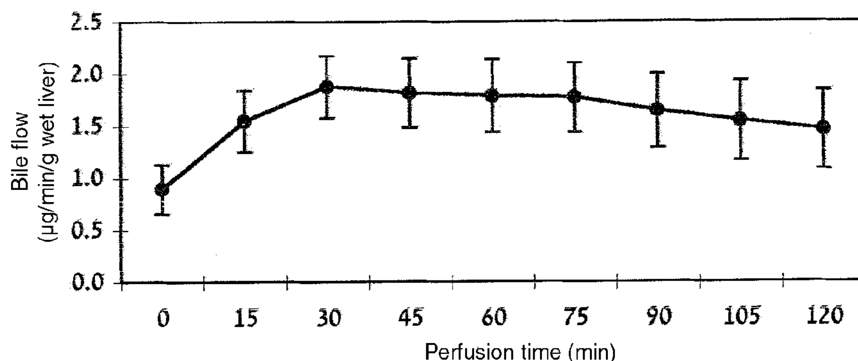

FIG. 1. Bile flow rate. Bile was collected into pretared tubes each 15 min. The bile flow was calculated as µg bile/min per gram wet liver. The results presented are the mean values ± SD of all bile samples collected during the study period (n = 12).

in PC. Biliary PE contained *ca.* 2–3%, and phosphatidylserine contained less than 1% (not shown).

Comparison of FA composition of liver and biliary PL. Table 3 compares the quantities and FA composition of liver and biliary PC and PE. Since the amounts of these PL and their FA composition were not affected by the addition of the different ^{14}C-FA to the perfusate, the mean results for all experiments are presented in the table. As can be seen from Table 3, biliary PL contained mainly PC (93%) and PE (6%); phosphatidylserine amounted to less than 1% (not shown).

Comparison of the incorporation of ^{14}C-FA into liver and biliary PC and cholesterol. Table 4 compares the specific radioactivity of liver and biliary PC and cholesterol. The specific radioactivity of biliary PC after administration of all saturated FA was 3.8 to 6.8 times higher than that of liver PC; for $C_{18:1}$ it was only 1.7-fold, suggesting a higher dilution of 18:1 by liver PC. The specific radioactivity of biliary cholesterol (as compared to liver cholesterol) was 12 times higher for C_2 and five times higher for MCFA. LCFA did not lead to ^{14}C-cholesterol synthesis.

From the data presented in Tables 1 and 2, it can be calculated that 200 nmol of acetate and 400–550 nmol of MCFA were incorporated into liver PC; 4.1 nmol acetate and 13–17 nmol of MCFA were incorporated into biliary PC. The incorporation of the LCFA into liver and biliary PC was considerably higher, 3312 and 85 nmol (into liver and bile, respectively) for ^{14}C-C_{16} and 2235 and 68 nmol (into liver and bile, respectively) for $C_{18:1}$. Although the incorporation of LCFA (16:0 and 18:1) was considerably higher, nevertheless in all experiments ^{14}C-labeled biliary PC amounted to 2.75 to 3.25% of the ^{14}C-labeled liver PC. Secretion of PL, TAG, and cholesterol into the perfusate reached 0.5–1% of that present in the liver. The distribution of radioactivity between the different lipids in the perfusate was generally similar to that de-

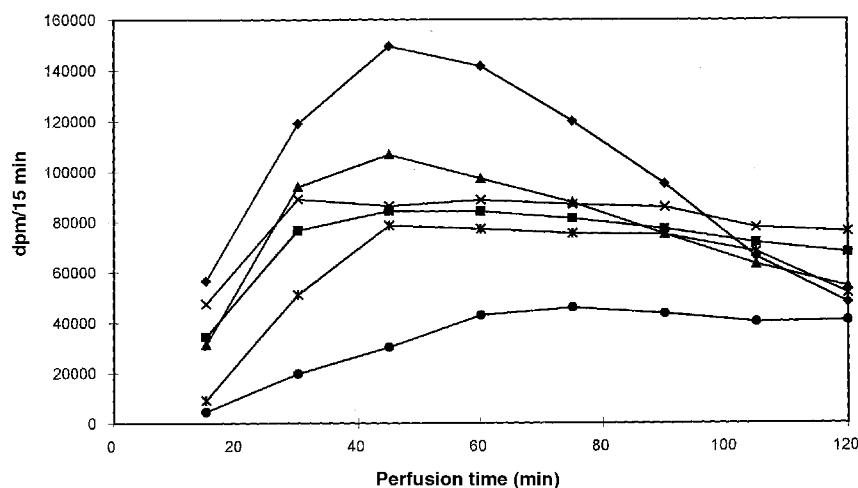

FIG. 2. Secretion of ^{14}C-labeled metabolites into bile. The secretion of ^{14}C-labeled metabolites into bile was measured for each fatty acid (FA): C_2 ◆, C_8 ■, C_{10} ▲, C_{12} X, C_{16} ✳, and $C_{18:1}$ ●. Bile samples were collected as described in the legend to Figure 1. A sample of 10 µL of bile from each time point was counted to determine ^{14}C-content.

TABLE 2
Incorporation of Different ^{14}C-FA into Biliary Lipids[a]

	^{14}C-FA in bile (nmol)	^{14}C incorporated (%)	
		Cholesterol	PC
C_2	17.02 ± 3.13	75.47 ± 6.8	23.85 ± 6.77
C_8	18.62 ± 10.24	17.54 ± 6.81	79.89 ± 6.99
C_{10}	19.00 ± 7.24	26.22 ± 12.04	71.34 ± 12.00
C_{12}	21.99 ± 1.25	21.22 ± 3.61	75.96 ± 3.99
C_{16}	89.18 ± 38.43	Trace	95.03 ± 0.91
$C_{18:1}$	70.65 ± 14.07	Trace	95.79 ± 0.39

[a]Lipids were extracted from 1 mL bile samples and separated by TLC as described in the legend to Table 1. The incorporation of ^{14}C-FA is expressed as nmol per total bile sample, assuming that the ^{14}C-FA reached the bile *via* the liver without processing. The results are the mean (±SD) of the two different bile samples analyzed. See Table 1 for abbreviations.

tected in the liver. Only when ^{14}C-acetate was added, the percentage of PL in the perfusate was higher and that of cholesterol was lower than in the liver.

Occurrence of C_{10} and C_{12} in liver and biliary lipids. ^{14}C-TAG and PL synthesized by the liver during perfusion with ^{14}C-C_2, -C_8 and -C_{16} were isolated and the ^{14}C-FA recovered after saponification were separated by TLC. Only ^{14}C-LCFA (^{14}C-C_{16} and -C_{18}) were identified. However, after perfusion with ^{14}C-C_{10}, saponification of ^{14}C-TAG yielded *ca.* 10% ^{14}C-C_{10} (Fig. 3) and 90% of ^{14}C-C_{16} and C_{18}; ^{14}C-C_{10} was not detected in liver PL. After perfusion with ^{14}C-C_{12} saponification of liver TAG yield *ca.* 35% ^{14}C-C_{12} and 65% ^{14}C-C_{16} and -C_{18}. Liver PC contained *ca.* 8% of ^{14}C-C_{12}. Biliary PC contained 3% ^{14}C-C_{10} and 18% ^{14}C-C_{12}.

For further identification of the newly incorporated FA, methyl-FA were separated on $AgNO_3$-impregnated plates. Less than 1% of the radioactivity was associated with monounsaturated FA (not shown), indicating that desaturation did not occur under our experimental conditions. After ^{14}C-$C_{18:1}$ perfusion, 98% of the radioactivity of the FA isolated from liver and biliary lipids was present in oleic acid.

DISCUSSION

In the present paper the metabolism of ^{14}C-labeled FA of different chain lengths by the isolated perfused rat liver was studied. This experimental approach permitted the simultaneous measurement of FA incorporation into liver and biliary lipids. We previously reported (23) that short-term infusion of MCT/LCT to patients resulted in significant changes in bile composition, whereas LCT alone did not elicit any changes. The aim of this and our previous studies (24) was to examine *in vitro* the mechanism underlying these *in vivo* effects. LCT (500 μL) which contained 113 μmol of soya-TAG and 0.53 μmol of (^{3}H-oleate)-labeled trioleylglycerol were added to the perfusate. After 2 h, 50% of the remaining TAG (12 μmol) were detected as free FA, indicating extensive lipolysis of the added LCT. Although the liver was perfused for 30 min with LCT-free buffer, most of the radioactivity isolated from the liver (61.4 μmol) was detected in TAG (39.8%), FFA (22.9%), and diacylglycerols (18.5%) and only 10.6% was incorporated into PL. The occurrence of large amounts of FFA in the perfusate and the liver suggests the role of FFA uptake under our experimental conditions. Since radioactively labeled MCT was not available to us, ^{14}C-labeled MCFA (C_8, C_{10}, and C_{12}) and LCFA (C_{16} and $C_{18:1}$) were added instead. ^{14}C Acetate was used in these experiments to represent acetyl CoA, the β-oxidation product of the added FA.

The major finding of the present investigation is that C_2, C_8, C_{10}, and C_{12} were largely used for the synthesis of saturated LCFA, which were subsequently incorporated into liver PL and TAG, whereas C_{16} and $C_{18:1}$ were used directly for PC and TAG synthesis. For C_8, C_{10}, and C_{12} to yield LCFA, they have to be either oxidized to C_2 units prior to being used for *de novo* FA synthesis or elongated after internalization. The occurrence of water-soluble metabolites in the bile after administration of ^{14}C-C_2 and ^{14}C-MCFA suggests that acetyl CoA was formed by β-oxidation of MCFA (32) and was further metabolized. We did not succeed in identifying the water-

TABLE 3
Lipid Content and PL-FA Composition of Liver and Bile[a]

	Content[b]	16:0	18:0	18:1	18:2	20:4	22:6
		FA composition (%)					
Liver PC	21.1 ± 4.2	19.3 ± 0.9	20.5 ± 1.0	8.5 ± 0.5	17.4 ± 1.0	20.5 ± 2.1	6.37 ± 0.6
PE	10.8 ± 2.4	15.3 ± 1.5	18.5 ± 1.8	8.2 ± 0.4	17.0 ± 4.0	22.5 ± 1.9	10.9 ± 0.9
Cholesterol	5.81 ± 1.01						
Bile PC	1.2 ± 0.2	34.7 ± 1.2	6.3 ± 0.3	9.4 ± 0.4	30.8 ± 1.5	10.1 ± 1.1	1.9 ± 0.3
PE	0.08 ± 0.01	27.9 ± 2.4	13.3 ± 1.0	8.4 ± 0.4	23.7 ±1.1	13.5 ± 0.8	5.6 ± 0.8
Cholesterol	0.26 ± 0.07						

[a]To determine the cholesterol and PL content and FA composition, lipids were extracted from liver and bile samples and separated by TLC as described in the Materials and Methods section and legend to Table 1. PL spots were collected and saponified with 0.5 M methanolic KOH. FA were recovered after acidification, methylated with diazomethane (Ref. 28) and separated by gas–liquid chromatography as described in the Materials and Methods section. Minor FA components such as 16:1, 18:3, 20:1, and 20:5 are not included in the table. The PL content and FA composition of liver and biliary lipids were similar for all samples analyzed. The results are the mean (±SD) of 12 independent experiments.
[b]Lipid contents of liver and bile are presented as μmol/g and μmol/mL, respectively. See Table 1 for abbreviations.

TABLE 4
Specific Radioactivity of Liver and Biliary PC and Cholesterol[a]

	PC		Cholesterol	
	Liver (dpm/μmol)	Bile (dpm/μmol)	Liver (dpm/μmol)	Bile (dpm/μmol)
C_2	3813	19572	14980	178208
C_8	7582	48395	2057	10870
C_{10}	7291	27815	4230	23040
C_{12}	6070	25844	1780	8414
C_{16}	54521	248931	—	—
$C_{18:1}$	23503	39032	—	—

[a]Liver and biliary PC and cholesterol were isolated and their concentration and radioactivity determined as described in Tables 1, 2, and 3. See Table 1 for abbreviations.

soluble compounds. The most likely metabolites, 3-hydroxybutyrate and acetoacetate, were not detected.

During the perfusion period, irrespective of the FA administered, 2.75 to 3.25% of the ^{14}C-labeled PC were secreted into bile. The specific radioactivity of biliary PC was higher than that of liver PC, indicating the secretion of the ^{14}C-labeled PC into bile prior to mixing with the liver pools of PC. Whereas after perfusion with MCFA, newly synthesized biliary PC amounted to 0.57–0.70% of total biliary PC, with LCFA newly synthesized PC reached 2.8 to 3.5%. Biliary secretion of PC by the perfused liver was previously studied by Robins *et al.* (18,33). In these experiments ^{14}C-choline was continuously added. Although PC secretion was four times higher than in our experiments, secretion of newly synthesized PC amounted to 3.4% of total biliary PC.

In contrast to the results obtained with HepG-2 cells (24) in which ^{14}C-C_{12} was incorporated into cellular lipids to the same extent as ^{14}C-C_{16}, in the perfused rat liver its incorporation was like that of C_8 and C_{10}. Furthermore, whereas in HepG-2 cells, C_{12} was largely incorporated unchanged (80–90% of all ^{14}C-FA in TAG; 50–60% in PL), in the perfused rat liver ^{14}C-C_{12} amounted to 30–40% of all ^{14}C-FA in TAG and 10% in PL. Unchanged ^{14}C-C_{10} was not detected in liver PL. Biliary PC contained 3% of unchanged ^{14}C-C_{10} and 18% unchanged ^{14}C-C_{12}. Thus the percentage of C_{10} and C_{12} in biliary PC was significantly higher than in liver PC.

Using GLC, we could not detect C_{10} and C_{12} in biliary PC. The calculated amount of C_{12} present in PC (500 ng) was only 0.04% of total biliary PC-FA and thus below our detection limit.

Robins *et al.* (18) previously showed that there were significantly greater amounts of the administered 16:1, 17:1, and 18:2 in the PC of bile than the liver leading to the secretion of 16:1-16:1, 17:1-17:1, and 18:2-18:2 species, indicating preferential secretion of newly synthesized PC. The occurrence of ^{14}C-C_{12} in biliary PC in our experiments is compatible with these observations.

^{14}C-Acetate, in contrast to MCFA, was preferentially incorporated into liver and biliary cholesterol (Table 2). However, since during β-oxidation of MCFA dilution of ^{14}C-acetyl CoA occurs, the actual incorporation is probably higher. During the 2-h infusion, 6.5 to 10% of the newly synthesized cholesterol was secreted into the bile. The specific radioactivity of biliary cholesterol, like that of PC, was higher than that of liver cholesterol, indicating secretion of newly synthesized

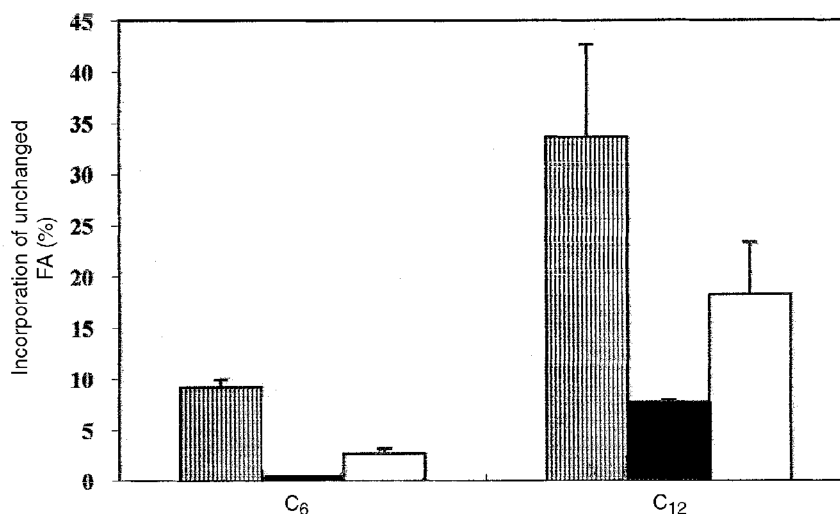

FIG. 3. Recovery of ^{14}C-C_{10} and ^{14}C-C_{12} in liver and biliary lipids. To determine the occurrence of ^{14}C-C_{10} and ^{14}C-C_{12}, lipids were extracted from liver and bile samples and separated by thin-layer chromatography as described in the Materials and Methods section. Liver triacylglycerols (TAG) ▥, liver phosphatidylcholine (PC) ■, and biliary PC □ were scraped off the plate and subjected to alkaline hydrolysis. FA were recovered after acidification and separated by reversed-phase thin-layer chromatography as described in the Materials and Methods section. FA spots were scraped into scintillation vials and the radioactivity determined. Each point is the mean of two to four different analyses ± SD. See Figure 2 for abbreviation.

cholesterol prior to its mixing with liver pools. The preferential secretion of newly synthesized cholesterol was reported previously (34,35). The percentage of newly synthesized cholesterol of the total biliary cholesterol reached 12.8% when acetate was administered and 3.2 to 5% in the presence of MCFA. Our present data on the secretion of ^{14}C-PC and ^{14}C-cholesterol and those previously reported by Robins *et al.* (18,33,35) indicate that most of the biliary PC and cholesterol were recruited from preexisting liver-lipid pools.

The effect of MCFA on the composition of biliary PC was not described previously. Feeding coconut oil (rich in 12:0 and 14:0) to hamsters (15,16) resulted in a reduction of biliary PL secretion and an increase in the lithogenic index. Occurrence of 12:0 and 14:0 in biliary PC was not recorded. Furthermore, the contribution of MCFA to biliary cholesterol and PC in the perfused rat liver as shown in the present communication may explain the mechanisms responsible for the different effects of MCFA and LCFA on bile composition as observed in our clinical studies as well others. Further studies are being conducted to clarify this issue.

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The Metabolism of Native and Randomized Butterfat Chylomicrons in the Rat Is Similar

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ABSTRACT: Reportedly, randomly rearranging the position of fatty acids (FA) in butterfat triacylglycerol (TAG) by interesterification, thereby lowering the proportion of saturated FA in the *sn*-2 position, reduces its hypercholesterolemic and hypertriglyceridemic properties when fed to humans. The aim of this work was to determine if these reductions in plasma cholesterol and TAG could be explained by an improved rate of clearance from the plasma of chylomicrons composed of randomized butterfat, using a rat model. Acute chylomicron clearance studies demonstrated no differences in fractional clearance rates of cholesteryl esters and TAG from the plasma of rats infused with chylomicrons produced from gastric feeding of either native (NBF) or randomized (RBF) butterfat. Although there was a 14% decrease in the level of saturated FA occupying the *sn*-2 position of TAG in RBF compared with NBF, this difference became negligible (~5%), following digestion of the fat and subsequent repackaging of TAG into chylomicrons. These observations suggest that the previously observed reduction in hypercholesterolemic properties of randomized butterfat in rat is unlikely to be explained by improved clearance of chylomicron TAG.

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The consumption of butterfat in the human diet was consistently demonstrated to cause the elevation of plasma total cholesterol levels, in particular low density (LDL)-cholesterol (1–3), and was also shown to lead to the development of atherosclerotic lesions of the aorta when fed to rats as part of a diet containing cholesterol and cholic acid (4). Mechanisms proposed to explain how fats rich in saturated fatty acid (FA), such as butterfat, result in these consequences include possibility that saturated FA modify the composition of LDL, resulting in the decreased binding, internalization, and degradation of LDL by tissues (5). Saturated FA can directly act to suppress receptor-mediated endocytosis of LDL by causing alterations in the free cholesterol pool in the liver which is thought to regulate LDL-receptor transcription within the cell as reviewed by Dietschy (6).

The physiological effects of dietary fat are influenced not only by the relative proportion of saturated and unsaturated FA in triacylglycerol (TAG) but also by their arrangement among the three available esterification sites present on the backbone of TAG molecules. In particular, Christophe *et al.* (7,8) described reduced hypercholesterolemic and hypertriglyceridemic properties of butterfat when fed to people in a randomized form when compared with native butterfat. These findings, along with evidence that randomized butterfat (RBF) was hydrolyzed *in vitro* by pancreatic lipase at a much faster rate than butterfat in the native form (9), demonstrated the possible importance of positional distribution of FA in lipid metabolism. Redgrave *et al.* (10) and Mortimer *et al.* (11) also demonstrated that the saturated FA in the *sn*-2 position of TAG directly affected the rate at which chylomicron remnant particles were cleared from the bloodstream by the liver.

In the present study, randomization of butterfat resulted in a ~14% decrease in the amount of saturated FA occupying the *sn*-2 position of TAG. This study set out to determine if this decrease affected the measured rate of clearance of chylomicron-borne TAG and cholesterol ester (CE) from the bloodstream of rats injected with chylomicrons produced during absorption of RBF vs. NBF, thereby providing a possible mechanism to explain the reduced lipidemic effects seen in the studies of Christophe *et al.* (7,8).

METHODS

Interesterification (randomization) of butterfat. Anhydrous butterfat was randomized using sodium metal as a catalyst as previously described (12). After the reaction, the butterfat was washed in water and then dried under vacuum at 110–120°C to clarify its appearance and finally bleached with 2% bleaching earth (bleaching earth and filter aid; Meadowlea Foods, Sunshine VIC, Australia) for 30 min at 110–120°C. The bleaching earth was then filtered off at 60–70°C in a Buchner funnel using a filter aid to give a clear oil. Native butterfat was bleached to obtain a clear fat similar to the randomized form before being used.

TAG and FA analysis of dietary fats. Dietary fats were examined for total FA composition and positional distribution

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Abbreviations: CE, cholesterol esters; FA, fatty acid; FAME, fatty acid methyl esters; LDL, low density lipoprotein; NBF, native butterfat; RBF, randomized butterfat; TAG, triacylglycerol.

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of FA in the backbone of TAG using pancreatic lipase digestion. About 20 mg of TAG was isolated by thin-layer chromatography (13), followed by reaction of ~5 mg of TAG with pancreatic lipase as described by Luddy *et al*. (14). The fatty acid methyl esters (FAME) of TAG were formed by saponification in KOH for 10 min followed by methylation with BF_3 in methanol for 10 min (13). FAME were analyzed by capillary gas–liquid chromatography (DANI 6500HR; DANI S.P.A., Monza, Italy), equipped with a flame-ionization detector, using a 60 m × 0.32 mm i.d. crossbonded 90% bis-cyanopropyl: 10% phenyl cyanopropyl capillary column (RTX 2330; Restec Corporation, Bellefonte, PA), programmed from 95°C for 3 min to 190°C at 10°C/min with helium as carrier gas at a flow rate of 5 mL/min. FAME were identified by comparison with standard mixtures of FAME, and the results were calculated using response factors derived from chromatographing standards of known composition. Indication of successful randomization of butterfat was obtained by examination of TAG species separated by high-performance liquid chromatography using a Supelco LC-18 column, dimensions 150 mm × 4.6 mm i.d. (Supelco Inc., Bellefonte, PA) and with detection by evaporative light-scattering (ACS Model 750/14; ACS Ltd.; Macclesfield, Cheshire, United Kingdom).

Animals and diets. Male Sprague-Dawley rats weighing 230–280 g, which had been allowed free access to water and commercial rodent diet, were used in the production of chylomicrons. Male rats of the same strain weighing 200–230 g fed commercial rodent diet were used in the reinjection studies.

Collection of lymph and isolation of chylomicrons. Chow-fed rats were infused gastrically with 300 mg of NBF or RBF or corn oil (control) along with 50 μCi [1α,2α(*n*)-^{3}H] cholesterol and 15 μCi [1-^{14}C]palmitic acid. Intestinal lymph was collected for 6 h *via* a vinyl tube (0.8 mm o.d. × 0.5 mm i.d.; Dural Plastics and Engineering, Auburn, NSW, Australia) surgically implanted in the cisterna chyli, and chylomicrons were isolated using the centrifugal method described by Redgrave *et al.* (10).

Characterization of chylomicrons. Relative proportions of TAG, cholesterol, phospholipid, and protein in chylomicron particles were measured enzymatically on an autoanalyzer (Hitachi autoanalyser 704; Tokyo, Japan), using commercially available kits (Boehringer Mannheim, Mannheim, Germany). Chylomicron TAG FA and *sn*-2 FA composition were determined as for dietary fat TAG (13). Electron microscopy of chylomicron particles was performed using a Jeol 2000 FX transmission electron microscope (Tokyo, Japan) following negative-staining of particles with 2% phosphotungstic acid in 0.15 M NaCl, pH 4.8. Briefly, 10 μL of diluted sample (<100 mg/mL of protein) was dropped onto a 150-mesh copper grid for 30 s. The pointed end of a very coarse blotting paper was used to touch the edge of the grid to draw off the excess fluid, leaving a layer of particles in the grid. Phosphotungstic acid (10 μL) was then dropped on the grid and left for 60 s before being drawn off using the blotting paper method. Particles were then viewed under electron microscopy after air drying for several minutes. Particle size was determined from the average diameter of 100 chylomicrons measured from high-quality photographs.

Acute chylomicron reinjection studies. Labeled chylomicrons were injected into the venous cannula of conscious rats. Blood samples of 0.35 mL were then taken from the arterial cannula (with physiological saline injected to replace the blood volume) at 3, 5, 8, 12, 20, 25, and 30 min. At 30 min, the rat was anesthetized by injecting 35 mg/kg of Nembutal *via* the venous cannula, the liver and spleen were excised, and the radioactive lipids were extracted from minced whole spleen and from minced 1-g pieces of liver. Lipids were extracted into 30 mL of chloroform/methanol 2:1. Aliquots were taken, the solvent was evaporated, and radioactivity was measured in Readyvalue liquid scintillation cocktail (Beckman Instruments Inc., Fullerton, CA) by liquid scintillation spectrometry (1215 RACKBETA Liquid scintillation counter; LKB-Wallac, Turku, Finland) as described by Redgrave *et al* (10). Radioactivity in plasma was measured in 150-μL aliquots without extraction. Plasma clearance kinetics were computed from exponential curves fitted by least squares procedures to samples taken during the first 12 min after injection. The fractional clearance rate then represents the slope of the exponential fit of the data (10).

RESULTS AND DISCUSSION

The total FA and *sn*-2 FA compositions of NBF and RBF and the total FA and *sn*-2 FA composition of chylomicron TAG are shown in Table 1. Total FA composition of the dietary fats was similar for both NBF and RBF forms of butterfat. Analysis of *sn*-2 position FA composition of NBF revealed that ~65% of FA was saturated. Randomization decreased the total level of saturates in the *sn*-2 position to 51%. This net decrease resulted from a 44% reduction in 14:0 and 29% decrease in 16:0. The amount of 18:0 present in the *sn*-2 position increased by 83% but only contributed 11% of *sn*-2 position FA in the randomized form (Fig. 1). The conditions of the interesterification reaction produced 100% randomization of FA for butterfat as judged by comparison of *sn*-2 position FA composition with total FA composition of the native fat, since the *sn*-2 position FA composition mirrored that of the total FA composition.

There were no significant differences in lipid composition between NBF and RBF chylomicrons, with TAG constituting ~89%, phospholipid ~9%, cholesterol ~1%, and protein ~1% of total weight (data not shown). Chylomicrons were viewed by transmission electron microscopy, and the mean diameters and appearance were similar for each dietary fat (data not shown). Total FA composition was similar for both NBF and RBF chylomicrons and reflected the total composition of the infused fats. The amounts of 18:2n-6 in the chylomicron TAG were increased, indicating supply of endogenous 18:2n-6 to intestinal TAG synthesis while the longer chain 20:4n-6 and 22:6n-3 polyunsaturated fatty acids, which were not present in the initial fats, also appeared in chylomicron TAG in small amounts. The *sn*-2 FA composition varied between dietary

TABLE 1
Fatty Acid Composition of Total TAG and TAG *sn*-2 in Fed Fats and Lymph Chylomicrons (% of total fatty acid, mean ± SD)[a]

	Native butterfat[a]		Randomized butterfat[a]		Native butterfat chylomicrons[b]		Randomized butterfat chylomicrons[b]	
FA	Total FA	TAG *sn*-2 FA	Total FA	TAG *sn*-2 FA	Total FA	TAG *sn*-2 FA	Total FA	TAG *sn*-2 FA
6:0	2.1 ± 0.0	—	2.0 ± 0.0	—	—	—	—	—
8:0	1.3 ± 0.0	—	1.3 ± 0.0	—	—	—	—	—
10:0	2.9 ± 0.0	0.4 ± 0.1	2.9 ± 0.0	0.8 ± 0.3	—	—	—	—
12:0	3.6 ± 0.0	3.2 ± 0.1	3.6 ± 0.1	2.3 ± 0.2	3.3 ± 0.2	2.9 ± 0.5	3.0 ± 0.2	3.0 ± 0.3
14:0	12.1 ± 0.4	18.3 ± 0.2	12.3 ± 0.0	10.3 ± 0.1	11.9 ± 1.0	16.2 ± 1.4[a]	11.4 ± 0.6	11.7 ± 0.2[b]
14:1	2.0 ± 0.0	2.0 ± 0.0	2.0 ± 0.0	1.9 ± 0.1	1.4 ± 0.1	1.7 ± 0.1	1.4 ± 0.1	1.6 ± 0.1
15:0	1.4 ± 0.0	—	1.4 ± 0.0	—	1.3 ± 0.1	1.6 ± 0.1	1.3 ± 0.1	1.4 ± 0.1
16:0	30.2 ± 0.4	36.5 ± 1.0	31.3 ± 0.2	25.9 ± 1.2	31.2 ± 1.5	32.8 ± 2.8	31.5 ± 0.9	30.1 ± 0.6
16:1	2.5 ± 0.1	3.5 ± 0.0	2.9 ± 0.5	3.2 ± 0.2	2.7 ± 0.2	2.4 ± 0.3	2.5 ± 0.2	2.2 ± 0.5
17:0	1.0 ± 0.0	—	1.0 ± 0.0	—	—	—	—	
18:0	11.3 ± 0.1	5.8 ± 0.0	11.3 ± 0.0	10.6 ± 0.2	9.4 ± 0.4	5.2 ± 0.2[b]	9.7 ± 0.7	7.7 ± 0.7[a]
18:1	24.4 ± 0.1	16.7 ± 0.1	23.8 ± 0.0	22.9 ± 1.5	23.4 ± 1.1	21.2 ± 0.9[b]	23.3 ± 1.1	26.2 ± 1.2[a]
18:2n-6	3.3 ± 0.1	2.5 ± 0.1	3.2 ± 0.1	3.7 ± 0.3	6.1 ± 2.6	9.3 ± 3.8	5.3 ± 1.4	8.9 ± 1.9
18:3n-3	0.8 ± 0.0	—	0.8 ± 0.0	—	0.7 ± 0.1	0.9 ± 0.1	0.7 ± 0.1	1.0 ± 0.1
20:0	0.1 ± 0.0	0.8 ± 0.0	0.1 ± 0.0	1.1 ± 0.1	0.8 ± 0.1	—	0.8 ± 0.1	—
20:1	1.0 ± 0.0	—	1.2 ± 0.1	—	1.0 ± 0.6	—	1.3 ± 0.6	—
20:4n-6	—	—	—	—	1.3 ± 0.8	1.2 ± 0.6	1.2 ± 0.5	1.3 ± 0.6
22:5n-3	—	—	—	—	0.2 ± 0.1	—	0.2 ± 0.1	—
22:6n-3	—	—	—	—	0.5 ± 0.3	—	0.5 ± 0.1	—
Total SFA	65.9	64.8	67.1	51.0	59.7	58.7	57.6	53.9

[a]$n = 2$.
[b]$n = 3$. Different roman superscripts indicate significant difference between native and randomized butterfat $P < 0.01$, paired *t*-test. SFA, saturated fatty acid; FA, fatty acid; TAG, triacylglycerol.

TAG and chylomicron TAG for both NBF and RBF with 18:1 and 18:2 present in increased proportions in the *sn*-2 position of chylomicron TAG compared with the dietary fat for both NBF and RBF. The chylomicrons from the RBF had decreased proportions of 14:0 and increased 18:0 and 18:1 in the 2-position compared with NBF chylomicrons which reflected the 2-position composition of the fed fats (Fig. 1). However, there was no significant difference in 16:0 levels in the *sn*-2 position in the chylomicrons which was unlike the fed fats where the RBF had a 29% lower 16:0 level than NBF. In fact, the proportion of 16:0 in the *sn*-2 position of the NBF chylomicrons was lower than in the fed fat (33% compared with 37%) while the proportion of 16:0 in the *sn*-2 position of RBF chylomicrons was higher than that in the RBF (30 vs. 26%). This suggests rearrangement of 16:0 in the 2-position of the RBF occurred during the formation of the chylomicrons (Fig. 1), unlike what was reported in humans (15). An earlier study found 75% conservation of the fatty acids located in the *sn*-2 position during absorption in the rat (16). Pufal *et al.* (17) reported that rats fed palmitate in the *sn*-2 position vs. the *sn*-1(3) position led to chylomicron TAG with very similar *sn*-2 position compositions compared with the test fats; however they did find that the difference in palmitate was less in the chylomicrons than in the test fats.

Conscious chow-fed rats were injected *via* the left jugular vein with a bolus dose of either NBF, RBF, or corn oil chylomicrons. Fractional clearance rates for injected chylomicrons were calculated from exponential curves fitted by least squares procedures to plasma samples taken during the first 12 min after injection and are shown in Table 2. The results show that there were no significant differences in rate of clearance of either chylomicron TAG or CE between NBF, RBF, or corn oil chylomicrons

While the recovery of radioisotopes in liver and spleen was based on one time point only, there were differences between the two test fats. There was a significantly increased labeling of NBF TAG in the liver and spleen compared with the corn oil control group ($P < 0.05$), while there was a significantly reduced labeling of CE in the RBF group in both

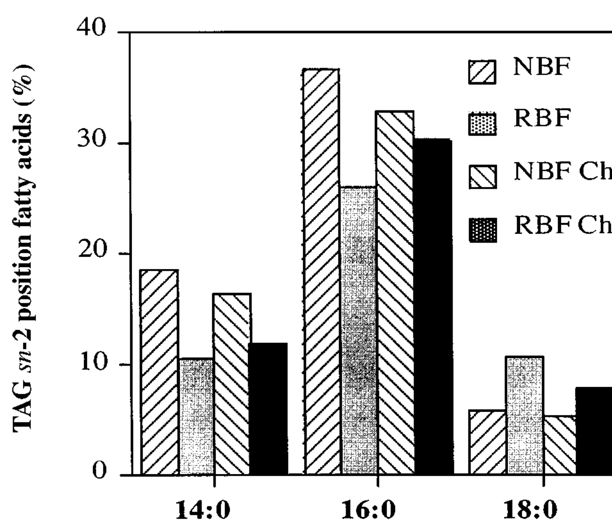

FIG. 1. Proportion of triacylglycerols (TAG) *sn*-2 14:0, 16:0, and 18:0 in fed diets and chylomicrons. Abbreviations: NBF, native butterfat diet; RBF, randomized butterfat diet; NBF Ch, native butterfat chylomicrons; RBF Ch, randomized butterfat chylomicrons.

TABLE 2
Removal from Plasma of Injected Lymph Chylomicrons Produced from Either Native Butterfat, Randomized Butterfat, or Corn Oil[a] (mean ± SD, $n = 6$).

			Organ uptake of lipid radioactivities (% of injected dose)			
	Fractional removal rate (min^{-1})		Liver		Spleen	
Lymph chylomicrons	TAG	CE	TAG	CE	TAG	CE
Native butterfat	0.200 ± 0.009	0.097 ± 0.016	19.5 ± 0.6[a]	67.7 ± 1.8[a]	0.8 ± 0.1[a]	1.9 ± 0.1[a]
Randomized butterfat	0.190 ± 0.007	0.090 ± 0.008	17.5 ± 0.8[a,b]	59.5 ± 2.5[b]	0.6 ± 0.1[a,b]	1.3 ± 0.1[b]
Corn oil	0.224 ± 0.021	0.100 ± 0.013	16.5 ± 0.1[b]	74.8 ± 2.4[a]	0.5 ± 0.0[b]	1.6 ± 0.1[a,b]

[a]Different roman superscripts in the same column are significantly different, $P < 0.05$, one-way analysis of variance, Duncan's new mutiple range test. CE, cholesterol esters. See Table 1 for other abbreviations.

tissues compared with the NBF group ($P < 0.05$). The physiological significance of these observations is not clear at the present since these data were collected in a very short-term study; however, it is possible that the reduced labeling of CE in the RBF group might signify a reduced activity of LDL receptors (6). While these data suggest the need for a long-term study using these test fats, Pufal *et al.* (17) reported that hamsters fed fat blends with palmitate in the *sn*-2 position vs. the *sn*-1(3) position for 28 d showed no significant difference in plasma lipoprotein levels.

In conclusion, this study did not demonstrate a significant difference in the fractional turnover rate of chylomicron lipids between animals fed NBF vs. RBF. Possibly the difference in the proportion of saturated fatty acids in the 2-position of TAG between the chylomicrons resulting from the two test fats was so small that it was not possible to detect the differences in chylomicron metabolism reported by Redgrave *et al.* (10) and Mortimer *et al.* (11) who used model TAG.

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Cholesterol Synthesis and Degradation in Normal Rats Fed a Cholesterol-Free Diet with Excess Cystine

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ABSTRACT: Feeding a diet with excess cystine to rats resulted in hypercholesterolemia. To understand the mechanism of the hypercholesterolemia, cholesterol synthesis and degradation, bile acid content of bile, and fecal steroids were determined. The *in vivo* incorporation of tritiated water into hepatic cholesterol, and activity of hepatic 3-hydroxy-3-methylglutaryl-CoA reductase in rats fed a high-cystine diet were significantly higher than those in rats fed a control diet. The activity of hepatic cholesterol 7α-hydroxylase was similar between two groups. Little effect of cystine supplementation was found on fecal sterol excretion although there were some changes in biliary excretion of cholic acid derivatives. These results indicate that hypercholesterolemia caused by feeding of a high-cystine diet may be due to the stimulation of hepatic cholesterol synthesis.

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The addition of high levels of single amino acids to the diet can cause growth retardation (1,2). Excess dietary cystine can cause hypercholesterolemia in rats after feeding for 2 mon (3–6). We also reported that the elevation in serum cholesterol by this diet was observed within 7 d (7–10), and this was prevented by the addition of dietary fiber (8). These findings were different from the results obtained when cholesterol was added to a 25% casein diet. In the latter study, blood cholesterol concentrations were increased by the addition of methionine, and this increase was partially reversed by the addition of cystine (11,12).

The hypercholesterolemia induced by excess dietary cystine was not due to increases in exogenous cholesterol because this diet was free of cholesterol. The mechanism for the elevation in blood cholesterol was investigated. Rukaj and Serougne (4) demonstrated that, except for absorption and external secretion, all other aspects of cholesterol turnover were increased when dietary cystine was increased. Serougne *et al.* (5) reported that the *in vivo* incorporation of [^{14}C]acetate into rat liver cholesterol was significantly higher in a cystine-supplemented group compared to controls. These results suggested that cholesterol synthesis was stimulated by excess dietary cystine.

The determination of cholesterol synthesis using ^{14}C-substrates may underestimate or overestimate the true rate of cholesterol synthesis (13–15). Therefore, in the present study, we used [^{3}H]water in order to examine *in vivo* the rate of cholesterogenesis in rats fed the high-cystine diet. It is well-known that 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase [EC 1.1.34] is the rate-limiting enzyme of cholesterol synthesis (16–18). The total activity of completely dephosphorylated HMG-CoA reductase was significantly higher in the livers of rats fed a cystine-excess diet than in those of rats fed a control diet (19). While cholesterol degradation in the liver was not studied when rats were fed the cystine-excess diet, cholesterol 7α-hydroxylase [EC 1.14.13.17] is the initial step in the conversion of cholesterol to bile acids and is rate-limiting for bile acid synthesis (20). Therefore, we assessed the *in vivo* synthetic rate of hepatic cholesterol using [^{3}H]water, hepatic activities of HMG-CoA reductase (dephosphorylated and native) and cholesterol 7α-hydroxylase, bile acid composition in bile, and fecal steroids to investigate their possible involvement in the elevation of blood cholesterol in rats fed a cystine-excess diet.

MATERIALS AND METHODS

Diets. Compositions of the basal and the cystine-excess diet are shown in Table 1. In the high-cystine diet, 3.5% by weight of L-cystine (Katayama Chemical Industries Co., Ltd., Osaka, Japan) was added to a basal diet at the expense of corn starch.

Animals. Male Wistar rats (Japan SLC, Inc., Hamamatsu, Shizuoka, Japan) were used. All rats were housed individually in stainless-steel wire-bottomed cages in an air-conditioned room kept at *ca.* 23°C, with a 12-h cycle of light (0800–2000 h) and dark (2000–0800 h). Animals were cared for under Guidelines for Animal Experimentation of the Committee of Experimental Animal Care, Nagoya University.

The rats (5 wk old) were fed a basal diet (Table 1) for 3 to 5 d, allowing them to adapt the shifted feeding pattern described below, and then were offered *ad libitum* either a basal or a high-cystine diet (Table 1). Pair-fed rats were offered the

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Abbreviations: DPS, digitonin-precipitable sterols; EDTA, ethylenediaminetetraacetic acid; GBq, giga becquerel; HMG-CoA, 3-hydroxy-3-methylglutaryl CoA.

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TABLE 1
Composition of the Experimental Diets

Ingredients	Basal diet (%)	High-cystine diet (%)
Casein[a]	25.0	25.0
L-Cystine[a]		3.5
Corn oil[b]	5.0	5.0
AIN-76A vitamin mixture[c]	1.0	1.0
Choline chloride[a]	0.2	0.2
AIN-76 mineral mixture[d]	3.5	3.5
Corn starch[e]	65.3	61.8

[a]Katayama Chemical Industries Co., Ltd. (Osaka, Japan).
[b]Nihon Syokuhin Kako Co., Ltd. (Fuji, Shizuoka, Japan).
[c]American Institute of Nutrition (1977) *J. Nutr. 107,* 1340–1348 and (1980) *J. Nutr. 110,* 1726.
[d]American Institute of Nutrition (1977) *J. Nutr. 107,* 1340–1348.
[e]Chuo Syokuryo Co., Ltd., Inazawa (Aichi, Japan).

same amount of a basal diet as consumed by rats fed the high-cystine diet for 7 d. Rats were allowed free access to water throughout the experimental periods.

Liver and serum components (Expt. 1). Rats were fed a basal *(ad libitum),* a basal diet pair-fed with the high-cystine group, and a high-cystine *(ad libitum)* diet for 7 d. Rats were sacrificed between 2300 and 2330 h by decapitation.

After sacrifice, blood was collected and allowed to clot at room temperature; serum was obtained by centrifugation. The livers were immediately excised and weighed. Serum and samples of liver were stored at −30°C until analysis. Serum cholesterol (21), phospholipids (22), and triacylglycerol (23) were measured by enzymatic methods. Liver lipids were extracted and purified according to the procedure of Folch *et al.* (24). Liver total lipid content was gravimetrically estimated after removing chloroform and methanol. Cholesterol (21) and triacylglycerol (23) in the liver were measured enzymatically. Liver phospholipids were calculated by the differences between liver total lipids minus the sum of cholesterol plus triacylglycerol.

In vivo *incorporation of [^{3}H]water into cholesterol (Expt. 2)*. Rats were fed a basal *(ad libitum),* a pair-fed basal, or a high-cystine *(ad libitum)* diet for 7 d. The rats were injected intraperitoneally between 2300 and 2330 h (25) with 250 µL of [^{3}H]water (1 mCi, 37 MBq) (Amersham Japan Ltd., Tokyo, Japan). At 60 min after injection, the rats were sacrificed by cardiac puncture. The liver and small intestine (0.5 cm below pylorus and 0.5 cm above cecum) were quickly removed. Intestinal contents were removed by rinsing with ice-cold saline. Both liver and small intestine were frozen in liquid nitrogen and stored at −20°C. Portions of the stored liver, small intestine, and serum were saponified in 15% ethanolic potassium hydroxide at 75°C for 60 min. The hydrolysate was then extracted four times with petroleum ether hydrocarbon (b.p., 30–60°C). An aliquot of the petroleum hydrocarbon fraction was counted for radioactivity in the unsaponifiable fraction. Sterols in another portion of the petroleum hydrocarbon fraction were purified as digitonin-precipitable sterols (DPS) by the method of Kelley and Tsai (26). The rate of synthesis was estimated by the formula of Turley *et al.* (27) using the serum ^{3}H-content. The rate of synthesis was expressed as nmoles or µmoles of [^{3}H]water incorporated into DPS or fatty acids per g of tissue per h or per whole organ per 100 g body weight per h.

HMG-CoA reductase activity (Expt. 1). Rats were fed a basal, a pair-fed basal, or a high-cystine diet for 7 d. The rats were sacrificed between 2300 and 2330 h by decapitation (25).

(i) Preparation of liver microsomes. An aliquot of liver (1.5 g) was weighed and homogenized with 4 vol of cold buffer containing 0.3 M sucrose, 10 mM 2-mercaptoethanol, 10 mM sodium EDTA (pH 7.4), and 50 mM sodium fluoride (28) using Potter-Elvehjem type Teflon homogenizer. The resulting homogenate was centrifuged at 12,000 × *g* at 4°C for 15 min, and the postmitochondrial supernatant was then centrifuged at 105,000 × *g* at 4°C for 60 min to sediment microsomes. The pellets were immediately stored at −80°C for 10 d without loss of enzyme activity and resuspended in 1 mL of incubation medium. Microsomal protein was assayed according to Lowry *et al*. (29) using bovine serum albumin as the standard.

(ii) Assay of microsomal HMG-CoA reductase activity. The activity of HMG-CoA reductase was assayed according to Brown *et al.* (28). To assay the total (dephosphorylated + phosphorylated) activity of HMG-CoA reductase, the microsomes were preincubated at 37°C for 60 min. The preincubation mixture contained the following concentrations of components in a volume of 45 µL: 20 mM imidazole chloride (pH 7.4), 5 mM dithiothreitol, 1.0–1.5 mg of microsomal protein, and 10 units of alkaline phosphatase *(Escherichia coli)* to dephosphorylate all of the HMG-CoA reductase to its active form. To assay the activity of (native) HMG-CoA reductase dephosphorylated *in vivo,* alkaline phosphatase was not added.

To the preincubation mixture, 45 µL of solution containing 0.2 M potassium phosphate (pH 7.4), 20 mM NADPH, 20 mM sodium EDTA, and 10 mM dithiothreitol was added. The HMG-CoA reductase assay was then initiated with the addition of DL-[3-^{14}C]HMG-CoA (2,500 dpm/nmol) (52 mCi/mmol, 1.92 GBq/mmol)(Amersham Japan, Ltd.) to a final concentration of 470 µM (final assay vol, 100 µL). After incubation at 37°C for 30 min, 50 µL of 6 N HCl and 10 µL of [^{3}H]mevalonate as carrier were added. The mixture was left overnight at room temperature in order to lactonize the formed [^{14}C]mevalonate and the added [^{3}H]mevalonate. After centrifugation of the lactonized mixture at 10,000 × *g* for 15 min, the supernatant was spotted on a thin-layer chromatographic plate and developed with acetone/benzene (1:1). The silica gel containing the [^{14}C]- and [^{3}H]mevalonolactone was scraped and counted. The recovery was corrected for by recovered [^{3}H]mevalonolactone. HMG-CoA reductase activity was expressed as the nmoles of [^{14}C]mevalonate formed per mg of microsomal protein per min, nmoles per g of liver per min, and nmoles per liver per 100 g body wt per min.

Cholesterol 7α-hydroxylase (Expt. 3). Rats were fed a basal *ad libitum* diet, a pair-fed basal diet, or a high-cystine *(ad libitum)* diet for 7 d. Rats were sacrificed between 2300 and 2330 h by decapitation (25).

(i) Preparation of liver microsomes. Aliquots of liver (1.0 g) were weighed and then homogenized with a Potter-Elvehjem-type homogenizer with a Teflon pestle in 4 vol of ice-cold 0.154 M potassium chloride isotonic solution. The homogenate was centrifuged at 12,000 × *g* at 4°C for 15 min, and the supernatant was then centrifuged at 105,000 × *g* at 4°C for 60 min. The pellets were finally resuspended in 0.1 M potassium phosphate buffer (pH 7.4) (30,31). Microsomal protein was assayed by the method of Lowry *et al*. (29) using bovine serum albumin as the standard.

(ii) Assay of microsomal cholesterol 7α-hydroxylase. The final incubation mixture (1 mL) contained 100 mM potassium phosphate buffer (pH 7.4), 20 mM cysteamine, 4 mM magnesium chloride, 5 mM NADPH, and 1.0–1.5 mg of microsomal protein. The substrate of [7(*n*)-^{3}H]cholesterol (5 µCi, 185 KBq) (7.45 Ci/mmol, 276 GBq/mmol) (Amersham Japan Ltd.) and 300 µM cholesterol were introduced into the incubation medium in trace amounts of saturating concentrations solubilized with help of a detergent, Tween-80. The incubations were run in a shaking bath at 37°C for 30 min. The enzymatic reaction was stopped by adding 3 vol of 20% trichloroacetic acid solution. The tubes were centrifuged at 3,000 × *g* for 5 min, and aliquots of the supernatants were transferred to tubes containing chloroform and mixed to remove nonmetabolized [7(*n*)-^{3}H]cholesterol. The procedures to remove nonmetabolized substrate were performed twice. The water layer finally obtained was counted for radioactivity.

The composition of bile acids in the bile (Expt. 4). Bile samples were collected for 60 min in the morning from rats treated by nembutal after feeding either a basal *(ad libitum)* or a high-cystine *(ad libitum)* diet for 7 d.

Each bile acid was analyzed by high-performance liquid chromatography (HPLC; TRIROTAR; Jasco, Tokyo, Japan), with an immobilized 3α-hydroxysteroid dehydrogenase column (Enzymepak-HSD) (32). Bile acids (33), cholesterol (21), and phospholipids (22) in bile were estimated by enzymatic methods.

Bile acids, coprostanol, and cholesterol in feces (Expt. 5). After feeding a basal *(ad libitum),* a pair-fed basal, or a high-cystine *(ad libitum)* diet for 7 d, bile was collected for 60 min in the morning from rats treated with nembutal. Feces were collected during the final 3 d of the study. Frozen fecal samples were lyophilized and weighed.

Aliquots of blended and powdered feces were used for the extraction of bile acids (34) and neutral steroids (35). Total bile acids were enzymatically estimated using 3α-hydroxysteroid dehydrogenase (33). For the determination of neutral steroids, the unsaponifiable fraction was transformed to trimethylsilyl derivatives by using the silylating mixture containing *N,N*-dimethylformamide, hexamethyldisilazane, and trimethylchlorosilane in proportions 40:40:1. The reaction for forming the trimethylsilyl derivatives was held at room temperature for 30 min. The derivatives were analyzed by gas–liquid chromatography on a 1.5% OV-17 column coated on silicon, 60–80 mesh (Shimadzu Corporation, Kyoto, Japan), which was slightly modified by the method of Miettinen *et al.* (35) using 5α-cholestane as an internal standard.

Statistical analysis. Data were analyzed by Student's *t*-test (36).

RESULTS

Changes in lipid composition in liver and serum of rats fed either a basal, a pair-fed basal, or a high-cystine diet (Expt. 1). Food intake, body weight gain, liver weight, and lipid composition of the liver and serum of rats fed for 7 d are summarized in Table 2. Food intake and body weight gain were suppressed by the high-cystine diet. Liver weight in rats fed the high-cystine diet was significantly higher than that of rats fed the basal diet *(ad libitum)*. The contents of liver total lipids and triacylglycerol in rats fed a basal diet *(ad libitum)* were higher than those of rats fed a high-cystine diet. The contents of liver cholesterol and phospholipids were similar for rats fed a basal *(ad libitum)* or a high-cystine diet. When liver lipid composition of rats fed a pair-fed basal and a high-cystine diet were compared, the liver total lipids and triacylglycerol contents were decreased in rats fed a high-cystine diet.

Serum cholesterol and triacylglycerol of rats fed a high-cystine diet increased compared with those fed a basal *(ad libitum)* or a pair-fed basal diet. The concentration of serum phospholipids in rats fed a basal diet *(ad libitum)* was significantly different from those on a pair-fed basal and high-cystine diets, the latter two of which values were similar.

Food intake and body weight gain of rats fed a high-cystine diet were lower than those of rats fed the basal diet *(ad libitum)*. Pair-fed rats were fed the same amount of food as that eaten by rats fed the cystine-excess diet, and the change in body weight of pair-fed rats was similar to those of rats fed the high-cystine diet.

In vivo *incorporation of [^{3}H]water into sterols (Expt. 2).* The effects of dietary excess cystine on the *in vivo* incorpora-

TABLE 2
Lipid Composition in the Liver and Serum[a]

	Basal *(ad libitum)*	Basal (pair-fed)	High cystine *(ad libitum)*
Food intake (g/7 d)	115 ± 2	57 ± 1[a]	59 ± 3[a]
Changes in body weight (g/7 d)	29.1 ± 1.2	4.4 ± 0.8[a]	0.5 ± 3.8[a]
Liver weight (g/100 g body wt)	4.08 ± 0.04	3.50 ± 0.04[a]	4.46 ± 0.04[a,b]
Liver (mg/g)			
Total lipids	53.3 ± 0.9	50.2 ± 0.7	44.9 ± 0.8[a,b]
Cholesterol	2.16 ± 0.03	2.33 ± 0.03[a]	2.21 ± 0.07
Phospholipids	34.8 ± 0.3	36.3 ± 1.3	33.6 ± 0.6
Triacylglycerol	16.4 ± 0.8	11.5 ± 0.6[a]	9.1 ± 0.6[a,b]
Serum (mg/100 mL)			
Cholesterol	113 ± 2	90 ± 2[a]	154 ± 8[a,b]
Phospholipids	207 ± 9	149 ± 2[a]	154 ± 16[a]
Triacylglycerol	115 ± 7	88 ± 3[a]	141 ± 7[a,b]

[a]Data represent means ± SEM for seven rats. Experiment 1. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

TABLE 3
***In vivo* Incorporation of Tritiated Water into Digitonin-Precipitable Sterols (DPS) and Fatty Acids in the Liver and Small Intestine[a]**

	Basal (*ad libitum*)	Basal (pair-fed)	High cystine (*ad libitum*)
Liver			
Weight (g/100 g body wt)	3.80 ± 0.10	3.02 ± 0.06[a]	3.90 ± 0.09[b]
[^{3}H]DPS			
(nmol/g liver/h)	247 ± 17	168 ± 19[a]	368 ± 28[a,b]
(nmol/liver/100 g body wt/h)	949 ± 85	514 ± 60[a]	1436 ± 114[a,b]
[^{3}H]Fatty acids			
(μmol/g liver/h)	16.9 ± 2.1	4.1 ± 0.2[a]	16.6 ± 2.2[b]
Small intestine			
Weight (g/100 g body wt)	2.42 ± 0.12	2.31 ± 0.05	1.92 ± 0.07[a,b]
[^{3}H]DPS			
(nmol/g tissue/h)	314 ± 21	215 ± 16[a]	334 ± 38[b]
(nmol/tissue/100 g body wt/h)	756 ± 53	500 ± 42[a]	634 ± 63
[^{3}H]fatty acids			
(μmol/g tissue/h)	5.50 ± 0.59	1.82 ± 0.05[a]	3.66 ± 0.39[a,b]

[a]Data represent means ± SEM for eight rats. Experiment 2. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

TABLE 4
Hepatic 3-Hydroxy-3-methylglutaryl-CoA Reductase Activities of Rats on High-Cystine Diet[a]

	Basal (*ad libitum*)	Basal (pair-fed)	High cystine (*ad libitum*)
Dephosphorylated			
(pmol/mg protein/min)	60.4 ± 3.6	40.4 ± 2.0[a]	99.7 ± 17.1[a,b]
(nmol/g liver/min)	0.68 ± 0.04	0.47 ± 0.02[a]	1.04 ± 0.18[a,b]
(nmol/liver/100 g body wt/min)	2.76 ± 0.16	1.64 ± 0.07[a]	4.80 ± 0.91[a,b]
Total			
(pmol/mg protein/min)	295 ± 21	197 ± 15[a]	463 ± 53[a,b]
(nmol/g liver/min)	3.27 ± 0.22	2.23 ± 0.12[a]	4.51 ± 0.49[a,b]
(nmol/liver/100 g body wt/min)	13.4 ± 0.8	7.8 ± 0.4[a]	21.5 ± 2.9[a,b]

[a]Data represent means ± SEM for seven rats. Experiment 1. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

tion of [^{3}H]water into DPS and fatty acids in the liver and intestine are summarized in Table 3. The *in vivo* incorporation of tritium from [^{3}H]water into liver DPS in rats fed a cystine-excess diet was significantly greater than that of rats fed either basal diet (*ad libitum* or pair-fed). The incorporation of tritium into DPS of small intestine in rats fed a high-cystine diet was not affected as compared with those in rats fed the basal diet *(ad libitum)*. When expressed as gram tissue per hour, the incorporation of tritium into DPS in small intestine of rats fed a cystine-excess diet was increased as compared with that of a pair-fed basal group. The tritium incorporation into DPS in the liver and small intestine by pair-feeding of the basal diet was reduced when compared with those of the basal diet. Whereas the incorporation into liver fatty acids in rats fed the high-cystine diet did not alter as compared with that of a basal diet *(ad libitum),* excess dietary cystine decreased the tritium incorporation into fatty acids of the small intestine as compared with those of the basal diet *(ad libitum)*.

Liver HMG-CoA reductase activity (Expt. 1). The activities of hepatic HMG-CoA reductase in rats fed a basal, a pair-fed basal, or a high-cystine diet are summarized in Table 4. Dephosphorylated and native liver HMG-CoA reductase activities in rats fed the cystine-excess diet were both higher than those in rats fed either amount of the basal diet (*ad libitum* or pair-fed). Dephosphorylated and native HMG-CoA reductase activities in the liver were decreased by pair-feeding as compared with the basal diet *(ad libitum)*.

Liver cholesterol 7α-hydroxylase (Expt. 3). Hepatic cholesterol 7α-hydroxylase activities of rats fed the high-cystine diet *(ad libitum)* were essentially similar to those of animals fed the basal diet *(ad libitum)*. The activities of this enzyme in livers of rats fed a basal *(ad libitum)* or a high-cystine diet were significantly ($P < 0.05$) higher than those of rats fed a pair-fed basal diet only when expressed as nmoles per liver per 100 grams body weight per hour (Table 5).

Composition of bile acids in bile (Expt. 4). Of bile acids analyzed, tauroursodeoxycholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid were increased by 59, 102, and 58%, respectively, when fed a high-cystine diet. Ursodeoxycholic acid was markedly decreased. Bile acid derivatives of cholic acid other than those described above were not affected by the diet. Total bile acid content was similar between two groups (Table 6).

Bile acids, cholesterol, and phospholipids in bile (Expt. 5). Bile flow rate and the content of bile acids and cholesterol in bile were similar between animals fed a basal *(ad libitum)* and a high-cystine diet, but the content of phospholipids in bile was increased by feeding the high-cystine diet. The content of phospholipids in bile of rats fed a high-cystine diet was higher than that of a pair-fed basal diet, but the contents of bile acids and cholesterol were similar between animals fed a pair-fed basal or a high-cystine diet (Table 7).

Bile acids, coprostanol, and cholesterol in feces (Expt. 5). Weight of feces from rats fed a high-cystine diet was decreased as compared with that of those eating a basal diet *(ad libitum)*. Fecal weight was lower still for rats fed a pair-fed basal diet. Excess dietary cystine did not alter the content of bile acids, coprostanol, or cholesterol in feces. Total amounts of bile acids and neutral steroids in the feces were similar

TABLE 5
Hepatic Cholesterol 7α-Hydroxylase Activity of Rats on High-Cystine Diet[a]

	Basal (*ad libitum*)	Basal (pair-fed)	High cystine (*ad libitum*)
(nmol/mg protein/h)	0.52 ± 0.03	0.45 ± 0.04	0.52 ± 0.03
(nmol/g liver/h)	12.7 ± 0.7	11.1 ± 1.1	12.4 ± 0.7
(nmol/liver/100 g body wt/h)	50.3 ± 2.5	36.4 ± 3.1[a]	51.7 ± 3.7[b]

[a]Data represent means ± SEM for eight rats. Experiment 3. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

TABLE 6
Bile Acid Composition (μmol/mL) in Bile[a]

Bile acids	Basal *(ad libitum)*	High cystine *(ad libitum)*	*P*
Glycoursodeoxycholic acid	0.025 ± 0.004	0.049 ± 0.014	NS
Tauroursodeoxycholic acid	0.427 ± 0.066	0.681 ± 0.096	<0.05
Ursodeoxycholic acid	2.247 ± 0.261	0.135 ± 0.037	<0.001
Glycocholic acid	0.431 ± 0.056	0.335 ± 0.044	NS
Taurocholic acid	7.790 ± 1.142	8.945 ± 1.173	NS
Cholic acid	N.D.	N.D.	
Glycochenodeoxycholic acid	0.046 ± 0.005	0.093 ± 0.013	<0.01
Taurochenodeoxycholic acid	1.523 ± 0.183	2.412 ± 0.319	<0.05
Glycodeoxycholic acid	0.055 ± 0.008	0.041 ± 0.012	NS
Taurodeoxycholic acid	0.901 ± 0.078	1.111 ± 0.164	NS
Chenodeoxycholic acid	N.D.	N.D.	
Deoxycholic acid	0.004 ± 0.003	0.001 ± 0.001	NS
Glycolithocholic acid	0.0014 ± 0.001	0.0002 ± 0.0002	NS
Taurolithocholic acid	0.027 ± 0.028	0.021 ± 0.004	NS
Lithocholic acid	N.D.	N.D.	
Total bile acids	13.46 ± 1.66	13.73 ± 1.63	NS

[a]Data represent means ± SEM for nine rats. Experiment 4. NS, not significant; N.D., not detected.

among the three dietary groups. Pair-fed rats excreted a lower amount of bile acids as compared with rats fed a basal diet *(ad libitum)* (Table 8).

DISCUSSION

Feeding of a high-cystine diet caused growth retardation, hepatomegaly, and hypercholesterolemia, consistent with previous reports (3–10). The large amount of cystine added to the diet may exert pharmacological and/or toxic effects, and may not occur in humans under normal conditions.

Dietschy and McGarry (13) and Stange and Dietschy (15) demonstrated that substrate labeling with ^{14}C such as acetate or octanoate can be used for the estimation of sterol synthesis (DPS). Using [^{14}C]substrate, the ability of the substrate to penetrate cell membrane and intracellular dilution may also have profound effects (14), suggesting that such methods using [^{14}C]substrates may underestimate or overestimate the true rate of cholesterol synthesis. Therefore, the use of [^{3}H]water seems to be a satisfactory way to assay the rates of cholesterogenesis. The results concerning hepatic cholesterol synthesis *in vivo* using either [^{3}H]water (Table 3) or [^{14}C]substrates (5) were essentially the same. Our overall results for hepatic cholesterogenesis *in vivo* (Table 3) and those of Serougne *et al*. (5) may parallel the activities of hepatic HMG-CoA reductase (both dephosphorylated and native, Table 4) and (native) (19). Indeed, the activities of hepatic HMG-CoA reductase were higher in rats fed the high-cystine diet than those eating the basal diet (Table 4).

Food intake of rats fed the cystine-excess diet was lower than that of rats fed the basal diet (Table 2). To exclude a confounding influence of reduced food intake by excess cystine on our results, the HMG-CoA reductase activity was also examined in a pair-fed basal group receiving the same reduced ration. Food intake of pair-fed rats was restricted to *ca.* 50% of that eaten by rats consuming the basal diet *ad libitum* (Table 2). Hepatic HMG-CoA reductase activities of rats fed the pair-fed basal diet were decreased as compared with those in rats fed the basal diet *(ad libitum)* (Table 4), suggesting that lower food intake in rats fed the high-cystine diet had the significant effect on the activities of this enzyme. Thus, the increased activity of this enzyme in livers of rats fed the high-cystine diet was not due to reduced food intake.

It is well-known that HMG-CoA reductase activity is modulated by both short- and long-term mechanisms (37–40). Short-term changes in enzyme activity depend on the modulation of this enzyme by phosphorylation and dephosphorylation (37,38), while long-term changes in activity of this enzyme may depend on the changes in the rate of protein synthesis and/or degradation. The total activity (native dephosphorylated + phosphorylated form) of HMG-CoA reductase is considered to reflect the total protein level of HMG-CoA reductase itself in the liver (40). In the present study, total HMG-CoA reductase activity in rats fed the high-cystine diet was significantly higher than that in rats fed the basal diet, suggesting that the content of HMG-CoA reductase protein may be higher in rats fed the high-cystine diet as compared

TABLE 7
Composition of Bile Acids, Cholesterol, and Phospholipids in Bile

	Basal *(ad libitum)*	Basal (pair-fed)	High cystine *(ad libitum)*
Bile flow (μL/100 g body wt/h)	218 ± 9	159 ± 14[a]	211 ± 20
Bile			
Bile acids (μmol/100 g body wt/h)	2.12 ± 0.12	1.75 ± 0.16	2.31 ± 0.32
Cholesterol (μg/100 g body wt/h)	24.4 ± 0.9	23.4 ± 2.1	27.0 ± 3.0
Phospholipids (μg/100 g body wt/h)	632 ± 51	733 ± 98	1047 ± 93[a,b]

[a]Data represent means ± SEM for seven rats. Experiment 5. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

TABLE 8
Composition of Sterols in Feces

	Basal *(ad libitum)*	Basal (pair-fed)	High cystine *(ad libitum)*
Weight (g/3 d)	2.10 ± 0.07	1.08 ± 0.05[a]	1.56 ± 0.09[a,b]
Bile acids (μmol/100 g body wt/3 d)	2.18 ± 0.17	1.63 ± 0.17[a]	1.75 ± 0.21
Coprostanol (μmol/100 g body wt/3 d)	6.98 ± 1.10	5.76 ± 0.70	5.93 ± 0.72
Cholesterol (μmol/100 g body wt/3 d)	5.14 ± 0.89	4.83 ± 0.55	5.52 ± 0.67
Bile acids + neutral steroids (μmol/100 g body wt/3 d)	14.3 ± 1.6	12.2 ± 0.9	13.2 ± 1.1

[a]Data represent means ± SEM for seven rats. Experiment 5. [a]Significantly different from a basal group *(ad libitum)* ($P < 0.05$). [b]Significantly different from a basal group (pair-fed) ($P < 0.05$).

with rats fed the basal diet (Table 4). Furthermore, the percentage of dephosphorylated form to total activity was constant (*ca.* 20%) whether excess cystine was fed or not (Table 4). HMG-CoA reductase has a short half-life of about 2 h (41), so its level can easily be changed by dietary conditions (40) or during its circadian rhythm (42). Although the cholesterol level was high in serum from rats fed the high-cystine diet, circulating cholesterol may not exert regulatory effects on hepatic HMG-CoA reductase under these circumstances.

The activities of hepatic cholesterol 7α-hydroxylase, the regulatory enzyme for the bile acid synthesis, were unchanged between a basal *(ad libitum)* and a high-cystine *(ad libitum)* diet (Table 5). Thus, from both the enhanced activities of hepatic HMG-CoA reductase and the lack of change in hepatic cholesterol 7α-hydroxylase activities, a possible mechanism by which excess dietary cystine produces an increase in circulating cholesterol may be the stimulation of cholesterol synthesis. Excess dietary cystine caused the stimulation of cholesterogenesis in the liver, but did not stimulate cholesterol synthesis in the small intestine (Table 3). Thus, cholesterol synthesized in the small intestine of rats fed a high-cystine diet may not contribute to an increase in circulating cholesterol.

As shown in Table 8, excess dietary cystine had no effects on bile acids, coprostanol, and cholesterol in feces. These results are supported by the facts that cholesterol 7α-hydroxylase activities in the liver were similar between rats fed the basal *(ad libitum)* and the high-cystine *(ad libitum)* diet (Table 5), and that the content of total bile acids in bile was not affected, although proportions of some bile acids were changed by excess dietary cystine (Table 6).

Although the amount of total bile acids in bile was not affected by excess dietary cystine (Table 6), ursodeoxycholic acid was markedly reduced. Ursodeoxycholic acid is one of the tertiary bile acids. Chenodeoxycholic acid is altered by enzymes of intestinal bacteria to form ursodeoxycholic acid (43). Thus, possibly excess dietary cystine altered the intestinal bacteria as compared with the basal diet, resulting in the decreased formation of ursodeoxycholic acid. The increased content in tauroursodeoxycholic acid, glycochenodeoxycholic acid, and taurochenodeoxycholic acid in the bile of rats fed the cystine-excess diet might be due to their increased synthesis from precursors or the decreased degradation of these bile acids.

The reason why serum triacylglycerol concentration was increased by feeding a cystine-excess diet (Table 2) remains obscure. It may be due to either the increased transport of triacylglycerol-rich particles from liver into the circulation or the decreased removal into extrahepatic tissues. Further work will be needed to elucidate the efforts of high dietary cystine on triacylglycerol metabolism.

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Regulation of HepG2 Cell Apolipoprotein B Metabolism by the Citrus Flavanones Hesperetin and Naringenin

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ABSTRACT: Our previous studies showed that replacing the drinking water of rabbits fed a casein-containing diet with either orange juice or grapefruit juice reduced serum low density lipoprotein cholesterol and hepatic cholesteryl ester concentrations. To determine whether the changes observed in rabbits were due to flavonoids present in the juices acting directly on the liver, the effects of hesperetin and naringenin on net apolipoprotein B (apoB) secretion by HepG2 cells were investigated. These flavanones dose-dependently reduced net apoB secretion by up to 81% after a 24 h incubation, while doses of 60 µg/mL reduced net apoB secretion by 50% after 4 h. Coincubation with the proteasome inhibitor, MG-132, did not alter the ability of the flavonoids to reduce net apoB secretion over 4 h, suggesting that the flavonoid-induced changes in apoB metabolism were not due to a direct increase in proteasomal activity. However, the flavonoids were unable to reduce net apoB secretion after 4 h in the presence of oleate, suggesting that these compounds may interfere with the availability of neutral lipids for lipoprotein assembly. Furthermore, our ^{14}C-acetate-labeling studies showed a 50% reduction in cholesteryl ester synthesis in the presence of either flavonoid, which could account for the reduction in net apoB secretion caused by incubation with these compounds. These *in vitro* studies suggest that hesperetin and naringenin may, in part, reduce net apoB secretion by HepG2 cells by inhibiting cholesteryl ester synthesis and that these compounds are good candidates for further *in vivo* studies to determine whether they are responsible for the cholesterol-lowering properties of dietary citrus juices.

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The concentration of low density lipoprotein (LDL) in the bloodstream is controlled partly by expression of LDL receptors in the liver (1). However, it has been known for some time that, in many patients, overproduction of LDL and very low density lipoprotein (VLDL) may cause the hyperlipidemias responsible for increased risk of coronary heart disease (2). Consequently, a surge of recent research focused on investigation of the regulatory mechanisms involved in the secretion of apolipoprotein B (apoB)-containing lipoproteins by the liver.

The assembly and secretion of apoB-containing lipoproteins from hepatocytes make up a complex and highly regulated process. As originally proposed by Alexander *et al.* (3), lipoprotein assembly is generally divided into two stages. During the first step, apoB is translated on the cytoplasmic surface of the rough endoplasmic reticulum (ER) and is either fully or partially translocated through the endoplasmic reticulum membrane. During this time, the nascent polypeptide is susceptible to rapid degradation by either the cytosolic proteasome or by ER lumenal proteases (reviewed in Ref. 4). Subsequently, newly formed apoB either is degraded or enters a secretion pathway, depending on the availability of cellular lipids (5). The addition of a small amount of lipid to apoB, catalyzed by microsomal triglyceride transfer protein (MTP) (reviewed in Ref. 6), leads to the formation of small, dense primordial lipoprotein particles and signifies the completion of the first step.

The addition of bulk quantities of triglycerides, cholesterol esters, and phospholipids to apoB is considered the second step of lipoprotein formation (3,7). Numerous studies showed that triglyceride availability is the major factor controlling posttranslational regulation of apoB secretion from hepatocytes (8–13). However, several reports also suggested a critical role for cholesteryl esters (14–17). Phospholipid synthesis is also required for normal apoB secretion (18,19), and perturbations of the normal phospholipid composition of the ER membrane reduce apoB secretion (20,21).

HepG2 human hepatoma cells, a commonly used model of human hepatocytes, have a defect in the second step of lipoprotein assembly which prevents the addition of substantial quantities of triglyceride to the primordial particle (10). As a consequence, these cells secrete small triglyceride- and apoB-containing particles with a density similar to that of LDL (22,23). However, as explained by Graham *et al.* (24), changes in triglyceride secretion in response to endogenous compounds suggest that these cells are an appropriate model for measuring qualitative changes in lipoprotein secretion. We therefore used the HepG2 cell line to investigate the ability of the citrus flavanones, hesperetin (from oranges) and naringenin (from grapefruit), to alter hepatic lipoprotein metabolism.

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Abbreviations: ACAT, acyl-CoA acyltransferase; apoB, apolipoprotein B; BSA, bovine serum albumin; DMSO, dimethyl sulfoxide; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; FBS, fetal bovine serum; LDL, low density lipoprotein; MEM, minimum essential medium; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; MTP, microsomal triglyceride transfer protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCA, trichloroacetic acid; VLDL, very low density lipoprotein.

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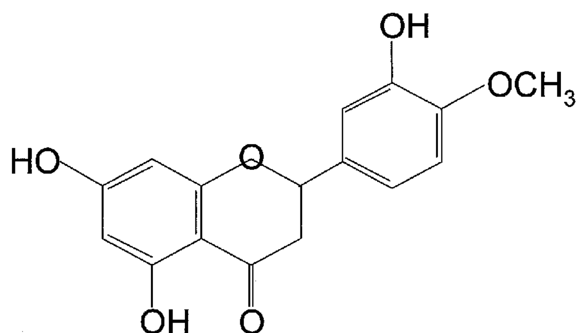
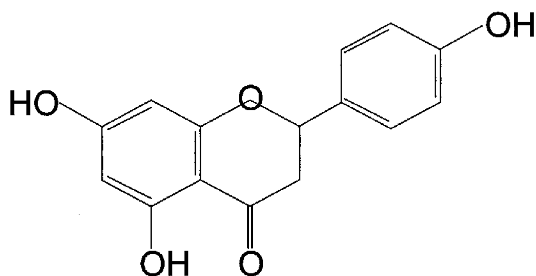


Hesperetin



Naringenin

SCHEME 1

Our recent studies demonstrated that dietary orange juice and grapefruit juice reduced serum LDL and hepatic cholesteryl ester concentrations in a rabbit model of endogenous hypercholesterolemia (25). We hypothesized that these effects could be due to the flavanone glycosides, hesperidin and naringin, which are present in significant quantities in orange juice and grapefruit juice, respectively. It has been postulated that these compounds are deglycosylated by bacteria in the gastrointestinal tract to hesperetin and naringenin (Scheme 1) prior to absorption (26,27). Thus, these aglycone flavonoids could act directly in the liver. In support of this hypothesis, previous studies showed that dietary hesperidin reduces serum cholesterol (28–30) and triglycerides (28), as well as hepatic free and esterified cholesterol concentrations (30) in rats. In the present studies, we investigated the ability of hesperetin and naringenin to reduce net apoB secretion by HepG2 cells. Further experiments were conducted to determine whether the observed effects were likely to be the result of increased proteasomal degradation or altered lipid availability during the first step of lipoprotein assembly.

MATERIALS AND METHODS

Cell culture. Minimum essential medium (MEM), fetal bovine serum (FBS), disposable Nunclon culture flasks, Falcon culture plates, and other tissue culture supplies were obtained from Life Technologies Inc. (Burlington, Ontario, Canada). Fatty acid-free bovine serum albumin (BSA) fraction V was obtained from Sigma (St. Louis, MO). HepG2 cells (American Type Culture Collection, Rockville, MD) were grown and maintained in MEM containing 10% FBS and 100 IU penicillin/streptomycin/amphotericin at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Flasks were subcultured at a 1:4 ratio every 7 d, using 0.25% trypsin in Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline for 10 min at 37°C. For experiments, cells were seeded in 6- or 24-well plates (6×10^5 cells/plate) and used at confluence (usually about 7 d after plating). Cell medium was changed every 2 to 3 d. Before each experiment, cells were preincubated for 24 h with MEM containing 1% BSA instead of FBS.

Hesperetin and naringenin obtained from Sigma were solubilized in dimethyl sulfoxide (DMSO) so that the final concentration of solvent in the media was not greater than 0.1% by vol.

ApoB determination. For the dose-response, time-course, reversibility, proteasome inhibitor, and oleic acid studies described below, apoB concentrations in the cell culture media following experimental incubations were determined by a competitive enzyme-linked immunosorbent assay (ELISA) developed by Young *et al.* (31) and modified by Ortho Diagnostics (La Jolla, CA). The coefficient of variance for this assay was *ca.* 3%. Human LDL for preparing standard curves and for coating microtiter plates was isolated from fresh human EDTA-plasma, by sequential density gradient ultracentrifugation (32). Soluble cellular protein was extracted with 0.1 N sodium hydroxide, measured using the Coomassie Plus Protein Assay (Pierce, Rockford, IL), and apoB content was calculated per mg of total cellular protein before conversion to percentage of control.

Dose-response and time-course studies. In order to determine whether hesperetin and naringenin could dose-dependently reduce net apoB secretion, HepG2 cells were grown to confluence in 24-well plates and incubated for 24 h in the absence or presence of 7.5–60 μg/mL of either hesperetin or naringenin. These flavonoid concentrations were determined to be noncytotoxic by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability assay (33). The time course of the flavonoid effects on net apoB secretion was determined by incubation of confluent HepG2 cells in the absence or presence of effective apoB-reducing concentrations (60 μg/mL) of either hesperetin or naringenin for 2–48 h. At the end of each incubation, culture media were collected and assayed for apoB content as described above.

Radiolabeling of intracellular and secreted proteins. To determine whether the flavonoids affect cellular protein synthesis and secretion, confluent HepG2 cells were washed with Hank's balanced salt solution and then incubated with or without 60 μg/L hesperetin or naringenin in the presence of 4,5-^{3}H-leucine (5 dpm/pmol) (ICN Pharmaceuticals, Irvine, CA) for 0–240 min. The media and cells were collected at the indicated time points, and aliquots were applied to 20-mm cellulose acetate filters. Proteins were precipitated with 10% trichloroacetic acid (TCA) for 30 min and then washed in 5% TCA as described by Everson *et al.* (34). Filters were dried,

and the precipitate was solubilized in 0.1 N NaOH and Scintigest (Fisher Scientific, Fairlawn, NJ). Incorporation of radioactivity into intracellular and secreted proteins was expressed as dpm ^{3}H-leucine incorporated/mg cell protein.

Reversibility studies. To investigate the ability of HepG2 cells to recover from flavonoid-induced reduction of net apoB secretion, cells were incubated for 24 h in the absence or presence of 60 µg/mL of either hesperetin or naringenin. Flavonoid-containing media were then removed and replaced with flavonoid-free medium for further 10 and 24 h incubations (postincubations). Aliquots of media collected after the initial 24 h incubation, as well as those collected after the postincubations, were assayed for apoB content as described above.

Proteasome inhibitor and oleic acid studies. To determine whether the flavonoids reduce net apoB secretion by increasing proteasomal degradation of the nascent protein, a highly selective inhibitor of the 26S proteasome, MG-132 (carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; Calbiochem-Novabiochem, LaJolla, CA), was used to prevent apoB degradation prior to secretion. MG-132 was solubilized in DMSO and diluted in MEM containing 1% BSA to a concentration of 10 µM, a concentration previously shown to inhibit the 26S proteasome in HepG2 cells effectively (35). As with the flavonoids, the final concentration of DMSO in the media was less than 0.1% by vol. Confluent cells were then preincubated for 1 h in the absence or presence of 10 µM MG-132, followed by a 4 h incubation with 60 µg/mL of either hesperetin or naringenin, in the absence or presence of 10 µM MG-132.

A similar protocol was used to determine the effect of increased lipid synthesis on flavonoid-induced reduction of net apoB secretion. Experimental medium was enriched with oleate by dissolving 0.8 mM sodium oleate (Sigma) in MEM containing 1% of fatty acid free BSA (Sigma), according to the method described by Dixon *et al.* (36). HepG2 cells were then preincubated for 1 h in the absence or presence of oleate-enriched medium, followed by a 4 h incubation in the absence or presence of 60 µg/mL of either hesperetin or naringenin, with or without 0.8 mM sodium oleate.

For both of these experiments, apoB content in the media was subsequently determined by ELISA, as described above.

Intracellular cholesterol and triglyceride metabolism. To determine whether exposure to flavonoids altered the metabolism of cholesterol and/or triglycerides, HepG2 cells grown to confluence in 6-well plates were incubated for 24 h in MEM plus 1% BSA or 5% lipoprotein-deficient serum with 60 µg/mL of either hesperetin or naringenin. During the final 5 h of incubation, 1-^{14}C-acetate (0.5 µCi/mL medium) (Amersham, Oakville, Ontario, Canada) was added to each well. Cells were washed three times with ice-cold phosphate-buffered saline, and radiolabeled lipids were subsequently extracted, using heptane/isopropyl alcohol (3:2, vol/vol). A small amount of ^{3}H-cholesterol (Amersham) was added to each extract as an internal standard. Free cholesterol, cholesterol esters, and triglycerides were then separated by thin-layer chromatography, using a hexane/ethyl ether/acetic acid (75:25:1, by vol) solvent system (37). Portions of silica-containing lipid fractions were then placed into scintillation vials. Radioactivity was measured with a Rackbeta 1214 scintillation counter and calculated as dpm/mg cellular protein before conversion to percentage of control.

Statistical analysis. All results were evaluated by one-way analysis of variance. Differences between groups were determined using Bonferroni and Tukey *t*-tests, where appropriate (SigmaStat; Jandel Corporation, San Rafael, CA). Significant differences were reported at $P < 0.05$.

RESULTS

Incubation of HepG2 cells with increasing, nontoxic concentrations (as determined by an MTT cell viability assay, data not shown) of either hesperetin or naringenin caused a dose-dependent reduction of net apoB secretion relative to cellular protein content (Fig. 1). The highest nontoxic dose of either hesperetin or naringenin (60 µg/mL) significantly lowered medium apoB by 76 and 81%, respectively, and naringenin, at a dose of 30 µg/mL, significantly reduced apoB content by 29%. The effect induced by incubation with 60 µg/mL of either flavonoid was rapid in onset. Net apoB secretion was reduced to *ca.* 50% of control values after a 4 h exposure to either hesperetin or naringenin (Fig. 2). As was expected, the reduction in net secretion became more pronounced with longer incubation times. DMSO at a concentration of 0.1% by vol, the highest concentration present in the media, had no effect on net apoB secretion.

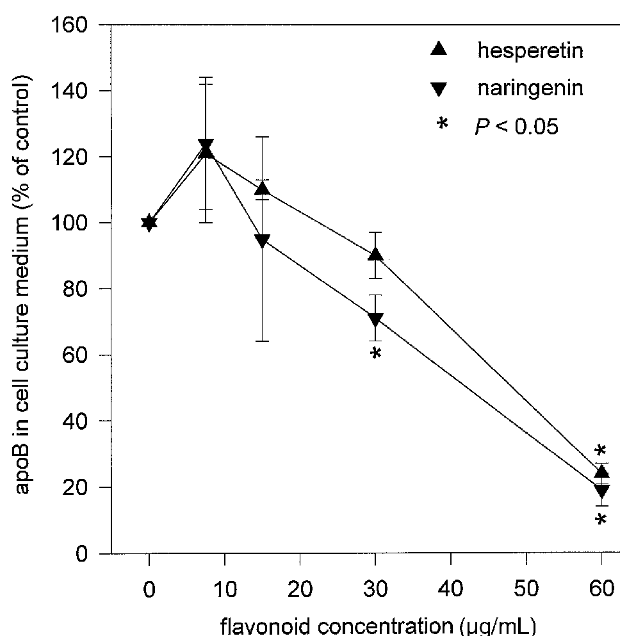


FIG. 1. Dose-response of flavonoid effects on apolipoprotein B (apoB) accumulation in the media of HepG2 cell cultures. Confluent HepG2 cells were incubated for 24 h in the presence of 0–60 µg/mL of either hesperetin or naringenin. ApoB in the cell culture media was quantified by enzyme-linked immunosorbent assay (ELISA) and expressed per mg cell protein before conversion to percentage of control. Values are means ± SEM, $n = 3$.

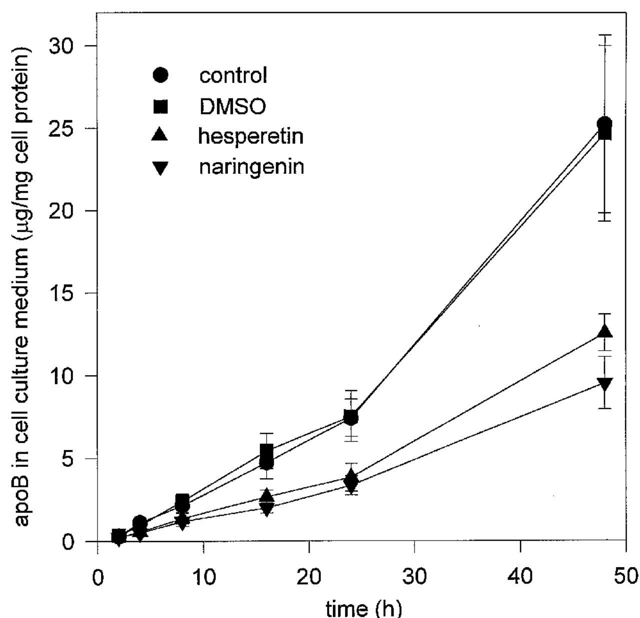


FIG. 2. Time-course of flavonoid effects on accumulation of apoB in the media of HepG2 cell cultures. Confluent HepG2 cells were incubated for 2–48 h in the absence or presence of 60 µg/mL of either hesperetin or naringenin. ApoB in the cell culture media was quantified by ELISA and expressed per mg cell protein. Values are means ± SEM, n = 4. All values for flavonoid treatments from 4–48 h were significantly lower than control values ($P < 0.05$). DMSO, dimethylsulfoxide; see Figure 1 for other abbreviations.

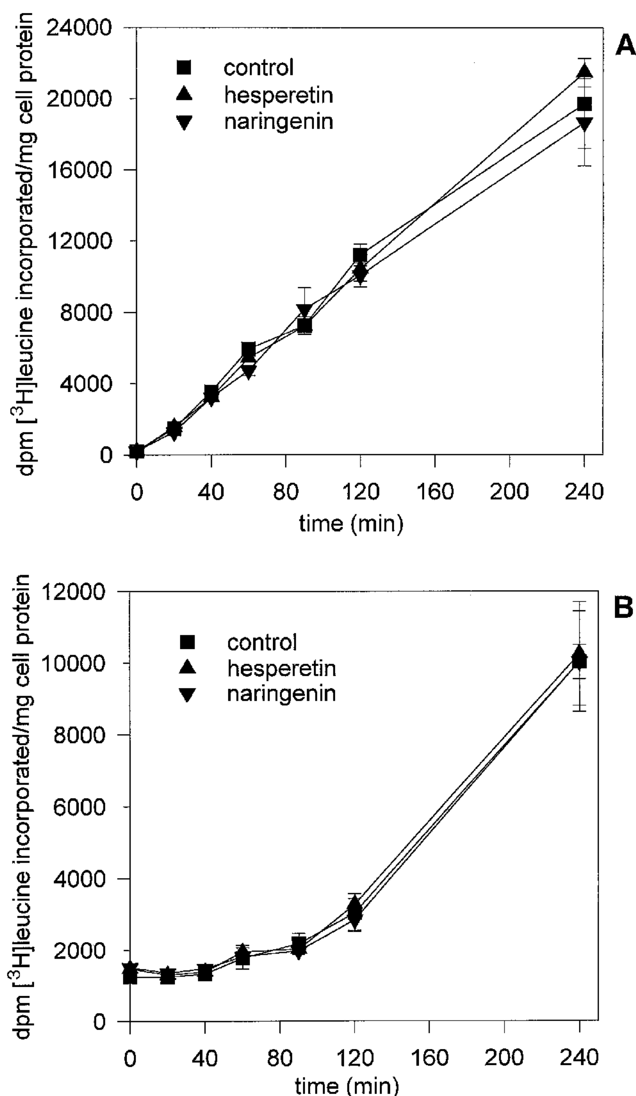


FIG. 3. Effects of hesperetin or naringenin on incorporation of ^{3}H-leucine into (A) nonsecreted and (B) secreted proteins. HepG2 cells were incubated for 0–240 min in serum-free medium with 1% bovine serum albumin in the absence or presence of 60 µg/mL of either hesperetin or naringenin. Radiolabeled intracellular and media proteins were precipitated with trichloroacetic acid and counted. Results of a representative experiment are expressed as mean dpm of ^{3}H-leucine incorporated per mg of cell protein ± SEM.

Exposure of HepG2 cells to either flavonoid at concentrations that substantially reduced net apoB secretion (60 µg/mL) did not affect incorporation of ^{3}H-leucine into cellular (Fig. 3A) and secreted (Fig. 3B) TCA-precipitable proteins over a 4 h period.

HepG2 cells were able to partially recover from the reduction in net apoB secretion induced by 24 h incubation with 60 µg/mL of either hesperetin or naringenin. After both 10 and 24 h postincubations in flavonoid-free medium, net apoB secretion returned to within 60–70% of control values (Fig. 4).

Incubation of HepG2 cells with the proteasome inhibitor, MG-132, had no effect on the reduction of net apoB secretion induced by incubation with 60 µg/mL of either hesperetin or naringenin (Fig. 5). Incubation with MG-132 for 4 h in the absence of either flavonoid nearly doubled net apoB secretion. However, after 4 h exposure to either flavonoid, net apoB secretion was reduced by *ca.* 50%, as compared to the untreated control, regardless of the presence of inhibitor.

Incubation with oleate-enriched medium, on the other hand, prevented the reduction of net apoB secretion induced by a 4 h incubation with 60 µg/mL of either hesperetin or naringenin (Fig. 6). Regardless of the presence of either flavonoid, oleate increased net apoB secretion by *ca.* 50%, as compared to the untreated control.

Incubation of HepG2 cells with 60 µg/mL of either hesperetin or naringenin decreased the incorporation of ^{14}C-acetate per mg protein into cholesteryl esters by *ca.* 50% (Table 1). Label incorporation into free cholesterol was slightly reduced in the presence of the flavonoids; however, this change was not statistically significant. Label incorporation into triglycerides was unaffected. DMSO at a concentration of 0.1% by vol had no effect on radiolabel incorporation and recovery for each sample was between 70 and 100%.

DISCUSSION

The results of these studies showed that both hesperetin and naringenin significantly reduced net apo B secretion by HepG2 cells in a dose-dependent manner (Fig. 1), without affecting the incorporation of ^{3}H-leucine into TCA-precipitable

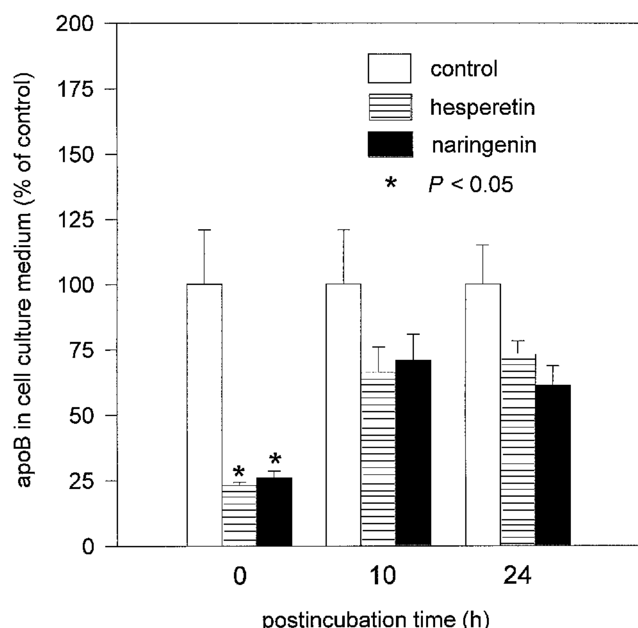


FIG. 4. Reversibility of flavonoid effects on net apoB secretion. Confluent HepG2 cells were incubated for 24 h in the absence or presence of 60 µg/mL of flavonoid. Medium was removed and assayed for apoB content (ELISA). The cells were then incubated for a further 10 or 24 h in flavonoid-free conditions; medium apoB content was again quantified and calculated per mg cell protein before conversion to percentage of control. Values are means ± SEM, $n = 3$. See Figure 1 for abbreviations.

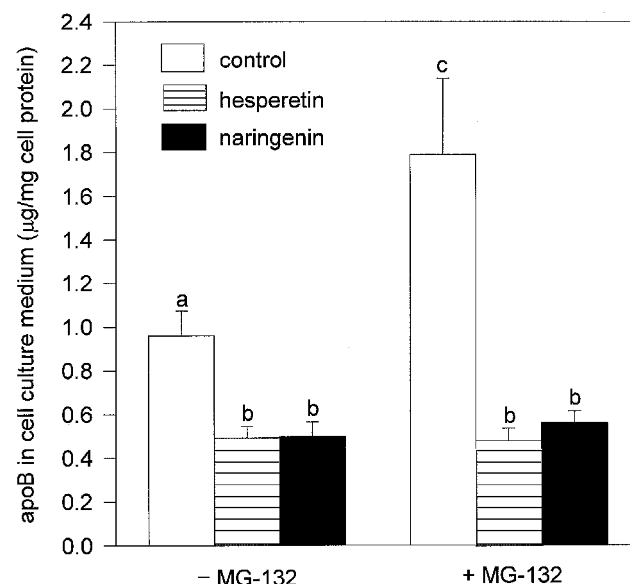


FIG. 5. Effects of citrus flavonoids on net apoB secretion in the presence of MG-132. Confluent HepG2 cells were preincubated for 1 h in the absence or presence of 10 µM MG-132, and incubated for a further 4 h in the absence or presence of flavonoid, with or without MG-132. Medium apoB was then quantified (ELISA) and expressed per mg cell protein. Values are means ± SEM, $n = 5$. a, b and c are significantly different from each other at $P < 0.05$. MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; see Figure 1 for other abbreviations.

cellular and secreted proteins (Fig. 3). This suggests that the flavonoids selectively inhibit net apoB secretion.

The flavonoid effect is relatively rapid in onset (Fig. 2). However, the 50% reduction in net apoB secretion after 4 h exposure to either hesperetin or naringenin does not exclude the possibility that these compounds could directly suppress the transcription and/or translation of apoB. Nevertheless, this mechanism of action is unlikely since most reports indicate that rapid changes in apoB secretion in both cultured hepatocyte and animal models are controlled posttranslationally (38–42).

As shown in Figure 4, the majority of the flavonoid effect on net apoB secretion is reversible after 10 and 24 h postincubations with flavonoid-free media. The inability of the cells to recover completely from exposure to the flavonoids could be due to cytotoxicity caused by either the parent compounds or their metabolites. However, MTT cell viability assays indicated no significant toxicity after 24 h incubations with 60 µg/mL of either flavonoid. A more likely explanation for the incomplete reversal may be that the flavonoids and/or their metabolites induce multiple biochemical changes which affect

TABLE 1
Percentage of Incorporation of ^{14}C-Acetate into Cellular Lipids of HepG2 Cells in the Presence of Citrus Flavonoids[a]

Treatment	Free cholesterol	Cholesteryl esters	Triglycerides
Control	100 ± 6	100 ± 6[a]	100 ± 6
Hesperetin	62 ± 14	48 ± 4[b]	118 ± 19
Naringenin	78 ± 7	53 ± 10[b]	109 ± 22

[a]Values are means ± SEM, $n = 3$. a and b are significantly different at $P < 0.05$.

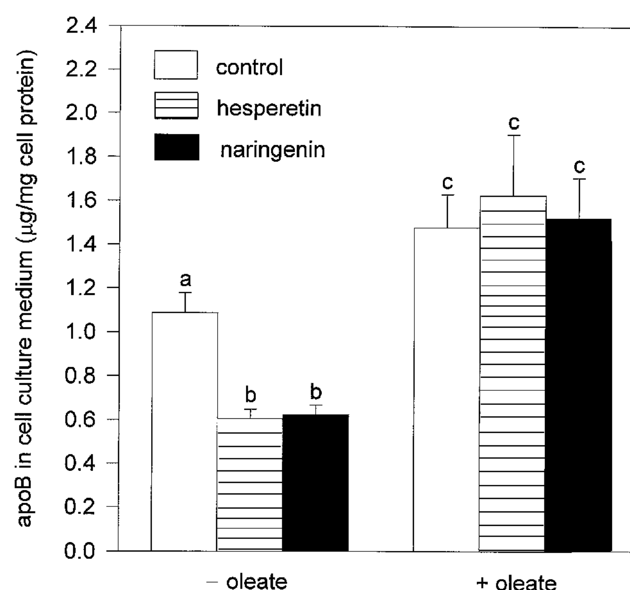


FIG. 6. Effects of citrus flavonoids on net apoB secretion in the presence of exogenous oleate. Confluent HepG2 cells were preincubated for 1 h in the absence or presence of 0.8 mM sodium oleate and incubated for a further 4 h in the absence or presence of flavonoid, with or without oleate-enriched media. Medium apoB was then quantified (ELISA) and expressed per mg cell protein. Values are means ± SEM, $n = 4$. a, b and c are significantly different from each other at $P < 0.05$. See Figure 1 for abbreviations.

lipoprotein secretion, some of which are irreversible. Further studies are needed to determine the nature of these changes.

Many studies showed that altered rates of degradation of newly synthesized apoB can produce rapid changes in net apoB secretion by hepatocytes (reviewed in Ref. 4). Wu *et al.* (43) proposed a two-site model for the degradation of apoB in HepG2 cells. According to this model, newly synthesized apoB associated with the ER membrane is degraded by cytosolic proteasomes (26S multicatalytic protease complex) at the first site, while fully translocated apoB is degraded by a second proteolytic pathway in the lumen of the ER. This second site may be responsible for the degradation of primordial apoB-containing particles which are inadequately lipidated during the second step of lipoprotein assembly. In order to determine whether a direct increase in proteasomal degradation could be responsible for the observed reduction in net apoB secretion in the presence of the flavonoids, we used a highly selective proteasome inhibitor, MG-132, to protect apoB from degradation at the first site. Our results showed increased net apoB secretion in the presence of MG-132 alone (Fig. 5). This is in agreement with data obtained by Sakata *et al*. (5) which showed increased net apo B secretion in the presence of *N*-acetyl-leucyl-leucyl-norleucinal which, however, is a less selective inhibitor of the 26S proteasome than MG-132 and has protease inhibitory activity which may account for part of the observed increase in net apoB secretion. More recent studies in HepG2 cells, using *N*-carbobenzoxy-leucyl-leucyl-norvalinal and lactacystin, showed that selective inhibition of the proteasome results in intracellular accumulation of ubiquitinated apoB, the substrate for proteasomal degradation (44), and that this accumulated apoB can be assembled into lipoproteins and secreted if sufficient lipid is available (35). Our results suggest that inhibition of the proteasome with MG-132 may allow the accumulation of secretion-competent apoB within HepG2 cells. In the case of the flavonoids, our results showed that inhibition of the proteasome by incubation with MG-132 had no effect on the ability of either hesperetin or naringenin to reduce net apoB secretion (Fig. 5). This suggests the flavonoids do not act by directly increasing the activity of the proteasome in degrading apoB during the earliest stages of lipoprotein assembly.

Since subsequent events in the process of lipoprotein assembly involve the addition of lipids to newly translated apoB, we studied the effect of exogenous oleate on the ability of hesperetin and naringenin to reduce net apoB secretion. Oleate added to the media of HepG2 cells rapidly increases net apoB secretion (8,9,11,13,36,39). This is thought to be due to increased triglyceride synthesis (9,11,13,39), but may also involve changes in cholesteryl ester synthesis (14,15). Our results showed that, in cells pre- and co-incubated with medium containing sodium oleate, the flavonoids were unable to reduce net apoB secretion (Fig. 6). This experiment suggests that the flavonoids did not limit lipid availability by directly inhibiting MTP, since recent studies showed that addition of exogenous oleic acid cannot overcome the reduced apoB secretion observed in MTP-inhibited cells (45). However, it should be noted that our experiments involved incubation with oleic acid prior to the addition of flavonoids. Therefore, further experiments are required to more directly assess flavonoid effects on MTP activity. The flavonoids could also act by inhibiting the synthesis of one or more cellular lipids. In support of this hypothesis, our experiments using ^{14}C-acetate to label cellular lipids showed a 50% decrease in cholesteryl ester synthesis in the presence of either hesperetin or naringenin (Table 1), which could account for the decrease in net apoB secretion caused by these compounds. These results are supported by recent studies by our collaborators which showed that naringenin substantially reduced apoB secretion and cholesterol esterification in HepG2 cells, as well as *in vitro* acyl-CoA acyltransferase (ACAT) activity (46). The slight reduction in label incorporation into free cholesterol in the presence of the flavonoids (Table 1), although not statistically significant, may also contribute to the observed reduction in apoB secretion. However, studies on the effects of specific ACAT inhibitors on apoB secretion also showed reduced incorporation of ^{14}C-acetate into cholesterol by HepG2 cells (16,17). Although phospholipid synthesis was not measured in the present studies, Wilcox *et al*. (46, abstract) showed no change in the synthesis of phospholipids in the presence of 200 μM naringenin after a 24 h incubation.

In summary, the studies presented in this report demonstrate that the citrus flavanones, hesperetin and naringenin, rapidly and selectively reduce net apoB secretion by HepG2 cells. This partially reversible effect may be, in part, the result of reduced cholesteryl ester synthesis, which suggests that these compounds may inhibit ACAT. However, since there have been reports of increased (13), decreased (14), and unchanged (8,11,12) secretion of apoB in response to direct ACAT inhibition, it is likely that hesperetin and naringenin have additional actions in HepG2 cells which contribute to and, perhaps, drive the effect on apoB secretion. Whether ACAT inhibition is a property of a number of classes of flavonoids remains to be determined. However, Yotsumoto *et al.* (47) reported that a Japanese–Chinese traditional herbal mixture containing the flavone, baicalein, significantly reduced the incorporation of ^{14}C-oleate into the cellular cholesteryl ester fraction of HepG2 cells over 6 and 24 h incubations. Furthermore, this report showed that baicalein, which is structurally similar to the flavonones, hesperetin and naringenin, dose-dependently reduced ACAT activity *in vitro*.

Since recent studies showed that hesperetin and naringenin are absorbed after consumption of orange juice and grapefruit juice, respectively (27), the ability of these compounds to reduce apoB secretion and cholesteryl ester synthesis could be responsible for the reductions in serum LDL and hepatic cholesteryl esters observed in endogenously hypercholesterolemic rabbits given either citrus juice (25). However, further studies are required to determine (i) whether hesperetin and naringenin, when given in isolation, can lower LDL cholesterol *in vivo* and (ii) whether dietary intake of citrus juices

leads to significant concentrations of these flavonoids in hepatic-portal blood and liver tissue.

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Cell Proliferation, Differentiation, and Apoptosis Are Modified by n-3 Polyunsaturated Fatty Acids in Normal Colonic Mucosa

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ABSTRACT: Supplementation with low doses of eicosapentaenoic (EPA) or docosahexaenoic (DHA) acid was used here to investigate changes in epithelial proliferation, differentiation, and apoptosis in normal rat colonic mucosa. ACI/T rats received by oral administration low doses of purified EPA or DHA ethyl esters (1g/kg body weight) and colonic mucosa was analyzed for cell proliferation, differentiation, and apoptosis. n-3 Polyunsaturated fatty acid incorporation into membrane phospholipids was investigated as reflections of fatty acid metabolism. Both EPA and DHA suppressed colonocyte proliferation and increased the numbers of differentiating and apoptotic cells without modification of the crypt morphology and the number of cells per crypt columns. A significant incorporation of the supplemented fatty acids into total phospholipids was observed. This enrichment was accompanied by a decreased content in arachidonic acid. The observation that EPA and DHA do not alter crypt morphology although they modify cell turnover in normal colonic mucosa suggests a possible use of these fatty acids as dietary chemopreventive agents.

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n-3 Polyunsaturated fatty acids (PUFA) were shown to inhibit tumor development in frequent experimental studies (1–3). We reported that dietary supplementation with n-3 PUFA normalized altered proliferative patterns in the colonic mucosa of human subjects at high risk for colon cancer (4,5). This was confirmed recently by Huang *et al.* (6), who found a decreased cell proliferation in the colonic mucosa of patients with colon carcinoma after treatment with n-3 PUFA. Moreover, we reported the inhibitory effects of oral administration of eicosapentaenoic acid (EPA, 20:5n-3) or docosahexaenoic acid (DHA, 22:6n-3), the main components of n-3 PUFA family, on the growth of a highly malignant hepatocarcinoma transplanted into rats (7). Both fatty acids exerted an antitumoral effect, with different mechanisms. EPA inhibited cell proliferation and DHA-induced apoptosis.

The findings overall suggest that n-3 PUFA supplement can be considered a candidate dietary agent for reduction of cancer risk. To determine whether the inhibition of growth by n-3 PUFA is specific for tumor cells or is a general effect in normal tissues with high degree of cell turnover, the present study investigated effects of dietary supplementation with EPA or DHA on cell proliferation, cell differentiation, and apoptosis in the normal colonic mucosa of rats. In this study n-3 PUFA were given separately as highly purified EPA or DHA to establish the specific effects of each fatty acid, and at doses comparable to the minimum used in our previous human studies (4,5).

The results showed both fatty acids have antiproliferative and proapoptotic effects on normal colonic mucosa, influencing cell differentiation without modification of the physiological homeostasis of the colonic crypts. EPA and DHA were found to be incorporated differentially in the main classes of phospholipids (PL).

MATERIALS AND METHODS

Animals and treatments. Sixty male inbred ACI/T rats (200 g) were housed in groups of two or three in wire cages. They had free access to a standard laboratory diet (Altromin-Rieper, Rieper Company, Bz, Italy) and water. The animals were divided randomly into three groups of 20. One group received in the morning, by oral administration, a single dose of 1 g/kg body weight/d of oleic acid-ethyl ester (OA, control); a second group received EPA-ethyl ester and a third DHA-ethyl ester. Preliminary experiments comparing OA-treated animals with untreated animals showed that OA treatment did not modify fatty acid composition of cells in the colonic mucosa (Table 1). No significant differences in body weight between control and treated animals were observed

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Abbreviations: BrdU, bromodeoxyuridine; DAB, diaminobenzidine-tetrachloride; DBA, dolichos biflorus agglutinin; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; LI, labeling index; OA, oleic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PUFA, polyunsaturated fatty acids; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end-labeling.

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TABLE 1
Fatty Acid Composition (% of total) of Phospholipids Isolated from Colonic Mucosa of Rats Supplemented with OA, EPA, or DHA (for 10 d)[a]

	None	OA	EPA	DHA
14:0	1.38 ± 0.15	1.27 ± 0.14	0.81 ± 0.10	0.90 ± 0.36
15:0	0.66 ± 0.02	0.78 ± 0.19	0.73 ± 0.16	0.63 ± 0.10
16:0	21.55 ± 1.31	22.93 ± 2.07	21.82 ± 1.11	22.55 ± 2.00
16:1n-7	3.50 ± 0.48[a]	3.40 ± 0.36[a]	2.05 ± 0.33[b]	2.88 ± 0.52[a]
17:0	0.89 ± 0.11	0.66 ± 0.02	0.56 ± 0.03	0.62 ± 0.04
18:0	13.40 ± 0.86	13.12 ± 0.41	14.21 ± 0.29	13.52 ± 0.37
18:1*	16.95 ± 0.71[a,b]	17.66 ± 0.30[a]	16.09 ± 0.12[b]	17.57 ± 0.30[a]
18:2n-6	13.48 ± 0.77	13.62 ± 0.89	14.76 ± 0.46	14.53 ± 0.10
18:3n-6	0.20 ± 0.00[a]	0.25 ± 0.00[a]	0.15 ± 0.03[b]	0.17 ± 0.01[b]
18:3n-3	0.14 ± 0.00	0.14 ± 0.03	0.20 ± 0.01	0.28 ± 0.09
18:4n-3	0.19 ± 0.01	0.40 ± 0.15	0.43 ± 0.09	0.39 ± 0.06
20:0	0.48 ± 0.03	0.50 ± 0.06	0.53 ± 0.04	0.48 ± 0.06
20:1n-9	0.24 ± 0.01	0.24 ± 0.02	0.24 ± 0.02	0.24 ± 0.02
20:2n-6	2.70 ± 0.21	2.33 ± 0.39	1.96 ± 0.19	1.39 ± 0.21
20:3n-6	2.67 ± 0.26	2.52 ± 0.32	1.96 ± 0.13	1.99 ± 0.20
20:4n-6	10.96 ± 0.84[a]	10.34 ± 0.17[a]	9.30 ± 0.52[a]	7.43 ± 0.36[b]
20:5n-3	0.65 ± 0.02[a]	0.61 ± 0.06[a]	3.15 ± 0.07[b]	2.04 ± 0.09[c]
22:0	0.71 ± 0.09	0.56 ± 0.03	0.53 ± 0.07	0.48 ± 0.11
22:1n-9	0.23 ± 0.00	0.23 ± 0.03	0.21 ± 0.05	0.17 ± 0.01
22:4n-6	1.25 ± 0.09	1.11 ± 0.18	0.87 ± 0.10	0.69 ± 0.18
22:5n-3	1.03 ± 0.12[a]	0.81 ± 0.13[a]	1.99 ± 0.25[b]	0.93 ± 0.41[a]
24:0	0.64 ± 0.04	0.53 ± 0.02	0.39 ± 0.06	0.49 ± 0.15
22:6n-3	2.73 ± 0.21[a]	2.58 ± 0.18[a]	2.30 ± 0.13[a]	4.49 ± 0.53[b]
Total saturated	39.67 ± 2.50	39.30 ± 1.47	38.70 ± 0.67	39.05 ± 1.75
Total monoenes	20.87 ± 1.08[a,b]	21.35 ± 0.35[a]	18.31 ± 0.67[b]	20.73 ± 1.47[a]
Total n-6 PUFA	30.26 ± 1.84	29.48 ± 0.86	28.80 ± 0.44	26.13 ± 0.90
Total n-3 PUFA	4.25 ± 0.32[a]	4.09 ± 0.24[a]	7.88 ± 0.46[b]	8.17 ± 0.97[b]
n-6/n-3 PUFA ratio	8.04 ± 0.92[a]	8.56 ± 1.16[a]	3.69 ± 0.20[b]	3.24 ± 0.20[b]

[a]Values are means ± SEM of seven separate determinations. Means in a row with a different superscript are significantly different at $P < 0.05$ (one-way analysis of variance followed by Fisher's test).Values without superscripts are not significantly different. *Isomers of n-9 and n-7 series are calculated together. OA, oleic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid.

during the study. All fatty acid ethyl esters (provided by Hoffmann-La Roche Ltd., Basel, Switzerland) contained 3 mg of α-tocopherol per g. Purity of 92% was verified by gas–liquid chromatography.

After 10-d treatment, the animals were sacrificed and colonic specimens were removed immediately, fixed in Metha-Carnoy, and then embedded in paraffin for histochemical processing (see below). The remaining tissue was excised, flushed with Ca-Mg free Hanks' solution pH 7.4, and freed of fecal material. Colonic mucosal cells were isolated as described by Bjerknes and Cheung (8).

The animal use protocol was approved by the Ministry of Health, Veterinary Service, Rome, Italy.

Analysis of fatty acids. Lipids were extracted from suspensions of colonic mucosal cells as described by Bligh and Dyer (9). Total PL were separated from neutral lipids by thin-layer chromatography with toluene/diethyl ether/ethanol (35:3.5:1, by vol) as a solvent system (7). All major PL [phosphatidylcholine (PC), phosphatidylethanolamine (PE), and phosphatidylinositol (PI)] were separated in chloroform/methanol/acetic acid/water (50:37.5:3.5:2, by vol) (10). After transmethylation, fatty acid methyl esters were analyzed by gas–liquid chromatography as previously described (7).

Cell proliferation, differentiation, and apoptosis. One hour prior to sacrifice, 10 animals from each group received an intraperitoneal injection of 50 mg/kg bromodeoxyuridine (BrdU, Sigma Chemical Co., St. Louis, MO). BrdU incorporated into S-phase DNA was determined immunoistochemically on deparaffinized colon sections by the method of avidine-biotine peroxidase complex using the mouse anti-BrdU monoclonal antibody (4IU; Lawrence Livermore National Laboratory, Livermore, CA) (7). The colon sections from the rats not injected with BrdU were processed for differentiation and apoptosis assay. Differentiation of epithelial colonocytes was assessed histochemically by measuring the binding of *Dolichos biflorus* agglutinin (DBA) to α-*N*-acetyl-galactosamine residues (11,12). The percentage of apoptotic cells in the crypts was determined by *in situ* nick-end labeling (TUNEL) (13). For determination of BrdU-, DBA- and TUNEL-labeling index (LI%), 20 well-oriented crypt sections displaying a U-shaped configuration per colon section were evaluated.

Statistical analysis. Data are shown as means ± SEM. One-way analysis of variance was used to determine significant differences among groups. When appropriate, a Fisher's test was used (Minitab software; Minitab Inc., State College, PA)

to determine the significance of differences between pairs of means. Statistical significance was accepted at $P < 0.05$.

RESULTS

Treatment for 10 d with low doses of EPA or DHA did not change the morphology and the average number of cells per crypt section (Fig. 1). The numbers were 91.7 ± 5.5 and 93.4 ± 8.4, respectively, as compared to 92.4 ± 6.8 for the OA group. The number of cells in S-phase, measured by the BrdU labeling index (LI%), was significantly decreased in EPA and DHA groups relative to the control group (Fig. 2A). Conversely, the percentage of differentiated cells, labeled by lectin histochemistry using *D. biflorus* (DBA LI%), increased significantly in colon mucosa from both EPA- and DHA-treated rats (Fig. 2B). Similarly, the number of apoptotic cells, measured by *in situ* nick-end labeling technique (TUNEL LI%) was significantly increased by both EPA and DHA treatment (Fig. 2C).

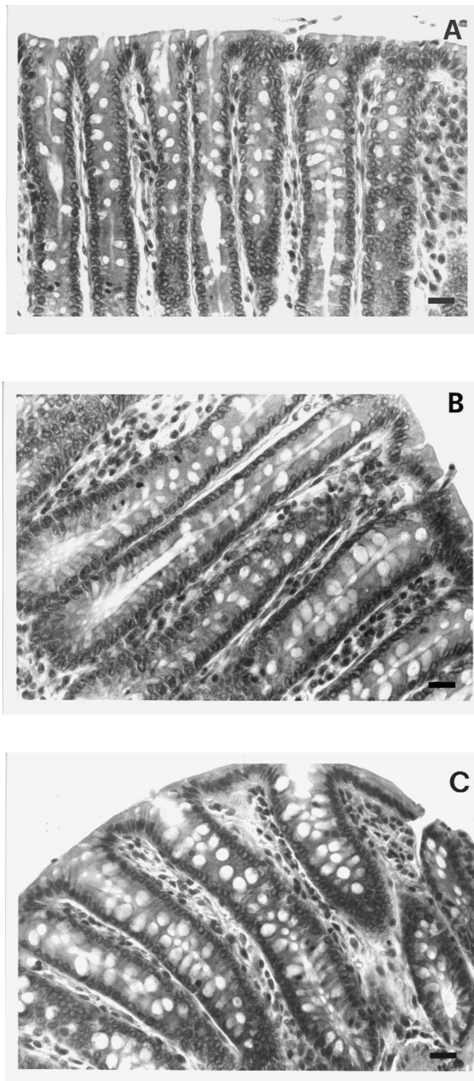

FIG. 1. Crypt morphology of colonic mucosa from rats treated with oleic acid (OA) (A), eicosapentaenoic acid (EPA) (B), and docosahexaenoic acid (DHA) (C). Specimens were stained with hematoxylin-eosin. Note the unchanged morphology and size of crypts after EPA and DHA treatments. Space bars, 20 µM.

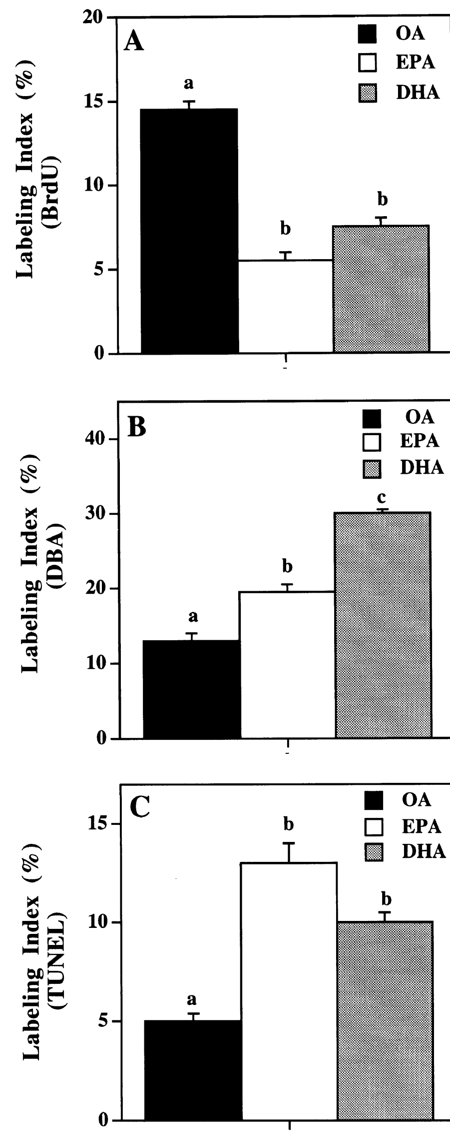

FIG. 2. Effect of EPA and DHA supplementation on cell proliferation, apoptosis, and differentiation in colonic crypts of rats. (A) Proliferation was evaluated as labeling index (%) in bromodeoxyuridine (BrdU)-immunostained specimens. (B) Differentiation was histochemically measured by binding of the lectin *Dolichos biflorus* agglutinin (DBA) to the colonocytes. (C) Apoptotic cells were evaluated with terminal deoxynucleotidyl transferase-mediated dutP-biotin nick end-labeling (TUNEL) method. Values are means ± SE of 10 determinations. Bars with different letters are significantly different ($P < 0.05$, one-way analysis of variance followed by Fisher's test). For other abbreviations see Figure 1.

Fatty acid composition of total PL obtained after dietary treatments is shown in Table 1. The content of saturated fatty acids was unaffected by supplementation with EPA or DHA. EPA treatment significantly reduced the level of both palmitoleic and oleic acids, as compared to the OA group. The content of total and individual PUFA was significantly affected by the treatment with the two n-3 fatty acids with respect to

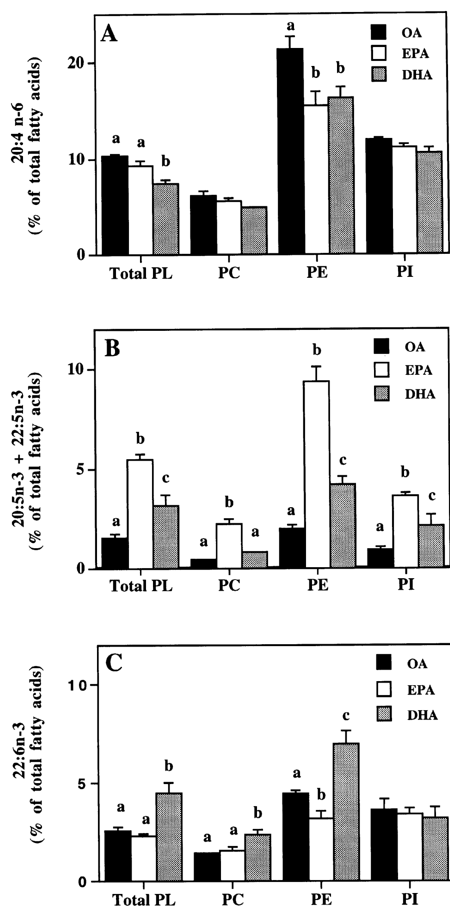

FIG. 3. Content of the principal polyunsaturated fatty acids in total phospholipids (PL) and in the different PL classes. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol. Values are means ± SE of six determinations. Bars in each group with different letters are significantly different ($P < 0.05$, one-way analysis of variance followed by Fisher's test). Bars in a group without superscripts are not significantly different from each other. For other abbreviations see Figure 1.

the OA group. EPA treatment significantly increased EPA (20:5n-3) and its metabolite docosapentaenoic acid (DPA, 22:5n-3) without modifying the level of DHA (22:6n-3). Conversely, DHA supplementation increased the amount of DHA itself (22:6n-3) and of EPA (20:5n-3), but not of DPA (22:5n-3). Linoleic acid was unchanged in both groups of animals, and arachidonic acid (20:4n-6) was significantly decreased only in the DHA group. All the observed modifications led to a large decrease in n-6/n-3 PUFA ratio in total PL of both EPA- and DHA-treated groups.

Modifications in the content of principal PUFA in the single PL classes (PC, PE, and PI) with respect to total PL after EPA and DHA treatment are illustrated in Figure 3. Arachidonic acid was very stable in PC and PI after both treatments. EPA and DPA were sensitive to treatments with both EPA and DHA in all PL classes, whereas DHA was not modified in PI even after DHA treatment.

DISCUSSION

The data show that dietary administration with either EPA or DHA does not modify the physiological homeostasis of normal rat colonic mucosa. Indeed, they do not alter crypt morphology and the number of cells per crypt section, although they inhibit cell proliferation and enhance cell differentiation and apoptosis. Inhibition of cell proliferation by n-3 PUFA was recently reported *in vitro* (14,15) and *in vivo* (4,16,17) in both normal and tumor cells. In particular, treatment with fish oils decreased cell proliferation in colonic mucosa of humans and rats (18,19). Fish oil induction of apoptosis was recently reported in rat colonic mucosa and in cell lines (11,15). Lai *et al.* (20) observed an enhancement of apoptosis by n-3 PUFA in pancreatic cancer cells *in vitro*, and Fernandes *et al.* (21) in lymphocytes of lupus-prone mice. Recently Chang *et al.* (22) reported that colon tumorigenesis was inhibited in rats treated with fish oil. This inhibition was related to an increase of cell differentiation and apoptosis rather than a decrease of cell proliferation. In our previous study we found that both EPA and DHA administered separately, in the same dose used in this study, decreased the growth of a highly malignant hepatocarcinoma after transplantation into rats by altering cell proliferation and apoptosis (7). In particular EPA significantly inhibited cell proliferation while DHA increased apoptosis. In the present study, in a normal tissue at high degree of cell turnover such as colonic mucosa, both fatty acids inhibited cell proliferation, induced apoptosis, and in addition increased the number of differentiating cells. Notably, however, in spite of these modifications, the tissue morphology or the number of cells per crypt section was unchanged. A possible explanation for this is that the primary effect of the two fatty acids in normal tissues is the expansion of the differentiated cellular pool and that the alterations on cell proliferation and apoptosis are a homeostatic response to this effect. This hypothesis is further supported by the observations of several authors who found an induction of differentiation in cultured cells treated with n-3 PUFA (23,24).

The results on fatty acid composition of total PL show an enrichment with the supplemented fatty acids and with 22:5n-3 that occurred only with EPA supplementation. Concerning the PL classes, PE was more sensitive than PC and PI to incorporate the supplemented n-3 fatty acids. We observed also a depressed content of 20:4n-6 that occurred primarily with DHA supplementation both in total PL and in PE. These modifications are similar to those observed by us and others in normal and tumor tissue and cells (7,25,26).

It is well known that arachidonic acid metabolites have a key role in signal transduction pathways involved in the control of cell growth. Therefore, it is possible that the effects of both n-3 PUFA on cell proliferation and apoptosis observed in our study are due to a decrease in arachidonic acid content in colonic PL. Recently the decrease of arachidonic acid in membrane PL induced by dietary n-3 PUFA was shown to inhibit the expression of cyclooxygenase-2, a key enzyme in

the conversion of arachidonic acid to prostaglandins (27). This enzyme appears to have a regulatory role in the colonic mucosa cell growth. Its selective inhibition decreases the growth of human colon cancer cells (28), and its increased expression is observed in human colorectal adenocarcinoma (29). Moreover, overexpression of cyclooxygenase-2 in rat intestinal epithelial cells cultured *in vitro* inhibits apoptosis (30). In our study, however, we observed that arachidonic acid was modified in total PL and in PE, but not in PC and PI, which represent the two major substrates for phospholipase activity. This suggests that in our conditions alteration of arachidonic acid metabolism may not be the only mechanism involved in PUFA effects. However, to better clarify this point, it would be of interest to explore the effects of alteration of colonic mucosa PL arachidonic acid levels by other dietary manipulation such as feeding ethyl arachidonate or providing a diet with almost equal proportions of linoleic and linolenic acids.

Consideration of the overall results of the study leads to the conclusion that dietary supplementation with EPA and DHA modifies the rate of cell proliferation, cell differentiation, and cell death in the colonic mucosa. The observation that EPA and DHA modify cell turnover without altering crypt morphology in normal colonic mucosa suggests a possible use of these fatty acids as dietary chemopreventive agents.

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Enhancement of Sterol Synthesis by the Monoterpene Perillyl Alcohol Is Unaffected by Competitive 3-Hydroxy-3-methylglutaryl-CoA Reductase Inhibition

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ABSTRACT: Monoterpenes such as limonene and perillyl alcohol (PA) are currently under investigation for their chemotherapeutic properties which have been tied to their ability to affect protein isoprenylation. Because PA affects the synthesis of isoprenoids, such as ubiquinone, and cholesterol is the end product of the synthetic pathway from which this isoprenoid pathway branches, we investigated the effects of this compound upon cholesterol metabolism in the colonic adenocarcinoma cell line SW480. PA (1 mM) inhibited incorporation of ^{14}C-mevalonate into 21–26 kDa proteins by 25% in SW480 cells. Cholesterol (CH) biosynthesis was assessed by measuring the incorporation of ^{14}C-acetate and ^{14}C-mevalonate into 27-carbon-sterols. Cells treated with PA (1 mM) exhibited a fourfold increase in the incorporation of ^{14}C-acetate but not ^{14}C-mevalonate into cholesterol. Mevinolin (lovastatin), an inhibitor of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase, at 2 μM concentration, inhibited CH synthesis from ^{14}C-acetate by 80%. Surprisingly, concurrent addition of mevinolin and PA did not significantly alter the stimulatory effects of PA. As observed differences in ^{14}C-acetate and ^{14}C-mevalonate precursor labeling could indicate PA affects early pathway events, the effects of this monoterpene on HMG-CoA reductase activity were evaluated. Unexpectedly, 1 mM PA did not stimulate activity of this enzyme. Consistent with its action as a reversibly bound inhibitor, in washed microsomes, 2 μM mevinolin pretreatment increased reductase protein expression causing a 12.7 (±2.4)-fold compensatory HMG-CoA reductase activity increase; concurrent treatment with 1 mM PA attenuated this to a 5.3 (±0.03)-fold increase. Gas chromatographic analysis confirmed CH was the major lipid present in the measured thin-layer chromatography spot. Since ^{14}C-acetate incorporation into free fatty acid and phospholipid pools was not significantly affected by PA treatment, nonspecific changes in whole acetate pool sizes were not indicated. Because increases in endogenous CH synthesis should result in compensatory changes in exogenous sterol utilization, the effects of PA upon low density lipoprotein (LDL) receptor activity were evaluated. Consistent with the observed increases in CH synthesis, 1 mM PA decreased ^{125}I-LDL internalization to 50% of the fetal bovine serum control; concurrent addition of 2 μM mevinolin attenuated this effect to a reduction of 80% of the control value. Data suggest that in certain colonic tumor cells PA strongly affects cholesterol metabolism *via* a mechanism of action that is insensitive to the HMG-CoA reductase inhibitor mevinolin.

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The primary rate-limiting step in cholesterol biosynthesis is catalyzed by 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase (EC 1.1.1.34) which directly yields mevalonate, an important precursor to cellular sterol and nonsterol isoprenoids such as dolichol, ubiquinone, and isopentenyl tRNA (1). Labeled mevalonate also incorporates as farnesyl or geranylgeranyl isoprenoid moieties into a number of isoprenylated proteins in mammalian cells, thereby imparting the hydrophobicity needed for membrane anchorage (2) and function or cellular localization (3). Examples include heterotrimeric G proteins, nuclear lamins, p21ras, and low molecular mass GTP-binding proteins (4).

Dysregulation of mevalonate metabolism in neoplasia is complex (5–8) and was reviewed (9). Studies by Maltese *et al*. (10) demonstrated reduction of neuroblastoma tumor growth in nude mice with the HMG-CoA reductase inhibitor mevinolin (MVN) (lovastatin). Other studies reporting a variety of effects induced by HMG-CoA reductase inhibitors followed (11–16), demonstrating the effectiveness of these compounds as inhibitors of human tumor growth.

Limonene is the predominant monoterpene in orange peel oil and was demonstrated to possess chemopreventive and chemotherapeutic activity against solid tumors (17,18). These actions result from the induction of hepatic phase I and phase II detoxifying enzymes during initiation and the suppression of growth in chemically initiated and transplantable tumors (17,18). In addition, d-limonene and PA (a hydroxyl derivative of limonene) were shown to cause apoptosis, induce differentiation and mammary carcinoma regression, and inhibit the posttranslational isoprenylation of cell growth-regulatory proteins, such as Ras (17–23).

Perillyl alcohol (PA) was reported to inhibit ubiquinone

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Abbreviations: CH, cholesterol; FBS, fetal bovine serum; HMG-CoA reductase, 3-hydroxy-3-methylglutaryl CoA reductase (EC 1.1.1.34); LDL, low density lipoprotein; LDL-R, LDL receptor; LPDS, lipoprotein deficient serum; MEM, minimal essential medium; MVN, mevinolin; 25-OH-CH, 25-hydroxy cholesterol; PA, perillyl alcohol; PBS, phosphate buffered saline; TLC, thin-layer chromatography.

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and cholesterol (CH) synthesis using a ^{14}C-mevalonate precursor (24). While mevalonate labeling does specifically target the isoprenoid pathway, its use cannot detect changes in early pathway enzyme activity including HMG-CoA reductase (1). For this reason the effects of this monoterpene on CH production were determined in SW480 colonic adenocarcinoma cells using a ^{14}C-labeled acetate precursor. This cell line was shown to contain a K-ras mutation and is highly sensitive to the action of the HMG-CoA reductase inhibitor MVN (12,25). While the mechanism(s) are unknown, PA was observed to enhance CH biosynthesis from a ^{14}C-labeled acetate precursor. Increased CH content, measured by an enzymatically based colorimetric assay system, was also observed in PA-treated cultures as compared to control groups. Intracellular CH homeostasis in humans is maintained by a balance among uptake of exogenous CH primarily *via* the low density lipoprotein receptor (LDL-R), endogenous synthesis (primarily regulated at the HMG-CoA reductase catalyzed step), and storage as cholesteryl-esters *via* the acyl-cholesteryl-acyl-transferase enzyme (26). While overall CH synthesis in human colonic adenocarcinomas is relatively insensitive to exogenous sterol presence, LDL-R expression, while low (12), remains sterol-regulated (27). Analysis of LDL-R activity indicated PA caused a decreased expression consistent with the observed increase in CH synthesis.

MATERIALS AND METHODS

Tissue culture. Cultures of the human colonic cancer cell line SW480, obtained from American Type Culture Collection (Rockville, MD), were grown and harvested as described (27). In brief, stock cultures were grown in monolayer in plastic 75 cm^2 flasks and incubated at 37°C in a 5% CO^2/95% air atmosphere. Culture medium comprised Eagle's minimal essential medium (MEM) containing antibiotics (penicillin, 200 units/mL; streptomycin, 0.2 mg/mL), glutamine (0.2 mg/mL), and nonessential amino acids and nonessential vitamins both at 10 mL of 100× solution (Gibco, Grand Island, NY), per 500 mL MEM and supplemented with 10% fetal bovine serum (FBS) (Sigma, St. Louis, MO). Cells were plated at a density of 10^6 cells per flask, and cultures divided weekly (1:19 or 1:20) using 0.05% trypsin/0.02% disodium EDTA. After removal of media, cells were rinsed with isotonic phosphate buffered saline (PBS) (pH 7.4), and 4 mL of trypsin was added. Flasks were incubated at 37°C until cells began to detach, after which 4–6 mL of fresh medium was added. Cell cultures were assayed for the presence of mycoplasma using the gen-Prove Mycoplasma T.C.11 Rapid Detection System (San Diego, CA).

Preparation of lipoprotein deficient serum (LPDS). Lipoproteins were removed from FBS by density gradient centrifugation (28) and prepared as described (12,27). FBS was adjusted to a density of 1.21 g/mL with solid potassium bromide (64.4 g KBr per 200 mL FBS) and centrifuged for 48 h at 5°C at 244,000 × g_{max} (45,000 rpm in a 50.2 ti rotor in a Beckman L8-70 ultracentrifuge). The top lipoprotein fraction (d < 1.21 g/mL) was removed, and the bottom lipoprotein-deficient fraction dialyzed extensively against 0.01% EDTA/double-distilled water (pH 8) and then PBS. The LPDS was sterilized by passage through a 0.22 μ filter.

Preparation of MVN. MVN (lovastatin) was kindly provided by A. Alberts of Merck Sharp & Dohme (Rahway, NJ) in the lactone form. This reagent was prepared as described and used at a concentration of 2 μM in MEM (12,27). Saponification was carried out by adding 0.1 mL absolute ethanol and 0.1 mL of 0.1 N NaOH to 4 mg of MVN and by heating at 50°C for 2 h. MVN was neutralized with 5% HCl to pH 7.3. Stock solutions (4 mg/mL) were prepared by bringing the volume to 1 mL with dimethyl sulfoxide and stored at −20°C. At the concentrations used, dimethyl sulfoxide was found to have no effect on cell growth.

Preparation of PA. PA stocks (Fluka, Ronkonkoma, NY) were made up in FBS before addition to the media. To control for osmotic stress, equivalent amounts of ethanol (dissolved in FBS) were added to control groups. PA was used in this study at a dose of 1 mM for a 24-h period. This dose was shown to inhibit growth of SW480 cells without causing toxicity (29).

Protein determinations. Proteins were determined by the bicinchoninic acid reagent method (Pierce Chemical Company, Rockford, IL). A standard curve was prepared using a stock bovine serum albumin standard in sample diluent. Samples and standard were mixed with the protein reagent and absorbance read at 562 nm by spectrophotometry.

CH synthesis. Assays were conducted using ^{14}C-acetate as described (27) or ^{14}C-mevalonate precursors, by the method of Brown *et al*. (28). Briefly, cells were seeded in 6-well (35 mm^2) dishes at 20,000 cells/well in triplicate per group studied per experiment, in MEM-supplemented media with 10% FBS. Cells were grown to subconfluency (≈50% of the plate was covered), rinsed with Dulbecco's PBS, and switched to a LPDS-based medium (1 mL/well). After 24 to 48 h, the media were replaced with media containing 5 μCi/mL of 2-^{14}C-acetate (51 mCi/mmole New England Nuclear, Boston, MA) or 7.5 μCi/mL of RS-[2-^{14}C]-mevalonate (54.1 mCi/mmol) (New England Nuclear) and the following supplements: (i) 10% LPDS, (ii) FBS, the basal medium for all subsequent treatments (each of which contains 10% FBS), (iii) MVN 2 μM, (iv) PA at 1 mM, and (v) MVN plus PA. After a 24-h incubation, the medium was removed and saved; monolayers were harvested in 1 mL 0.1 N NaOH and pooled with their respective saved media (whole culture assessment) to be saponified following the methodology of Brown *et al.* (28). Briefly, these mixtures were treated with 0.5 mL 50% KOH, 3 mL ethanol, and (1,2) ^3H-CH (10^5 cpm, 50 Ci/mmole) as internal standard. After saponification at 80°C for 2 h, unsaponifiable lipids were extracted three times with petroleum ether followed by two washes with NaOH. Samples were dried with nitrogen gas, resuspended in chloroform, spotted on silica gel "G" thin-layer chromatography (TLC) plates (Analtech Inc., Newark, DE) and separated using chloroform (EM Science Inc., Gibbstown, NJ) as a running solvent to iso-

late the ^{14}C-CH with appropriate CH markers. Lipid spots were scraped and collected in liquid scintillation vials (VWR Scientific, Philadelphia, PA) containing 7 mL of econofluor scintillation fluid (New England Nuclear). Radioactivity was counted in an LKB Model 1209 Rackbeta Liquid Scintillation Counter using a program simultaneously counting the two isotopes [^{3}H] and [^{14}C]. The samples were subsequently corrected for quenching. Results were expressed as nanomoles of acetate incorporated into CH per mg cellular protein per 24-h period, taking into account the efficiency of the machine for each isotope and the specific activity of the label. Losses during the extractions were accounted for using the internal standard ^{3}H-CH. These values were then normalized to the LPDS control within each experiment. Results of various treatments were expressed as percentage of LPDS control, which was given a value of 100.

Enzymatic colorimetric CH measurement. To determine actual levels of CH in samples, a colorimetric assay system purchased from Sigma was adapted to use with tissue culture-derived samples. This assay was based on three enzymatic reactions: (i) CH esters were converted to CH by the action of CH esterase; (ii) CH was converted to a CH oxide by CH oxidase, a reaction which generates peroxide; and (iii) the peroxide was then coupled to the chromagen 4-aminoantipyrine and *p*-hydroxybenzenesulfonate by a peroxidase causing a color change measurable at 490 nm. Cells were seeded, grown, treated, and harvested as described above for the CH synthesis assay, except that no radioactive precursor was used, and cells and media were assessed separately. Samples were saponified and extracted, as described above. One-half of the extract was brought up in (200 µL) absolute ethanol, and 25–50 µL of this was used to determine actual CH levels in the colorimetric assay. A standard curve was generated from CH (Sigma) dissolved in absolute ethanol. Samples and standards were placed in a 96-well plate, and the ethanol solvent was evaporated overnight at room temperature. To each well, 100 µL of assay buffer containing the enzymes (actual contents are unknown) was added. Reactions occurred for 30 min, and wells were read in an ELISA plate reader at 490 nm. Absorbance values of the standards were plotted to generate a standard curve, which was used to obtain corresponding CH concentrations for the absorbance values of the samples. Data were normalized to protein as determined by the BCA assay.

Derivatization procedure: Esters of benzoic acid. Cells were cultured and treated as above for the CH biosynthesis experiments. After saponification and separation by TLC, lipids were visualized using the fluorescent dye 1,6-diphenyl-2,3,5,-hexatriene (Sigma) by ultraviolet light. The CH spots were then scraped, and lipids were extracted by the addition of 1 mL of chloroform. Samples were vortexed and centrifuged at 2,000 rpm to remove the silica. To dried chloroform extracts was added 300 µL of a solution of 4-dimethylaminopyridine (Sigma) dissolved in pyridine (1 g/300 mL) (30). Benzoyl chloride (50 mL; Sigma) was then added, and the samples were capped, vortexed vigorously, and allowed to stand overnight at room temperature. Next, 300 µL of 0.12 N HCl (American Bioanalytical, Natick, MA) was added, the samples vortexed again, and then chloroform (300 µL) for extracting the newly-formed sterol esters was added. To facilitate separation of the organic and aqueous layers, the samples were centrifuged in a Beckman (Fullerton, CA) tabletop GPR centrifuge equipped with a swinging-bucket rotor at 800–1000 rpm for 5 min. The top aqueous layer was then removed by aspiration, and the organic layer was reduced to a thick, oily liquid under a gentle stream of nitrogen gas while the samples were secured in a low-temperature water bath. Reconstitution of the sterol esters for gas chromatography was with 50 mL of chloroform.

Lipid analysis by gas chromatography. The gas chromatograph (GC-14A; Shimadzu Corporation, Kyoto, Japan) was fitted with an SE-30 capillary column (length, 30 m; internal diameter, 0.25 mm; Alltech, Deerfield, IL). Helium was the carrier gas at a volumetric flow rate of 1.0 mL/min, and a split ratio of 1:20 was utilized. Oven, injector port, and detector temperatures were maintained isothermally at 300°C. Retention times for the various sterol esters on the SE-30 column were determined from freshly derivatized standards prepared from relatively pure chemicals (Sigma). The retention times of the derivatized compounds were as follows: CH, 21.2 min; 7-dehydrocholesterol, 22.2 min; desmosterol, 22.5 min; and lathosterol, 23.7 min. Peak areas and retention times were determined using a CR501 Chromatopac integrator (Shimadzu Corporation, Kyoto, Japan) electronically linked to the gas chromatograph.

^{125}I-LDL binding to colonic adenocarcinoma cell lines. The ^{125}I-LDL binding assays were conducted using the following methodology adapted from Goldstein *et al.* (31). Cells were seeded in 6 wells of 6-well (35 mm^{2}) dishes (Costar, Cambridge, MA) at 2.5×10^{5} cells/well in MEM supplemented with 10% FBS. A duplicate plate for each experiment received only MEM 10% FBS, as a control for nonspecific binding to plastic. After 48 h of growth, cells were washed with PBS and changed to media supplemented 10% LPDS, which we prepared in-house from FBS (Sigma) for 24 h to generate maximal responses in terms of receptor activity. After 24 h, the cells received the following treatments in FBS-based MEM for a duration of 24 h: FBS alone, MVN 2 µM, PA 1 mM, and MVN + PA. Following treatments, the cells were incubated in 1 mL LPDS/MEM with 5–10 µg/mL ^{125}I-LDL (Biomedical Technologies Inc., Stoughton, MA) for 5 h. Typically, four wells of a 6-well dish were used to measure hot or total binding per experiment. To control for nonspecific binding, two wells received 25–40-fold increase of cold LDL (125–200 µg/mL) above the level of the hot LDL in the media. One empty, cell-free, 6-well plate was treated with media, washed, and incubated just as the other groups, and used as a blank.

Surface binding. After incubation for 5 h, cells were shown to reach a steady state in which binding, internalization, and degradation of the lipoprotein are equilibrated. The cells were then placed on an ice bed and rinsed: five times with 2 mL ice-cold Tris-buffered saline with albumin buffer [50 mM Tris/150

mM NaCl/(2 mg/mL albumin) @ pH 7.4], one time with ice-cold Tris-buffered saline, and finally incubated with 1.5 mL of Dextran sulfate release buffer [50 mM NaCl/10 mM HEPES/(4 mg/mL dextran sulfate) @ pH 7.4] for 1 h in a 4°C cold room. Polyanionic macromolecules such as dextran sulfate or heparin sulfate cause LDL to be released from its receptor; thus the activity of the released tracer is a measurement of the surface binding capacity for the cells. Following this incubation, 750 µL aliquots were taken for gamma counting.

Internalization. To measure internalization, the cells were returned to the ice bed and washed two times with 2 mL Tris-buffered saline. Then, the monolayers were removed to a benchtop and dissolved in 1.5 mL hot 60°C NaOH (0.1 N), and aliquots were taken for gamma counting (750 µL) and protein (20 µL) measurement.

Incorporation of ^{14}C-mevalonate into isoprenylated proteins in PA treated cells. Isoprenylation of proteins was assessed following the methodology of Repko and Maltese (32) with some modifications as described below. Briefly, cells were seeded at a density of 5×10^5/35 mm^2 dish (Costar). When cells reached 50% confluence, they were divided into two groups. One group was labeled with 2 µCi of ^{14}C-mevalonate at 54.1 mCi/mmol (New England Nuclear), and subjected to the following treatments for 24 h in MEM + 10% FBS: (i) FBS, (ii) PA 1 mM, (iii) MVN 2 µM, and (iv) PA + MVN. Another set of cells was subjected to each of the above for 24 h and then labeled with 15 µCi of ^{14}C-mevalonate for three additional hours in the presence of cycloheximide at 20 µg/mL. Cells were harvested after trypsin treatment, centrifuged at 3,000 × *g*, and washed twice with PBS. Pellets were then suspended in 150 µL of electrophoresis sample buffer containing 2 M urea, 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, 0.05% bromphenol blue, and 0.0625 M Tris-HCl pH 6.8. Equal amounts of protein were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described by Laemmli (33) using 12% separating and 4% stacking polyacrylamide gels. Instruments were from Hoeffer Scientific Instruments (San Francisco, CA) and reagents from Biorad Life Science (Hercules, CA). Gels were then stained with Coomassie blue, permeated with fluorographic enhancer (En^{3}Hance, New England Nuclear), and dried with a Biorad Gel Dryer for 2.5 h at 70°C. They were then left to cool for an additional 30 min, followed by exposure to preflashed X-OMAT AR film (Eastman-Kodak Co., Rochester, NY) at –70°C for 14 d. Film was then developed using an X-ray film processor (Fuji, Photofilm USA Inc.), and fluorograms were scanned with a phosphorimager:SI (Molecular Dynamics, Sunnyvale, CA).

Basic lipogenesis assay. Cells were cultured and treated and labeled with 2.5–5 µCi/mL [1-^{14}C]acetate (51 mCi/mmole, New England Nuclear), as described for CH synthesis experiments. After labeling was complete (24 h), cells were harvested as described for whole culture assessment of CH synthesis, but saponified overnight at 70°C. After extraction of CH under alkaline conditions with petroleum ether as described above, the samples were acidified with 500 µL of concentrated HCl, vortexed and incubated for 30 min at 70°C to maximize protonization of COOH groups. The samples were extracted twice with 4 mL of petroleum ether and the ether evaporated. Samples were then brought up in 200 µL ether, and 100 µL of this was spotted on silica "G" TLC plates. These were run in hexane/diethyl ether/HOAc (70:30:1), allowed to dry, and incubated in an iodine chamber to visualize the lipids. Standards were: for phospholipids, phosphatidylcholine (R_f 0), and for free fatty acids, oleic acid (R_f 0.39). Spots corresponding to the standards were marked, iodine was allowed to dissociate, and spots were scraped and counted in econofluor. Results are reported as nanomoles of acetate incorporated into lipid type per mg cell protein.

HMG-CoA reductase activity assay in washed microsomal preparations. Cells were grown to subconfluence in 150 mm dishes, and treated for 24 h with MEM/10% FBS (control and basal media for all subsequent groups), 2 µM MVN, 1 mM PA, or a combination of the two treatments. Cells were rinsed once with PBS, scraped into 4°C PBS, and pelleted at 250 × *g*. The enzymatic preparation essentially follows the methodology of Ness *et al.* (34). In this process competitive inhibitors, such as MVN, are removed from the HMG-CoA reductase enzyme preparation. Pellets were homogenized in ice-cold solution 1 (0.25 M sucrose with 5 mM EGTA, 1 mM dithiothreitol, and 40 mM leupeptin, pH 7.1), in a Dounce homogenizer. The homogenate, after removing unlysed cells by centrifugation at 12,000 × *g* (supernatants were reserved), was centrifuged at 100,000 × *g*. The pellet was resuspended in solution 1 and centrifuged again at 100,000 × *g* to generate a washed microsomal preparation. The pellet was resuspended in solution 2 (solution 1 with 33% glycerol). Aliquots for Lowry protein determination were taken, and 10 to 100 µg of protein was used in the standard assay described below.

Specific activity measurements were made as described by Shapiro *et al.* (35) and represent the cell's total amount of reductase enzyme (36). Reductase activity was measured by incubating 10–100 µg of protein in 50 µL of a 100 mM phosphate buffer, pH 7.1, with 200 mM NaCl, 1mM EDTA, 3 mM NADPH, 30 mM glucose 6-phosphate, one unit of glucose 6-phosphate dehydrogenase, and 50 mM [3-^{14}C]HMG-CoA (3.3 mCi/mmole) at 37°C for 60–120 min. The reaction was stopped by the addition of 10 mL of 6 N HCl. [^{3}H]-mevalonic acid (0.015 µCi) was then added and the mixture was incubated for 20 min to allow for lactonization of mevalonic acid. Isolation of the product was achieved by TLC on silica gel "G" in benzene/acetone (1:1, vol/vol). The silica containing mevalonolactone was visualized against normal light, and scraped off into a counting vial, and radioactivity determined in a beta scintillation counter. Corrections for losses were accounted for by the tritium-mevalonate internal standard. Results are expressed as pmoles of mevalonate formed per milligram of protein per minute.

RESULTS AND DISCUSSION

Limonene and PA, are terpene inhibitors of protein isoprenylation, which are synthesized in plants from geranyl pyrophos-

phate, a component of the mevalonate pathway (23). These compounds were demonstrated to inhibit growth and proliferation of tumors (13,17–23). Monoterpenes were demonstrated to suppress hepatic HMG-CoA reductase activity (37) and to inhibit cellular protein isoprenylation, specifically for the small molecular weight 21–28 kDa proteins (19,22,23). These findings are consistent with the observation that PA can inhibit the enzymatic action of protein/farnesylpyrophosphate transferase (38). As nonsterol isoprenoid intermediates are responsible for a posttranscriptional control of HMG-CoA reductase activity (26) at both translational and degradational levels (39), the impact of monoterpene treatment on CH metabolism was investigated in the colonic adenocarcinoma cell line SW480.

PA was found to increase the incorporation of ^{14}C-acetate into CH fourfold as compared to the FBS or LPDS controls (Fig. 1). While MVN treatment significantly ($P < 0.001$) inhibited acetate incorporation into CH 80% relative to the controls, the combination of PA and MVN treatment resulted in an increased incorporation which was similar to that with PA treatment alone. Thus, the presence of a strong HMG-CoA reductase competitive inhibitor did not block PA's action. Control studies showed the same effects at 1.5 and 3 h of treatment, even when the specific activity was diluted to one-third of the original values used in this study (data not shown).

To investigate whether the observed changes in ^{14}C-acetate label incorporation into CH as a result of PA treatment were resulting in actual changes in steady-state levels of CH, an enzymatically based colorimetric assay system was employed to measure CH content in treated cultures. Unlike the radioactive precursor assays, cell monolayers and culture media were separately assessed. Results are depicted in Figure 2. As compared with either the FBS-treated control or the MVN-treated group, increases in the cellular fraction were only significant in the combination group, while both PA and combination treatments caused a significant accumulation of CH in the media fraction of the culture ($P < 0.01$ by analysis of variance; Bonferoni test for multiple comparisons). While these results are consistent with the findings of increased CH production seen using an acetate precursor, unfortunately, we cannot say with certainty that residual PA in the media or the cells did not have an effect on the enzymes or reactions used to drive the colorimetric reaction. Results from the MVN group indicated that 24-h treatment did not result in an overall net change in total CH in either cell or media fractions of

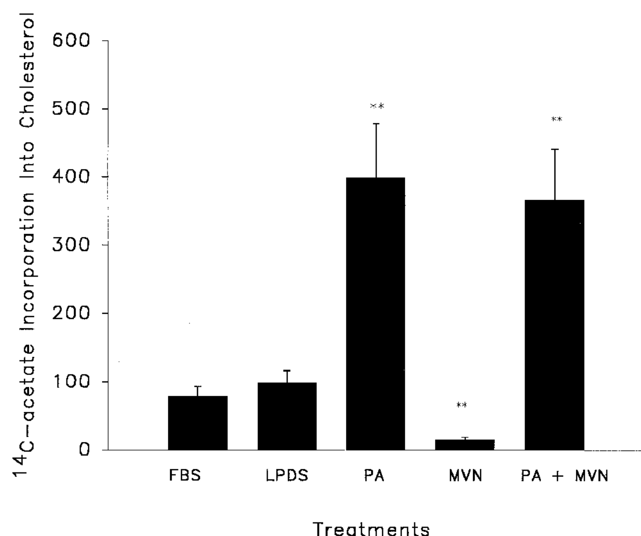

FIG. 1. Effects of mevinolin (MVN) and perillyl alcohol (PA) treatments upon the incorporation of ^{14}C-acetate into cholesterol. Cells were grown in minimum essential medium (MEM) + 10% fetal bovine serum (FBS) to 80% confluency and incubated with ^{14}C-acetate. Nonsaponifiable lipids were extracted and subjected to thin-layer chromatography as indicated in the Materials and Methods section. Endogenous cholesterol synthesis, as estimated by nanomoles of acetate-incorporated per mg cellular protein, was determined per sample. Values were normalized to the lipoprotein-deficient serum (LPDS) control within each experiment. Results of challenge for cells treated in FBS, LPDS, FBS plus PA 1 mM, FBS plus MVN (2 μM), and FBS plus MVN + PA, are expressed as percentage of LPDS control and depicted as bar graphs, where the filled area represents the mean of three different experiments carried out in triplicate. Error bars represent the standard deviation of the mean. Significance of findings was evaluated by general linear model analysis of variance comparing treatments with the LPDS control. **Significantly different from FBS and LPDS groups ($P < 0.001$).

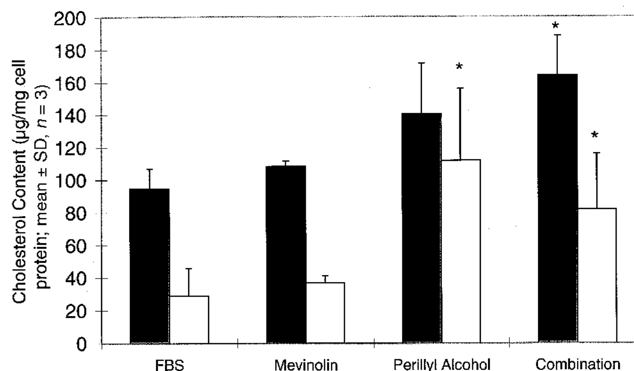

FIG. 2. Effects of MVN and the monoterpene PA upon total cholesterol levels in SW480 cells. Cells were seeded, grown, treated, and harvested as in the cholesterol synthesis assay described in the Methods section, except that no radioactive precursor was used, and cells and media were assessed separately. Treatments depicted under the bars were FBS/MEM (basal medium for all subsequent groups), 2 μM MVN, 1 mM PA, and a combination of the two treatments. Media (open bars) and cellular (solid bars) samples were saponified and extracted, as described in the Materials and Methods section. One-half of the extract was brought up in (200 μL) absolute ethanol, and 25–50 μL of this was used to determine actual cholesterol levels in the colorimetric assay. This assay is based on three enzymatic reactions: (i) cholesterol esters are converted to cholesterol by the action of cholesterol esterase; (ii) cholesterol is converted to a cholesterol oxide by cholesterol oxidase, a reaction which generates peroxide; and (iii) the peroxide is then coupled to the chromagen 4-aminoantipyrine and *p*-hydroxybenzenesulfonate by a peroxidase causing a color change measurable at 490 nm. A standard curve was generated from standards dissolved in absolute ethanol. Reactions occurred in a 96-well plate, and samples were read in an ELISA plate reader at 490 nM. Differences between groups (* = $P < 0.01$ vs. control and MVN groups) were determined by analysis of variance as described in the Materials and Methods section. See Figure 1 for abbreviations.

the culture. Thus the cells were either able to overcome the CH biosynthetic block imposed by MVN at a 2 μM concentration to generate their required sterols, or they decreased sterol usage to preserve sterol stores. This was consistent with the continued growth of the cells, albeit at a 50% reduced rate (12), and the dramatic compensatory increase in reductase activity generated by the cells to overcome the MVN imposed blockage of the pathway (26). Indeed, the disparity between results with MVN-treated cells between the ^{14}C-acetate incorporation assay (strong inhibition of synthesis) and the colorimetric assay (no change in content) illustrates pointedly the disadvantage of using either method alone. Presumably part of the reason total CH levels do not drop in 24-h treatment with MVN is a decrease in sterol utilization, particularly to meet cellular growth needs. This level of MVN treatment permits enough CH to be made to support a reduced growth rate (12). Thus the colorimetric results, which indicated no change in CH, taken alone, would imply there were no differences in CH metabolism as a result of MVN treatment. However, the results of ^{14}C-acetate incorporation assays in MVN-treated cells indicate that CH biosynthesis was greatly reduced by the drug. Yet total CH levels remain the same. When both sets of results are evaluated together, it is suggested that MVN treatment, which was ultimately compensated for by the cells, results in a dramatic upregulation of at least the early steps of the pathway.

Gas chromatography was employed to evaluate the content of the TLC spot where C_{27}-sterols from cells in the various treatment groups migrate (Fig. 3). Chloroform-extracted sterols from the TLC scrapings were derivatized to benzoyl esters and run using a SE-30 column. The major peak constituting >90% of the injected lipid corresponded to cholesteryl-ester. Depicted is a representative chromatograph of sterols from the TLC spot derived from PA-treated cells. Minor peaks included desmosterol and lathosterol, and these were never greater than 6 and 1.9%, respectively, of the CH peak area.

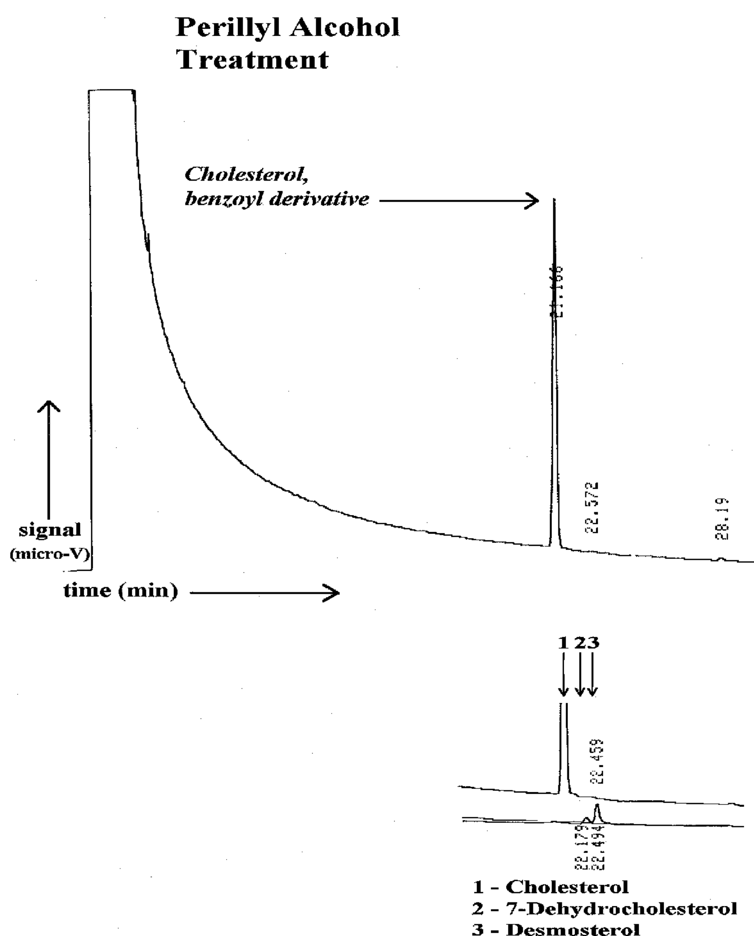

FIG. 3. Gas chromatography of PA treated cells. SW480 cells were treated with an FBS control or with 1 mM PA. Lipids were extracted, saponified, and unsaponifiable lipids were separated by thin-layer chromatography along with appropriate standards. Lipids were visualized, scraped, and derivatized for analysis by gas chromatography as described in the Materials and Methods section. The retention times of various sterol-ester derivatized standard compounds (depicted in the inset below the chromatograph) were as follows: cholesterol, 21.2 min; 7-dehydrocholesterol, 22.2 min; desmosterol, 22.5 min; and (not depicted) lathosterol, 23.7 min. Chromatograph of sterol spot from PA-treated cells is depicted; this was virtually identical to that from FBS-treated cells (not shown). See Figure 1 for abbreviations.

To determine if the PA-mediated enhancement of sterol synthesis could affect expression of sterol-controlled proteins, the activity of LDL receptors was assessed in treated cells by measuring ^{125}I-LDL binding and internalization. LDL-R activity is reported as ng ^{125}I-LDL bound per mg cell protein, and one representative assay is depicted in Table 1. The major count found in this assay represents the LDL that is internalized by the cells which has not yet been degraded. In normal cells this will typically be 80–90% of the total cell-associated radioactivity. Because the same cell line under the same treatment, when measured on different days, can vary by as much as tenfold (31), we concentrated on the relative differences observed between treatment groups within distinct assays. All findings were statistically significant by $P <$ 0.001 unless otherwise indicated. Under conditions where synthesis of C_{27}-sterols was enhanced fourfold, LDL receptor activity was markedly reduced (See Table 1), a finding consistent with the sterol-repressible nature of the gene for this protein. As the enhancement of CH synthesis by PA is unaffected by MVN, the ability of this inhibitor to ameliorate the effects of PA pretreatment upon LDL-R activity was evaluated. MVN, which usually causes an increase in LDL-R activity, had only a slight effect upon LDL-R expression in PA-treated cells, bringing it from 50 to 80% of the control value. Treatment with LPDS-supplemented media, which enhanced LDL-R activity threefold relative to the FBS controls, provided a basis to determine if removal of exogenous sterols would alter the ability of PA to affect LDL-R expression. The LDL-R activity of cells cultured in PA/LPDS was reduced to 47% of the LPDS-treated cells, indicating the ability of PA to cause sterol-mediated suppression in even sterol-poor environments.

At this point the data suggested that PA treatment caused an increase in CH derived from acetate. The inability of MVN to block this increase indicated PA could be acting at a later step than HMG-CoA reductase, the primary control point of the pathway (26). To test this hypothesis, the direct product of this enzyme, mevalonate, was added in radiolabeled form to determine if increases in incorporation of ^{14}C-acetate into CH, as compared to untreated cells, was due to enzymes subsequent to mevalonate production in the CH biosynthetic pathway. However, this was not found to be the case; when ^{14}C-mevalonate was added as a label (Fig. 4), sterol synthesis from this metabolite was inhibited by 65% under PA treatment, as compared to the FBS control. A combination of MVN with PA similarly resulted in a decreased synthesis relative to the controls to an extent that was even greater than PA treatment alone.

We hypothesized that an increase in endogenous mevalonate synthesis may reflect as decreases in ^{14}C-mevalonate incorporation into CH or isoprenylated proteins. An analogy for this mechanism of action can be found in the effects of MVN on protein isoprenylatation where the drug, which acts by depleting endogenous mevalonate, results in increased incorporation of ^{14}C-mevalonate into isoprenylated proteins (19). To test this hypothesis, the effects of these inhibitors alone and in combination upon protein isoprenylation in the

TABLE 1
Representative Experiment Measuring Surface-Bound and Internalized LDL in SW480 Cells

	LDL (ng/mg cell protein)			
	Surface-bound[a]		Internalized	
Treatments[b]	Mean[c]	SD	Mean	SD
FBS	1.99	0.30	14.9	1.5
PA/FBS	0.98	0.31	8.5	0.7
PA + mevinolin/FBS	1.07	0.14	11.9	1.4
LPDS	4.42	0.27	46.9	2.1
PA/LPDS	2.14	0.73	23.8	1.7

[a]Cells were evaluated as described in the Materials and Methods section for their ability to bind (surface activity) and internalize ^{125}I-human low density lipoprotein (LDL). PA, perillyl alcohol.
[b]SW480 cells were treated for 24 h with the basal media supplemented with fetal bovine serum (FBS) and various agents as depicted, unless lipoprotein-deficient serum (LPDS) was used as basal media and indicated in the group designation.
[c]Activity is reported as ng of radiolabeled LDL per mg cellular protein (quadruplicate samples). All groups were statistically different from the respective basal media controls by analysis of variance ($P < 0.001$; Bonferoni test for multiple comparisons).

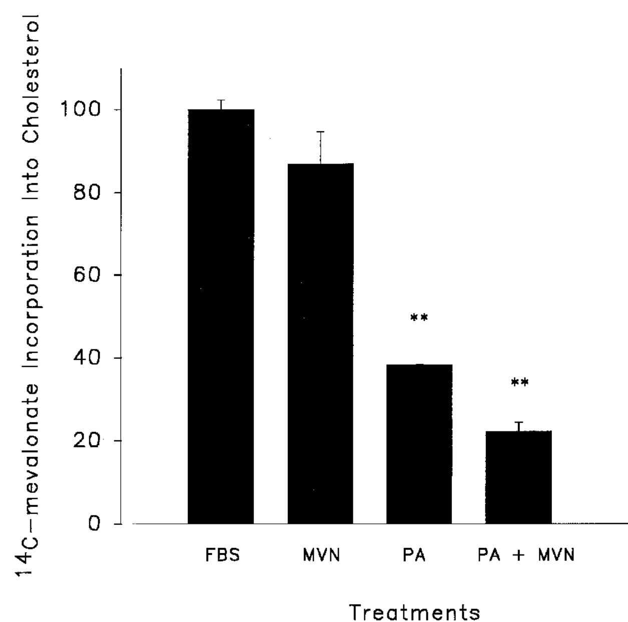

FIG. 4. Effects of MVN and PA treatments upon the incorporation of ^{14}C-mevalonate into cholesterol. Cells were grown in MEM + 10% FBS to 80% confluency and incubated with ^{14}C-mevalonate. Nonsaponifiable lipids were extracted and subjected to thin-layer chromatography as indicated in the Materials and Methods section. Endogenous cholesterol synthesis, as estimated by nanomoles of mevalonate incorporated per mg cellular protein, was determined per sample. Values were normalized to the FBS control within each experiment. Treatments in MEM + 10% FBS comprised: (i) FBS alone, (ii) MVN (2 μM), (iii) PA 1 mM, and (iv) MVN + PA. Results are expressed as percentage of FBS control and depicted as bar graphs, where the filled area represents the mean of two different experiments carried out in triplicate. Error bars represent the standard deviation of the mean. Significance of findings was evaluated by general linear model analysis of variance comparing treatments with the FBS control. **Significantly different from LPDS control ($P < 0.001$). See Figure 1 for abbreviations.

adenocarcinoma cell line SW480 were evaluated. Protein isoprenylation was assessed by measuring the incorporation of ^{14}C-mevalonate into proteins as described in the Materials and Methods section. Figure 5 shows an autoradiogram of ^{14}C-mevalonate-labeled proteins under the following treatments: FBS, 1 mM PA, 2 µM MVN, and PA + MVN. Densitometric analysis of ^{14}C-mevalonate incorporation into the 21–28 kDa proteins showed a 25% decrease in label incorporation in 1 mM PA-treated cells as compared to the FBS control (lane 3 vs. lane 1). In contrast, 2 µM MVN treatment resulted in a 2.2-fold increase in labeling (lane 2 vs. lane 1). Treatment with MVN left a pool of unisoprenylated proteins which resulted in incorporated label when chased with 15 µCi/mL of ^{14}C-mevalonate in the presence of cycloheximide at 20 µg/mL, as compared to untreated cells (lane 6 vs. lane

TREATMENT	LANES							
	1	2	3	4	5	6	7	8
MVN 2 µM	-	+	-	+	-	+	-	+
PA 1 mM	-	-	+	+	-	-	+	+
CHX 20 µg/	-	-	-	-	+	+	+	+

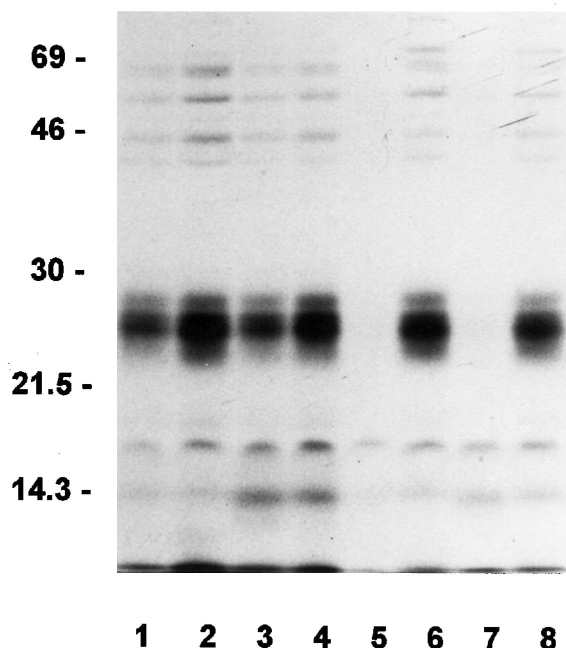

FIG. 5. Inhibition of protein isoprenylation in SW480 under MVN and PA treatments. SW480 cells were seeded at a density of 5×10^5/35 mm^2 dish and cultured for 2 d in MEM + 10% FBS. Cells were then subjected to the following treatments and divided into two groups. Group A [to which no cycloheximide (CHX) was added] was labeled with 2 µCi of ^{14}C-mevalonate with (i) FBS, (ii) MVN (2 µM), (iii) PA (1 mM), and (iv) PA + MVN. In Group B, cells were treated as above with no label for 24 h after which they were labeled with 15 µCi of ^{14}C-mevalonate in the presence of CHX at 20 µg/mL. Cells were harvested after trypsin treatment, centrifuged, and washed twice with phosphate buffered saline, and suspended in electrophoresis buffer in the presence of 2 M urea. Equal aliquots of protein from whole cell extracts were then analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis in 12% polyacrylamide gels, stained, and enhanced. Incorporation of label into 21–28 kDa proteins was analyzed by densitometry. Results are depicted in lanes (1,5) FBS; (2,6) MVN; (3,7) PA; (4,8) PA + MVN. See Figure 1 for other abbreviations.

5, respectively). PA at 1 mM did not result in incorporation of label in the presence of cycloheximide (see lane 7). Concurrent addition of PA and MVN to cells with cycloheximide further decreased the label by ≈20% as compared to its respective MVN treated group (Fig. 2, lane 8 vs. lane 6), suggesting that PA inhibited protein isoprenylation at a step in the pathway subsequent to mevalonate synthesis. In fact, PA was suggested to be an inhibitor of the enzymatic action of protein/farnesylpyrophosphate transferase (38). Finally, as studies indicated that PA treatment can cause decreased protein expression for such isoprenylated proteins as Ras, the authors note that small decreases in ^{14}C-mevalonate incorporation into proteins may be reflective of a more generalized effect on protein synthesis (20,29).

Together, these results suggest that terpenes affect CH synthesis at an early step in the pathway *via* a mechanism of action that is not affected by HMG-CoA reductase competitive inhibition. This effect was also observed in the murine colonic carcinoma cell line CT-26 (13). This decrease in labeled mevalonate incorporation into CH is consistent with (i) a dilution by endogenous mevalonate diverted from isoprenoid synthesis or (ii) an actual endogenous enhancement of mevalonate production caused by this terpene. Because increased incorporation of ^{14}C-acetate into CH is not a direct measurement of CH synthesis, as differences in intracellular acetate uptake, acetate pools, or activation of acetate to acetyl-CoA can occur, rates of sterol synthesis based on this assay could be regarded as estimates only. However, preliminary studies showed that the action of PA upon sterol synthesis was not blocked by MVN when ^{14}C-octanoate was utilized as a source of cytosolic acetyl CoA derived from the rapid intramitochondrial oxidation of this medium-chain fatty acid (data not shown). Nevertheless, we used ^{14}C-acetate and not ^{14}C-octanoate for our experiments, because intestinal cells preferentially use this substrate as a source of acetyl CoA to synthesize CH (40). While the possibility that this alcohol may affect membrane permeability cannot be dismissed, PA-treated cells were viable at 1 mM concentration for at least 4 d (data not shown), and all treatments received equal amounts of ethanol to control for osmotic differences between groups.

Despite the procedures employed to determine the putative identity of the radioactive material generated by PA-treated cells from a ^{14}C acetate precursor, some doubts remained as to whether this apparent enhancement of CH synthesis could be the result of a change in acetate pool sizes. If a treatment causes the general cellular levels of acetate in a cell to decrease, this would cause a nonspecific increase in the specific activity of ^{14}C-acetate label within the cells, subsequently resulting in apparent increases in all compounds that draw substrate from the affected acetate pool. As this type of artifact is nonspecific, a good means of detecting it is to assess the incorporation of acetate label into other compounds, to determine if there is a broadly based enhancement of apparent synthesis. Toward this end, SW480 cells were treated with MVN and PA, labeled with ^{14}C-acetate, and harvested for determination of CH synthesis according to the

standard methodology. Samples were then acidified with HCl to protonate-charged lipids, and reextracted. The free fatty acids and phospholipids in the resultant extracts were isolated and identified by TLC, and counted to determine the extent of ^{14}C-acetate incorporation. Results depicted in Figure 6 indicated that these three lipid classes, CH, free fatty acids and phospholipids, incorporated ^{14}C-acetate in a manner consistent with specific effects of the treatments. Nonspecific changes in whole acetate pool sizes were not indicated.

Although terpenes were reported to suppress hepatic HMG-CoA reductase activity (37) and decrease CH synthesis from ^{14}C-mevalonate in MVN-pretreated NIH 3T3 cells (19), this may not be the case for all tumor cells. The fact that PA treatment caused increased incorporation of ^{14}C-acetate into CH and decreased incorporation of ^{14}C-mevalonate implies that PA may be affecting as early a step as mevalonate synthesis, most probably increasing the activity of HMG-CoA reductase. Such stimulation could yield a buildup in intracellular CH. It is known that nonsterol derivatives of mevalonate affect HMG-CoA reductase activity post-transcriptionally (26).

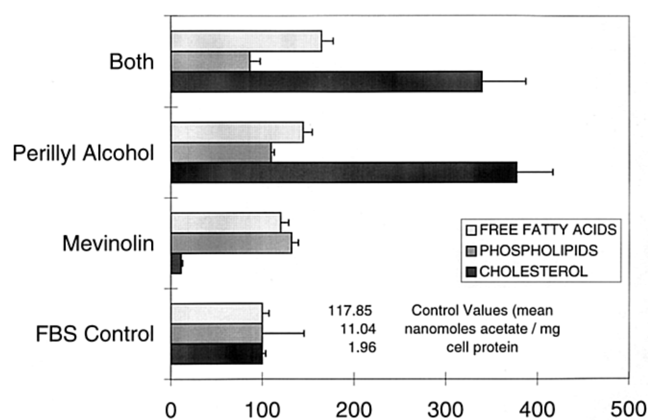

FIG. 6. Effects of MVN and the monoterpene PA upon total lipogenesis in SW480 cells. Cells were cultured and treated and labeled with 2.5–5 µCi/mL of [1-^{14}C]-acetate (51 mCi/mmole; New England Nuclear, Boston, MA), as described in the Materials and Methods section for cholesterol synthesis experiments. Cells were treated with FBS/MEM (basal medium for all subsequent groups), 2 µM MVN, 1 mM PA, and a combination of the two treatments. After labeling was complete (24 h), cells were harvested as described in the Materials and Methods section for whole culture assessment of cholesterol synthesis, with overnight saponification @ 70°C. After extraction of cholesterol under alkaline conditions with petroleum ether, the samples were acidified with 500 µL of concentrated HCl, vortexed, and incubated for 30 min at 70°C to maximize protonization of COOH groups. The samples were extracted twice with 4 mL of petroleum ether, and the ether evaporated. Samples were then brought up in 200 µL ether, and 100 µL of this was spotted on silica G thin-layer chromatography plates. These were run in hexane/diethyl ether/HOAc (70:30:1), allowed to dry, and incubated in an iodine chamber to visualize lipids. Standards were: for phospholipids, phosphatidylcholine (R_f 0), and for free fatty acids, oleic acid (R_f 0.39). Spots corresponding to the standards were marked, iodine was allowed to dissociate, and spots were scraped and counted in scintilant. Results are reported as nanomoles of acetate incorporated into lipid-type per mg cell protein, normalized to the FBS control group values within each group which were: free fatty acids, phospholipids, cholesterol. See Figure 1 for abbreviations.

Studies indicate that the mevalonate-derived mediator required for the accelerated degradation of HMG-CoA reductase enzyme is derived from farnesyl diphosphate and/or squalene (41). Maximal degradation requires synergistic action of both sterols and this mediator in the endoplasmic reticulum. A digitonin-permeabilized cell system was then utilized to identify farnesol as the nonsterol derivative that can specifically initiate and promote this degradation (42). Because PA is derived from the farnesol precursor geraniol, and was recently shown to inhibit farnesyl pyrophosphate transferase (38), we hypothesized that this terpene may act in a fashion similar to that of farnesyl pyrophosphate, by sending regulatory signals to HMG-CoA reductase, thereby inhibiting its degradation. Alternatively, PA treatment could stimulate an HMG-CoA reductase isoform that expresses different enzymatic responses to certain mevalonate-derived nonsterol isoprenoids. This enzyme could originate either from different genes, as occurs in yeast (43), or from different HMG-CoA reductase transcripts, whose translation could be regulated by a mevalonate-derived substance (26), as occurs in eukaryotic cells. Thus, a second transcript of HMG-CoA reductase could be predominant in neoplastic cells, as a result of increased demands of mevalonate-derived products in cancer cells.

For these reasons, and to investigate whether the ability of MVN to inhibit HMG-CoA reductase in the presence of PA is abrogated, the impact of PA upon HMG-CoA reductase was examined in washed microsomal preparations from pretreated SW480 cells. In this assay system, inhibitors such as MVN, which bind HMG-CoA reductase protein reversibly, are washed free of the reductase in the microsomal preparation. For this reason, groups treated with strong HMG-CoA reductase inhibitors, which cause strong compensatory increases in HMG-CoA reductase protein synthesis to overcome the effects of the inhibitor, increased activity relative to untreated control groups. Results depicted in Figure 7 indicate that MVN pretreatment caused a 12.7 (±2.4)-fold compensatory increase in microsomal HMG-CoA reductase activity, consistent with this concept. Unexpectedly, PA did not stimulate activity of this enzyme. Cells treated with both PA and MVN showed a 5.3 (±0.03)-fold compensatory increase in activity relative to the control group, but this increase was only 42% of that seen with MVN alone. It is suggested that this attenuation occurs because of the nature of the compensatory increase in reductase activity seen as the result of MVN treatment. This compensation results from the increased cellular need for all products of mevalonate metabolism—of which sterols are the bulk product. If the sterol availability in PA-treated cells is sufficient to downregulate sterol-controlled genes, then the ability of MVN to cause an upregulation in HMG-CoA reductase protein expression (measured as an increase in activity) would be attenuated. This is precisely what the "internalized" ^{125}I-LDL receptor activity data suggest (Table 1), where the PA-treated group demonstrates a rough decrease in receptor activity to 50% of the FBS control, while, in the presence of both MVN and PA, there is only a decrease to 80% of the FBS control. This is

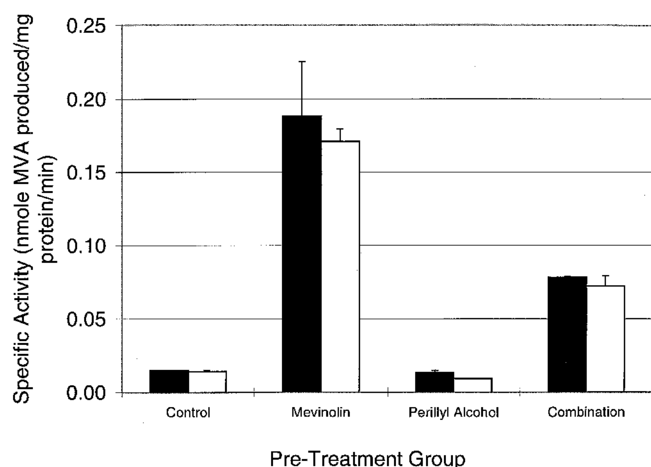

FIG. 7. Effects of pretreatment of SW480 cells with MVN and the monoterpene PA on 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity in washed microsomal preparations. Cells were grown to 70–80% confluence in 150 mm dishes. Cells were treated for 24 h with FBS/MEM (basal medium for all subsequent groups), 2 μM MVN, 1 mM PA, and a combination of the two treatments. Cells were harvested for preparation of microsomes, and samples were evaluated in the HMG-CoA reductase activity assay as described in the Materials and Methods section. Results are reported by listed treatment group as mean (± SD) specific activity (nmoles mevalonate formed/mg protein/min) after 60 min (solid bars) and 120 min (open bars) of incubation with substrate. MVA, mevalonic acid.For other abbreviations see Figure 1.

consistent with the increases in CH seen by CH synthesis from ^{14}C-acetate and in the colorimetric assay. Finally, when 12 nM MVN was tested in extracts from control and PA-treated cells, HMG-CoA reductase activity was similarly inhibited, indicating that the reductase protein did not aquire, as the result of cellular PA exposure, a resistance to effects of MVN (data not shown).

It is concluded that while the mechanisms for its action are unknown, PA increases sterol biosynthesis. Primarily, this occurs *via* a step sensitive to discrimination with ^{14}C-acetate precursor labeling but not with ^{14}C-mevalonate precursor labeling. Within the known paradigm for CH biosynthesis regulation, this step may involve: (i) Increased enzyme activity at an early step in the pathway above mevalonate production, such as HMG-CoA synthase or other earlier enzymes, causing an enhancement of endogenous mevalonate synthesis that leads to an overall increase in sterol production as measured by a labeled acetate precursor, or (ii) nonspecific effects on acetate pools. However, the latter possibility seems unlikely because synthesis of other lipids from ^{14}C-acetate was not affected by PA treatment, and this terpene caused an increase in ^{14}C-acetate incorporation into sterols when a mitochondrial source of ^{14}C-acetate was used, such as ^{14}C-octanoate (data not shown). In addition, increased culture CH content upon PA treatment was also verified using an enzymatic colorimetric assay to measure CH, and the decreases in LDL receptor activity caused by PA treatment were consistent with sterol repression. It is attractive therefore to suggest that PA, due to its structural analogy to isoprenes of the CH biosynthetic pathway such as geraniol (23), may somehow enter the CH biosynthetic pathway, possibly by a salvage pathway similar to that described by Crick *et al.* (44). To explain labeling increases which result from ^{14}C-acetate vs. ^{14}C-mevalonate precursors, we suggest that the PA-derived isoprenoids incorporate an acetate-radiolabeled carbon as part of the process (i) of either entering the pathway or (ii) at a latter step as sterol products, in either case by unknown mechanisms which do not involve mevalonate. An enhancement of the CH biosynthetic pathway by PA which cannot be detected through mevalonate labeling would be consistent with earlier observations in which PA was found to cause decreased incorporation of ^{14}C-mevalonate into isoprenylated proteins (19), ubiquinone, and CH (24). However, the interpretation of these prior studies using a labeled mevalonate precursor (that PA treatment was inhibitory of the studied processes) may be in question. Understanding this mechanism may enable eventual therapeutic exploitation of altered pathways for CH metabolism in certain tumors.

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Biosynthesis of *R*-(+)-Octane-1,3-diol. Crucial Role of β-Oxidation in the Enantioselective Generation of 1,3-Diols in Stored Apples

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ABSTRACT: The biosynthesis of *R*-octane-1,3-diol and *R*-5(*Z*)-octene-1,3-diol, two natural antimicrobial agents in apples and pears, was investigated in stored apples after application of [9,10,12,13-^{3}H]linoleic acid, [9,10,12,13,15,16-^{3}H]linolenic acid, [1-^{14}C]linoleic acid, [U-^{14}C]oleic acid, lipoxygenase-derived metabolites of [9,10,12,13-^{3}H]linoleic acid, $^{13}C_{18}$-labeled linoleic acid hydroperoxides, and ^{2}H-labeled octanol derivatives. Analysis of the products and quantification of incorporation and labeling pattern were achieved by high-performance liquid chromatography-radiodetection, capillary gas chromatography (GC)-isotope ratio mass spectrometry, and GC-mass spectrometry analysis. Almost all the applied precursors were partly transformed into *R*-octane-1,3-diol. Linoleic acid derivatives, still containing the 12,13 *cis* double bond, and octanol derivatives oxy-functionalized at carbon 3 were the most efficient precursors of the 1,3-diol. The data imply that *R*-octane-1,3-diol is generated in stored apples in the course of the β-oxidation from *R*-3-hydroxy-octanoyl-SCoA originating from 2-*cis*-octenoyl-SCoA by enoyl-CoA hydratase. In an analogous fashion, *R*-5(*Z*)-octene-1,3-diol is formed from the unsaturated intermediate.

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Several apple cultivars such as Peau de Chien, Douce Moen, and Rheinischer Bohnapfel accumulate high concentrations of the antimicrobial β-glycols *R*-octane-1,3-diol and its unsaturated analog *R*-5(*Z*)-octene-1,3-diol, as well as their glucosylated forms during storage (1–3). The diols are effective in controlling microorganisms associated with infections in humans and animals (4). Studies indicated that inhibition of amino acid transport is the primary antimicrobial effect of the β-glycols (5). The diols are harmless to humans at concentrations exhibiting antimicrobial effects. In humans, *R*-octane-1,3-diol is completely resorbed and metabolized as previously observed for butane-1,3-diol (6). Due to their chemical structures, both diols are considered to be intermediates of fatty acid metabolism, and their biosynthesis may be explained by at least three possible pathways: (i) generation *via* fatty acid synthesis (*de novo*) (1), (ii) a catabolic route of formation (β-oxidation) (7), or (iii) a lipoxygenase-like reaction (7). Recently, we investigated the biogenesis of *R*-octane-1,3-diol and its glucosylated form by administering [1-^{14}C]hexanoic acid, [1-^{14}C]octanoic acid, and [U-^{14}C]linoleic acid into intact ripe apple fruits (8). The study presented evidence that both *R*-octane-1,3-diol and its glucoside are derived primarily from linoleic acid. In the present work, we demonstrate the crucial role of β-oxidation in the enantioselective generation of 1,3-diols in stored apples.

MATERIALS AND METHODS

Materials. Linoleic acid and soybean lipoxygenase were purchased from Sigma (St. Louis, MO); linoleic acid-$^{13}C_{18}$ from Isotec (Emmerich, Germany); [1-^{14}C]linoleic acid (58 mCi/mmol) from DuPont (Boston, MA); [U-^{14}C]oleic acid (768 mCi/mmol) from DuPont; [9,10,12,13-^{3}H]linoleic acid (60 Ci/mmol) from Biotrend (Cologne, Germany); [9,10,12,13,15,16]linolenic acid (120 Ci/mmol) from Biotrend; silica gel thin-layer chromatography (TLC) plates (0.25 mm, 60 F254) from Merck (Darmstadt, Germany). Solvents or reagents were of analytical or high-performance liquid chromatography (HPLC) grade. Fresh, ripe apple fruits (cv. Douce Moen) were kindly provided by Pernod Ricard (Créteil, France).

Synthesis of $^{13}C_{18}$-labeled linoleic acid hydroperoxides. A solution consisting of 50 mg $^{13}C_{18}$-linoleic acid, 18 mg methylene blue, and 20 mL methanol was irradiated with a mercury lamp at −10°C for 24 h with 300 W. A similiar reaction was conducted with 1 g of unlabeled linoleic acid. The solvent was removed *in vacuo,* and the residue was dissolved in diethyl ether. The precipitate consisting of methylene blue was filtered off. TLC analysis showed a yield of 75% hydroperoxides, which were separated from linoleic acid by flash chromatography on silica gel with hexane/diethyl ether/acetic acid (85:15:1). The individual compounds 9-hydroperoxy-10(*E*), 12(*Z*)-octadecadienoic acid (9-HPOD), 10-hydroperoxy-8(*E*),

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Abbreviations: df, thickness of the film; EIMS, electron impact mass spectrometry; ELSD, evaporative light-scattering detector; GC, capillary gas chromatography; GC–IRMS, capillary gas chromatography–isotope ratio mass spectrometry; GC–MS, capillary gas chromatography–mass spectrometry; HODE, hydroxy-octadecadienoic acid; HPLC, high-performance liquid chromatography; HPOD, hydroperoxy-octadecadienoic acid; KODE, keto-octadecadienoic acid; NMR, nuclear magnetic resonance spectroscopy; R_F, retention factor (TLC); R_i, retention index (GC); RP18, octadecyl reversed-phase; TLC, thin-layer chromatography; UV, ultraviolet; XAD, polystyrene resin.

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12(*Z*)-octadecadienoic acid (10-HPOD), 12-hydroperoxy-9(*Z*), 13(*E*)-octadecadienoic acid (12-HPOD), 13-hydroperoxy-9(*Z*), 12(*E*)-octadecadienoic acid (13-HPOD), and 13-hydroperoxy-9(*E*), 12(*E*)-octadecadienoic acid [13(*E,E*)-HPOD] were isolated by analytical normal-phase HPLC analysis according to Reference 9.

Synthesis of $^2H_2$-labeled octanol derivatives. 2,3-$^2H_2$-octenal: Lindlar catalyst (Pd/$CaCO_3$) (300 mg) was added to a solution of 1,1-diethoxy-2-octyne (12.5 mmol, 2.48 g) and pentane (75 mL). The flask was evacuated and closed with a balloon filled with H_2 or $^2H_2$. The suspension was stirred overnight. The catalyst was removed by filtration. (2,3-$^2H_2$)-1,1-Diethoxy-2-octene: yield: 86% by gas chromatography (GC) analysis. Retention index (R_i) 1448, $R_{i(\text{deuterated})}$ 1446. ^1H nuclear magnetic resonance (NMR) ($CDCl_3$, 250 MHz) δ 5.63 (1H, *m*, C-3*H*), 5.46 (1H, *m*, C-2*H*), 5.29 (1H, *dd*, *J* = 7.5 Hz, 1.0 Hz, C-1*H*), 3.57 (4H, *m*, OCH_2), 2.11 (2H, *m*, C-4H_2), (6H, *m*, C-5H_2, C-6H_2, C-7H_2), 0.89 (9H, *m*, C-8H_3, OCH_2CH_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 135.3 (C-3), 127.6 (C-2), 98.1 (C-1), 60.9 (OCH_2CH_3), 32.3 (C-6), 29.5 (C-5), 28.9 (C-4), 23.1 (C-7), 15.7 (OCH_2CH_3), 14.5 (C-8); electron impact mass spectrometry (EIMS) *m/z* (%) 85 (100), 155 (95), 57 (80), 129 (64), 103 (63), 62 (62), 109 (62), 83 (61), 75 (58), 55 (55), 73 (54), 47 (49), 69 (47), 101 (38), 111 (25), 156 (20), 171 (5), 199 (4) $[M-H]^+$, 200 (1) $[M]^+$; deuterated: 59 (100), 87 (97), 157 (95), 58 (78), 103 (76), 75 (76), 131 (72), 86 (70), 111 (68), 57 (63), 85 (55), 47 (58), 72 (50), 69 (49), 56 (45), 113 (27), 158 (25), 130 (17), 156 (13), 173 (5), 201 (4) $[M-H]^+$, 202 (1) $[M]^+$. 4-Toluenesulfonic acid (100 mg) and (2,3-$^2H_2$)-1,1-diethoxy-2-octene (10 mmol, 2.0 g) were dissolved in acetone (40 mL), and the mixture was stirred overnight (10). The solvent was removed *in vacuo,* and the product was purified by flash chromatography on silica gel with pentane/diethyl ether (8:2). Yield: 71% by GC analysis. R_i 1426, $R_{i(\text{deuterated})}$ 1423, retention factor (R_F) 0.39 (pentane/diethyl ether 8:2). ^1H NMR ($CDCl_3$, 250 MHz) δ 9.50 (1H, *d*, *J* = 8 Hz, C-1*H*), 6.85 (1H, *m*, C-3*H*), 6.11 (1H, *m*, C-2*H*), 2.33 (2H, *m*, C-4H_2), 1.25–1.4 (6H, *m*, C-5H_2, C-6H_2, C-7H_2), 0.89 (3H, *t*, *J* = 7 Hz, C-8H_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 194.5 (C-1), 159.5 (C-3), 133.4 (C-2), 33.1 (C-6), 31.7 (C-5), 27.9 (C-4), 22.8 (C-7), 14.3 (C-8) ; EIMS *m/z* (%) 70 (100), 83 (76), 55 (68), 57 (50), 67 (45), 84 (26), 97 (24), 82 (24), 93 (19), 79 (15), 111 (6), 108 (5), 125 (2) $[M-H]^+$; deuterated: 41 (100), 57 (85), 55 (75), 59 (68), 85 (60), 72 (57), 70 (57), 71 (53), 83 (30), 69 (27), 99 (18), 95 (9), 113 (5), 110 (4), 127 (1) $[M-H]^+$.

2,3-$^2H_2$-Octenol. Sodium borohydride (1.6 mmol, 60.5 mg) was carefully added to a solution containing (2,3-$^2H_2$)-2-octenal (4 mmol, 504 mg) and methanol (10 mL). The mixture was stirred overnight. After addition of 2 N HCl (5 mL), the solution was extracted (3×) with diethyl ether (20 mL). The combined organic layer was dried (Na_2SO_4) and was concentrated *in vacuo*. Purification by flash chromatography on silica gel with pentane/diethyl ether (2:1) afforded pure product. Yield: 83% by GC analysis. R_i 1615, $R_{i(\text{deuterated})}$ 1612, R_F 0.33 (pentane/diethyl ether, 2:1). ^1H NMR ($CDCl_3$, 250 MHz) δ 5.64 (2H, *m*, C-2*H*, C-3*H*), 4.08 (2H, *d*, *J* = 5.5 Hz, C-1H_2), 2.02 (2H, *m*, C-4H_2), 1.2–1.4 (6H, *m*, C-5H_2, C-6H_2, C-7H_2), 0.88 (3H, *t*, *J* = 7 Hz, C-8H_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 133.5 (C-2), 128.8 (C-2), 63.8 (C-1), 32.1 (C-6), 31.4 (C-5), 28.8 (C-4), 22.5 (C-7), 14.0 (C-8); EIMS *m/z* (%) 57 (100), 55 (60), 81 (59), 68 (50), 67 (45), 71 (24), 95 (20), 85 (15), 110 (8) $[M-H_2O]^+$, 99 (6), 128 (2) $[M]^+$; deuterated: 59 (100), 56 (67), 69 (64), 83 (53), 57 (50), 68 (47), 82 (31), 84 (23), 97 (14), 112 (7) $[M-H_2O]^+$, 130 (4) $[M]^+$.

5,6-$^2H_2$-Ethyl-3-oxooctanoate. Ethyl-3-oxo-5(*Z*)-octenoate (6.1 mmol, 1.12 g), synthesized according to Reference 2, was dissolved in degassed benzene (25 mL), and tris (triphenylphosphine)-chlororhodium (I) (0.07 mmol, 70 mg) was added (11). The flask was evacuated and closed with a balloon filled with H_2 or $^2H_2$. The suspension was stirred for several days until complete conversion was detected by TLC and GC. Yield: 98% by GC analysis. R_i 1859, $R_{i(\text{deuterated})}$ 1851, R_F 0.5 (pentane/diethyl ether, 9:1). ^1H NMR ($CDCl_3$, 250 MHz) δ 4.18 (2H, *q*, *J* = 7 Hz, OCH_2CH_3), 3.42 (2H, *s*, C-2H_2), 2.51 (2H, *t*, *J* = 7 Hz, C-4H_2), 1.28 (3H, *t*, *J* = 7 Hz, OCH_2CH_3), 1.1–1.65 (8H, *m*, C-5H_2, C-6H_2, C-7H_2), 0.87 (3H, *t*, *J* = 7 Hz, C-8H_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 202.0 (C-3), 167.2 (C-1), 61.2 (OCH_2CH_3), 49.2 (C-2), 42.8 (C-4), 30.6 (C-6), 22.7 (C-5), 22.2 (C-7), 13.8 (C-8), 13.9 (C-9); EIMS *m/z* (%) 43 (100), 99 (55), 84 (42), 71 (42), 88 (39), 130 (33), 69 (20), 55 (15), 115 (13), 97 (12), 87 (10), 56 (10), 102 (9), 143 (8), 144 (3), 168 (2), 186 (1) $[M]^+$; deuterated: 43 (100), 44 (90), 101 (75), 45 (65), 73 (33), 88 (31), 84 (30), 130 (24), 131 (20), 85 (17), 56 (15), 115 (15), 69 (14), 102 (12), 98 (12), 144 (9), 145 (3), 170 (2), 188 (1) $[M]^+$.

5,6-$^2H_2$-Ethyl-3-hydroxyoctanoate. Sodium borohydride (1.9 mmol, 73 mg) was carefully added to a solution of (5,6-$^2H_2$)-ethyl-3-oxooctanoate (1.6 mmol, 298 mg) and ethanol (10 mL). The mixture was stirred overnight. After addition of 2N HCl (5 mL), the solution was extracted (3×) with diethyl ether (20 mL). The combined organic layer was dried (Na_2SO_4) and was concentrated *in vacuo*. Purification by flash chromatography on silica gel with pentane/diethyl ether (1:1) afforded pure product. Yield: 43% by GC analysis. R_i 1887, $R_{i(\text{deuterated})}$ 1883, R_F 0.33 (pentane/diethyl ether, 1:1). ^1H NMR ($CDCl_3$, 250 MHz) δ 4.18 (2H, *q*, *J* = 7 Hz, OCH_2CH_3), 3.97 (1H, *m*, C-3*H*), 2.42 (2H, *m*, C-2H_2), 1.28 (3H, *t*, *J* = 7 Hz, OCH_2CH_3), 1.2–1.6 (8H, *m*, C-4H_2, C-5H_2, C-6H_2, C7H_2); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 173.6 (C-1), 68.3 (C-3), 60.3 (OCH_2CH_3), 41.5 (C-2), 36.8 (C-4), 31.9 (C-6), 25.4 (C-5), 22.8 (C-7), 14.2 (OCH_2CH_3), 13.9 (C-8). EIMS *m/z* (%) 43 (100), 117 (98), 71 (62), 44 (57), 55 (50), 89 (49), 88 (42), 45 (37), 56 (26), 99 (25), 83 (24), 60 (19), 125 (17), 75 (16), 101 (12), 97 (12), 96 (12), 141 (4), 143, (2), 170 (1) $[M-H_2O]^+$, 187 (1) $[M-1]^+$; deuterated: 43 (100), 117 (98), 71 (44), 89 (43), 44 (43), 45 (49), 88 (47), 101 (23), 58 (23), 56 (21), 84 (19), 70 (16), 75 (15), 99 (11), 83 (11), 61 (11), 103 (8), 127 (11), 143 (3), 145 (2), 172 (1) $[M-H_2O]^+$, 189 (1) $[M-1]^+$.

1,1-$^2H_2$-3-Oxooctanol. 4-Toluenesulfonic acid (100 mg),

ethyl 3-oxooctanoate (40 mmol, 7.5 g) (2), and ethyleneglycol (40 mmol, 2.5 g) were dissolved in benzene (25 mL) and refluxed until formed water was completely removed (12). The solution was concentrated *in vacuo*. Ethyl 2-pentyl-(1,3-dioxolan-2-yl)acetate: yield: 61% by GC analysis. R_i 2007, R_F 0.49 (pentane/diethyl ether, 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 4.17 (2H, *q*, *J* = 7 Hz, O$CH_2$$CH_3$), 3.95 (4H, *m*, O$CH_2$$CH_2$O), 2.62 (2H, *s*, C-2$H_2$), 1.81 (2H, *m*, C-4$H_2$), 1.28 (3H, *t*, *J* = 7 Hz, O$CH_2$$CH_3$), 1.1–1.5 (6H, *m*, C-5$H_2$, C-6$H_2$, C-7$H_2$), 0.89 (3H, *t*, *J* = 7 Hz, C-8H_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 170.0 (C-1), 109.9 (C-3), 65.5 (O$CH_2$$CH_2$O), 60.9 (O$CH_2$$CH_3$), 38.1 (C-4), 32.3 (C-6), 23.5 (C-7), 23.0 (C-5), 14.6 (O$CH_2$$CH_3$), 14.4 (C-8). EIMS *m/z* (%) 159 (100), 143 (95), 89 (31), 117 (30), 71 (28), 99 (19), 144 (17), 160 (17), 86 (16), 55 (10), 97 (6), 131 (3), 185 (2), 187 (1). A suspension consisting of $LiAlH_4$ or $LiAlD_4$ (1.8 mmol, 72 mg) and absolute diethyl ether (10 mL) was carefully added to a solution of ethyl 2-pentyl-(1,3-dioxolan-2-yl)acetate and absolute diethyl ether (10 mL). The suspension was stirred for 4 h at room temperature. After addition of 2 N HCl (5 mL), the solution was extracted (3×) with diethyl ether (20 mL). The combined organic layer was dried (Na_2SO_4) and was concentrated *in vacuo*. (2′,2′-$^2H_2$)-2′-Hydroxyethyl-2-pentyl-1,3-dioxolan: yield: 64% by GC analysis. R_i 2073, $R_{i(\text{deuterated})}$ 2070, R_F 0.38 (pentane/diethyl ether, 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 3.96 (4H, *m*, O$CH_2$$CH_2$O), 3.73 (2H, *m*, C-1$H_2$), 1.91 (2H, *t*, *J* = 5.5 Hz, C-2H_2), 1.62 (2H, *m*, C-4H_2), 1.2–1.45 (6H, *m*, C-5H_2, C-6H_2, C-7H_2), 0.88 (3H, *t*, *J* = 7 Hz, C-8CH_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 112.3 (C-3), 64.7 (O$CH_2$$CH_2$O), 58.8 (C-1), 38.0 (C-2), 37.0 (C-4), 32.0 (C-6), 23.4 (C-5), 22.5 (C-7), 13.9 (C-8). EIMS *m/z* (%) 117 (100), 143 (82), 99 (48), 43 (33), 71 (28), 73 (20), 55 (19), 87 (9), 144 (9), 118 (8), 115 (5); deuterated: 119 (100), 143 (78), 101 (75), 43 (62), 71 (55), 75 (46), 99 (39), 55 (37), 86 (35), 144 (21), 120 (19), 117 (12). A solution consisting of (2′,2′-$^2H_2$)-2′-hydroxyethyl-2-pentyl-1,3-dioxolan (2.8 mmol, 530 mg), acetone (25 mL), and pyridinium 4-toluenesulfonic acid (211 mg) was refluxed for 2 h (13). The solvent was removed *in vacuo,* and the residue was dissolved in diethyl ether (25 mL). The extract was washed successively with 5% HCl (25 mL), saturated $NaHCO_3$ solution (25 mL), and saturated NH_4Cl solution. Purification by flash chromatography on silica gel with pentane/diethyl ether (1:1) afforded pure product. Yield: 74% by GC analysis. R_i 1907, $R_{i(\text{deuterated})}$ 1903, R_F 0.34 (pentane/diethyl ether, 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 3.82 (2H, *m*, C-1H_2), 2.65 (2H, *t*, *J* = 5.5 Hz, C-2H_2), 2.42 (2H, *t*, *J* = 7 Hz, C-4H_2), 1.56 (2H, *m*, C-5H_2), 1.2–1.4 (4H, *m*, C-6H_2, C-7H_2), 0.87 (3H, *t*, *J* = 7 Hz, C-8H_3). ^{13}C NMR ($CDCl_3$, 63 MHz) δ 211.0 (C-3), 57.8 (C-1), 44.2 (C-2), 43.3 (C-4), 31.2 (C-6), 23.2 (C-5), 22.3 (C-7), 13.8 (C-8). EIMS *m/z* (%) 43 (100), 55 (73), 70 (71), 88 (61), 73 (58), 99 (55), 71 (50), 58 (27), 97 (17), 83 (15), 101 (11), 115 (5), 144 (2); deuterated: 72 (100), 75 (67), 43 (65), 90 (57), 99 (55), 57 (48), 58 (37), 55 (32), 47 (29), 85 (21), 103 (15), 117 (5), 146 (3).

4,5-$^2H_2$-3-Hydroxyoctanal. 2(*E*)-Hexenal (18 mmol, 1.8 g) was dissolved in degassed benzene (75 mL), and tris (triphenylphosphine)chlororhodium (I) (0.216 mmol, 200 mg) was added (14). The flask was evacuated and closed with a balloon filled with H_2 or $^2H_2$. The suspension was stirred for several days until complete conversion was detected by GC and TLC. Benzene and the catalyst were removed by flash chromatography on silica gel with pentane. Product was eluted with pentane/diethyl ether (9:1). (2,3-$^2H_2$)-Hexanal: yield: 53% by GC analysis. R_i 1074, $R_{i(\text{deuterated})}$ 1070, EIMS *m/z* (%) 56 (100), 44 (95), 57 (87), 72 (45), 55 (38), 45 (38), 82 (33), 67 (25), 58 (22), 71 (19), 100 (2) $[M]^+$, 99 (2); deuterated: 57 (100), 45 (95), 58 (67), 46 (60), 59 (53), 56 (35), 72 (34), 84 (17), 83 (17), 73 (15), 68 (12), 69 (10), 55 (10), 102 (2) $[M]^+$, 101 (2). A Grignard reagent prepared from Mg (8.8 mmol, 235 mg) and allylbromide (8.8 mmol, 1.07 g) in absolute diethyl ether (15 mL) was added to a solution of (2,3-$^2H_2$)-hexanal or hexanal (8.8 mmol, 862 mg) and diethyl ether (5 mL). The mixture was refluxed for 1 h. Saturated NH_4Cl solution was added, and the product was extracted with diethyl ether (25 mL) and successively washed with saturated $(NH_4)_2CO_3$ solution (25 mL) and brine (25 mL). Purification by flash chromatography on silica gel with pentane/diethyl ether (8:2) afforded pure product. (5,6-$^2H_2$)-4-Hydroxy-1-nonene: yield: 86% by GC analysis. R_i 1502, $R_{i(\text{deuterated})}$ 1499, R_F 0.18 (pentane/diethyl ether, 8:2). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.82 (1H, *m*, C-2H_2), 5.13 (1H, *d*, *J* = 4 Hz, *cis* C-1*H*), 5.10 (1H, *s*, *trans* C-1*H*), 3.63 (1H, *m*, C-4*H*), 2.28 (1H, *m*, C-3H_{2a}), 2.12 (1H, *m*, C-3H_{2b}), 1.2–1.5 (8H, *m*, C-5H_2, C-6H_2, C-7H_2, C-8H_2), 0.88 (3H, *t*, *J* = 7 Hz, C-9H_3); ^{13}C NMR ($CDCl_3$, 63 MHz) δ 135.3 (C-2), 118.4 (C-1), 71.1 (C-4), 42.3 (C-3), 37.2 (C-5), 32.3 (C-7), 25.8 (C-6), 23.0 (C-8), 14.4 (C-9). EIMS *m/z* (%) 55 (100), 83 (95), 101 (35), 43 (35), 71 (21), 57 (20), 56 (13), 84 (11), 67 (6), 102 (4), 69 (4), 124 (1); deuterated: 85 (100), 56 (93), 103 (40), 71 (25), 45 (20), 59 (19), 46 (18), 104 (15), 57 (14), 69 (12), 68 (11), 73 (10), 54 (10), 86 (9), 102 (5), 101 (5), 82 (4), 126 (1). A solution consisting of (5,6-$^2H_2$)-4-hydroxy-1-nonene (1.4 mmol, 200 mg) and methanol (5 mL) was treated with O_3 at −60°C until the mixture remained blue (15). The solution was stirred for 10 min, purged with nitrogen, and dimethylsulfide (2 mmol) was added at −10°C. The mixture was stirred for 1 h at 0°C, then overnight at room temperature. Methanol was removed *in vacuo,* and the residue was dissolved with diethyl ether (20 mL) and was washed with brine (5 mL). Yield: 86% by GC analysis. R_i 1867, $R_{i(\text{deuterated})}$ 1862, R_F 0.64 (pentane/diethyl ether, 1:1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 9.84 (1H, *m*, C*H*O), 4.14 (1H, *m*, C-3H_2), 2.61 (2H, *m*, C-2H_2), 1.2–1.6 (8H, *m*, C-4H_2, C-5H_2, C-6H_2, C-7H_2), 0.87 (3H, *t*, *J* = 7 Hz, C-8H_3). ^{13}C NMR ($CDCl_3$, 63 MHz) δ 202.5 (C-1), 68.1 (C-3), 50.6 (C-2), 36.7 (C-4), 31.5 (C-6), 24.6 (C-5), 22.5 (C-7), 13.9 (C-8). EIMS *m/z* (%) 55 (100), 73 (94), 56 (89), 45 (82), 70 (67), 57 (61), 83 (57), 69 (35), 82 (22), 98 (19), 67 (19), 84 (13), 88 (10), 58 (10), 101 (8), 111 (2), 126 (1), 143 (1) $[M-1]^+$; deuterated: 45 (100), 57 (90), 73 (85), 56 (56), 58 (45), 71 (43), 55 (30), 85 (29), 72 (25), 84 (23), 70 (20), 59 (18), 83 (15), 100 (13), 69 (12), 86

(6), 99 (5), 89 (5), 101 (4), 103 (3), 113 (2), 128 (1), 145 (1) $[M - 1]^+$.

Synthesis of ^{3}H-labeled linoleic acid transformation products. An ethanolic solution (10 µL) of [9,10,12,13-^{3}H]linoleic acid (14.5 µCi) was injected into an apple, and the apple was worked up after 1 d of incubation as described in the *General work-up procedure* below. The diethyl ether extract was separated by reversed-phase HPLC analysis, and the four major radioactive peaks were collected. The individual fractions were diluted with the same amount of water and were subjected to RP18 solid-phase extraction (500 mg cartridge). The column was rinsed with water (3 mL), dried with nitrogen, and the compounds were eluted with 3 mL of diethyl ether. Fraction 1 consisted of a mixture of 13-keto-9(*Z*), 11(*E*)-octadecadienoic acid (13-KODE) and 9-keto-10(*E*), 12(*Z*)-octadecadienoic acid (9-KODE); fraction 2 contained a mixture of 13(*R*)-hydroxy-9(*Z*), 11(*E*)-octadecadienoic acid (13-HODE) and 9(*S*)-hydroxy-10(*E*), 12(*Z*)-octadecadienoic acid (9-HODE); fraction 3 was tentatively identified as a mixture of 11-hydroxy-12,13-epoxy-9-octadecenoic acid and 11-hydroxy-9,10-epoxy-12-octadecenoic acid; and fraction 4 was a mixture of the positional isomers of 9,10,13- and 9,12,13-trihydroxyoctadecenoic acid.

Application of substrates. Ethanolic solutions (up to 100 µL) of [1-^{14}C]linoleic acid (2.0 µCi), [1-^{14}C]oleic acid (2.0 µCi), [9,10,12,13-^{3}H]linoleic acid (15.7 µCi), [9,10,12,13,15,16-^{3}H]linolenic acid (23.0 µCi), $^{13}C_{18}$-linoleic acid (20 µg), unlabeled linoleic acid (20 µg), ^{3}H-labeled linoleic acid transformation products (0.25–0.47 µCi), $^{13}C_{18}$-9-HPOD (20 µg), $^{13}C_{18}$-10-HPOD (20 µg), $^{13}C_{18}$-12-HPOD (20 µg), $^{13}C_{18}$-13-HPOD (20 µg), $^{13}C_{18}$-13(*E,E*)-HPOD (20 µg), 2,3-$^2H_2$-2-octenol (5 mg), 2,3-$^2H_2$-2-octenal (5 mg), 5,6-$^2H_2$-ethyl-3-oxooctanoate (5 mg), 5,6-$^2H_2$-ethyl-3-hydroxyoctanoate (5 mg), 1,1-$^2H_2$-3-oxooctanol (5 mg), and 4,5-$^2H_2$-3-hydroxyoctanal (5 mg) were injected subepidermally with a syringe into ripe apples (*ca*. 40 g). Apples were kept up to 4 mon at 4°C connected to a hood.

General work-up procedure. Apples were cut into small pieces and homogenized with 100 mL of water. After centrifugation (4000 × *g*, 15 min), the solid residue was washed with 100 mL of water (3×). The supernatants were combined and passed through a conditioned polystyrene resin (XAD) column. After being rinsed with 500 mL of water, the column was eluted with 500 mL of diethyl ether and 750 mL of methanol. The extracts were concentrated and were analyzed by HPLC with ultraviolet (UV) and radiodetection, UV and evaporative light-scattering detection (ELSD), as well as GC–MS analysis and GC-isotope ratio mass spectrometry (IRMS). Solid residues were dried at 80°C for 24 h. Aliquots of liquid samples were added to 10 mL of scintillation cocktail (Emulsifier-Safe). Solid samples were combusted in a biological oxidizer. The formed $^{14}CO_2$ was absorbed in 12 mL of the scintillation cocktail, Oxysolve 400 (Zinsser, Frankfurt, Germany). Recoveries of ^{14}C as $^{14}CO_2$ from test combustions fortified with ^{14}C standards, immediately before combustion, were greater than 90%. All measurements were carried out by means of liquid scintillation counting using corrections for chemiluminescence.

GC–MS analysis. Analysis of the synthesized substrates and quantification of the labeling pattern of formed *R*-octane-1,3-diol was performed with a Fisons MD 800 Quadrupol mass spectrometer coupled to a Fisons GC 8000 with split injector (1:20) equipped with Fisons MassLab software (version 1.3). A J&W DB Wax 20 M fused-silica capillary column [30 m × 0.25 mm i.d.; thickness of the film (df) = 0.25 µm], which was programmed from 50°C for 3 min, then to 240°C at 4°C/min, was used with helium gas at a flow rate of 3 mL/min. The EIMS-operating parameters were: ionization voltage, 70 eV (electron impact ionization); ion source and interface temperature, 230 and 240°C, respectively. Chemical ionization (CI)–MS was performed with methane as reagent gas at 5 psi head pressure, source temperature 150°C and 70 eV.

On-line GC/combustion/IRMS (*GC–IRMS*). GC–IRMS analyses were performed on a Hewlett-Packard 5890 gas chromatograph connected to a Finnigan MAT delta S isotope mass spectrometer *via* a combustion interface. Isotope ratios were expressed as δ-values [‰] vs. the PDB standard ([^{13}C]/[^{12}C] isotope ratio = 0.0112372 for CO_2, yielded by the reaction of fossil $CaCO_3$ (Pee Dee Belemnite). The GC was equipped with a polyethylene glycol fused-silica capillary column (DB-Wax 20M 25 m × 0.32 mm i.d.; df 0.3 µm). Helium was used as carrier gas (14 psi head pressure). Samples were injected in the cool on-column mode. The following temperature program was applied: starting isothermal at 50°C for 3 min, then programmed to 240°C at 4°C/min, and finally held for 20 min.

HPLC analysis. Reversed-phase HPLC was carried out on an Eurospher 100 C-18 column (250 × 4 mm, 5 µm; Knauer, Berlin, Germany), using a flow rate of 1 mL/min, employing a Knauer HPLC pump MaxiStar coupled on-line to a Knauer multiwavelength UV/VIS detector (205, 234, or 254 nm) and an ELSD (SEDERE) at 40°C and 2.4 bar compressed air. Data acquisition was achieved using Eurochrom 2000 software (Knauer). Radio-HPLC was conducted using a Waters HPLC system equipped with a variable wavelength detector and a Canberra Packard A100 radioactivity detector with a 300 µL YSi scintillation tube. The following gradient was applied: solvent A (0.05% formic acid), solvent B (CH_3CN): 0–30 min, 5–80% B, 30–40 min, 80–100% B, 40–45 min, 100% B.

Normal-phase HPLC analysis was performed on an Eurospher Si 100 column (250 × 4.6 mm, 5 µm; Knauer), using a mixture of isohexane/2-propanol/acetic acid (98:2:0.5, by vol) at a flow rate of 1 mL/min.

NMR. ^{1}H and ^{13}C NMR spectra were recorded on a Bruker WM 250 spectrometer with $CDCl_3$ ^{1}H (δ $CDCl_3$ 7.27 ppm) ^{13}C (δ $CDCl_3$ 77.0 ppm) (Merck, Darmstadt, Germany) as solvent. NMR data of the nonlabeled compounds were determined.

RESULTS

Application of [1-^{14}C]linoleic acid and [9,10,12,13-^{3}H]-linoleic acid. [1-^{14}C]Linoleic acid and [9,10,12,13-^{3}H]-

TABLE 1
Time Course and Distribution of Recovered Radioactivity (expressed as percentage of applied ^{14}C) in Different Fractions After the Application of [1-^{14}C]Linoleic Acid to Apple Fruits[a]

	Incubation time (d)					
	0.5	1	2	4	9	64
XAD flowthrough	29.5	34.4	38.9	35.9	32.6	26.7
Diethyl ether extract	19.7	7.6	3.9	1.8	1.6	2.7
Methanol extract	1.9	1.9	2.1	1.8	1.0	1.2
Nonextractable residue	18.0	14.8	19.9	19.8	20.1	17.7
CO_2 (calculated)	30.9	41.3	35.2	40.7	44.7	51.7

[a]XAD, polystyrene resin.

linoleic acid were injected subepidermally into ripe apples, and the fruits were stored for up to 4 mon at 4°C. Apples were taken after defined periods and were subjected to solid-phase extraction. Tables 1 and 2 show the ^{14}C and ^{3}H recovery data, respectively, for the different fractions as a percentage of the applied radioactivity. After 0.5 d, *ca*. 31% of the applied ^{14}C is lost as $^{14}CO_2$, indicating a rapid degradation of linoleic acid by β-oxidation (Table 1). The further release of $^{14}CO_2$ is probably caused by the degradation of transformation products formed from linoleic acid, e.g., 13(*R*)-hydroxy- and 13-keto-9(*Z*), 11(*E*)-octadecadienoic acid (13-HODE and 13-KODE), 9(*S*)-hydroxy- and 9-keto 10(*E*), 12(*Z*)-octadecadienoic acid (9-HODE and 9-KODE), and the stereoisomers of the 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids were identified as major products in the diethyl ether extract after 0.5 d (data not shown). Already after 12 h, linoleic acid was completely degraded. The values for ^{14}C quantified in the methanol extract and the nonextractable residues remained stable at *ca*. 2 and 17% of the applied radioactivity, respectively (Table 1). The XAD flowthrough containing highly polar compounds, which do not adsorb to XAD, showed high amounts of radioactivity at days 2 and 4. ^{14}C-Acetate formed by β-oxidation accounts probably for the majority of this radioactivity. This result is consistent with the data obtained after the application of [9,10,12,13-^{3}H]linoleic acid (Table 2), as 40.3, 45.0, and 45.2% of applied ^{3}H were recovered in the XAD flowthrough at days 2, 4, and 9, respectively. The radioactivity in the diethyl ether extract gradually decreased during the incubation period of 129 d. In contrast, the content of ^{3}H gradually increased in the methanol extracts. In the diethyl ether extract obtained after 0.5 and 1 d 13- and 9-HODE (12:1), 13- and 9-KODE (4:1), and the stereoisomers of the 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids were identified as the major degradation products of [1-^{14}C]- and [9,10,12,13-^{3}H]linoleic acid. However, *R*-octane-1,3-diol was the major compound detected by HPLC analysis in the diethyl ether extract obtained after 9, 16, 32, 64, and 129 d of incubation with [9,10,12,13-^{3}H]linoleic acid. Furthermore, 3-hydroxyoctyl β-D-glucoside accounted for more than 30% of the radioacivity in the methanol extract after 9, 16, 32, 64, and 129 d. Although 26.9% of the applied ^{3}H is recovered in the nonextractable residues after 12 h, this value constantly decreased to 14.4% after 129 d (Table 2). We assume that linoleic acid degradation products with chain lengths shorter than 18 carbon atoms are still attached to the insoluble β-oxidation complex, and therefore they contribute significantly to the radioactivity found after 0.5 d in the nonextractable residues (Tables 1 and 2). After 4 d, the majority of the ^{3}H linoleic acid pulse was degraded, and the rate of decrease slowed for ^{3}H recovered in the nonextractable residues.

Application of [U-^{14}C]oleic acid and [9,10,12,13,15,16-^{3}H]linolenic acid. Radio-labeled *R*-octane-1,3-diol was also detected after the application of [U-^{14}C]oleic acid to apples and 64 d of storage. The distribution of ^{14}C in the different fractions is shown in Table 3. The total incorporation into octane-1,3-diol, 3-hydroxyoctyl β-D-glucopyranoside and diol released by glycosidase from the nonextractable residue was *ca*. 9%. The respective values obtained for [1-^{14}C]hexanoic acid, [1-^{14}C]octanoic acid, and [U-^{14}C]linoleic acid were 5.2, 4.0, and 20.6% of applied radioactivity, respectively (8). Radio-labeled *R*-5(*Z*)-octene-1,3-diol was formed after the application of [9,10,12,13,15,16-^{3}H]linolenic acid. The recovery of radioactivity in the different fractions after solid-phase extraction is displayed in Table 3. *R*-5(*Z*)-Octene-1,3-diol, *R*-5(*Z*)-3-hydroxyoctenyl β-D-glucopyranoside, and *R*-octene-1,3-diol released by glycosidase from the nonextractable residue accounted for *ca*. 11% of the applied radioactivity. A comparable value was obtained for the incorporation of [9,10,12,13-^{3}H]linoleic into tritium-labeled octane-1,3-diol derivatives. The incorporation for [9,10,12,13-^{3}H]linoleic acid into *R*-octane-1,3-diol was smaller than the

TABLE 2
Time Course and Distribution of Recovered Radioactivity (expressed as percentage of applied ^{3}H) in Different Fractions After the Application of [9,10,12,13-^{3}H]Linoleic Acid to Apple Fruits[a]

	Incubation time (d)								
	0.5	1	2	4	9	16	32	64	129
XAD flowthrough	35.2	38.3	40.3	45.0	45.2	37.8	37.1	33.5	23.3
Diethyl ether extract	35.6	32.8	27.1	26.6	21.4	18.0	17.8	13.9	11.6
Methanol extract	9.5	6.5	7.3	7.1	7.6	9.7	12.0	10.8	12.5
Nonextractable residue	26.9	22.0	19.8	16.1	16.0	17.8	17.6	14.9	14.4
Sum	107.2	99.6	94.5	94.8	90.2	83.3	84.5	73.1	61.8

[a]See Table 1 for abbreviation.

TABLE 3
Distribution of Recovered Radioactivity (expressed as percentage of applied ^{14}C and ^{3}H) in Different Fractions After the Application of [U-^{14}C]Oleic Acid and [9,10,12,13,15,16-^{3}H]Linolenic Acid to Apple Fruits[a]

	^{14}C-$C_{18:1}$	*R*-Octane-1,3-diol	^{3}H-$C_{18:3}$	*R*-5(*Z*)-Octene-1,3-diol
XAD flowthrough	23.7		33.6	
Diethyl ether extract	6.4	5.9	18.8	6.0
Methanol extract	2.6	0.9	16.2	5.0
Nonextractable residue	47.8	2.0	23.6	0.3
CO_2 (calculated)	19.5			
Sum		8.8	92.2	11.3

[a]See Table 1 for abbreviation.

incorporation of [U-^{14}C]linoleic acid into the diol. This implies that during the transformation a higher percentage of ^{3}H is lost compared to ^{14}C.

Application of ^{3}H-labeled linoleic acid transformation products. Tritium-labeled 13- and 9-HODE, 13- and 9-KODE and a mixture of the stereoisomers of the 9,10,13- and 9,12,13-trihydroxyoctadecenoic acids were produced by application of [9,10,12,13-^{3}H]linoleic acid to apples and were separated by semi-preparative HPLC analysis. Three fractions containing a mixture of ^{3}H-13- and 9-HODE (12:1), a mixture of ^{3}H-13- and 9-KODE (4:1), and a mixture of ^{3}H-labeled trihydroxyoctadecenoic acids, respectively, were injected into apples, and radioactively labeled *R*-octane-1,3-diol was determined after 9 d of incubation. Incorporation of HODE, KODE, and trihydroxy acids amounted to 3.6, 3.1, and 3.1% of the applied radioactivity, respectively.

Application of $^{13}C_{18}$ hydroperoxides. Uniformly labeled ^{13}C hydroperoxides 9-HPOD, 10-HPOD, 12-HPOD, 13-HPOD, and 13(*E,E*)-HPOD were synthesized according to Reference 9, and the individual compounds were separated by normal-phase HPLC analysis. After application of $^{13}C_{18}$-linoleic acid and $^{13}C_{18}$-HPOD to apples, δ ^{13}C values of formed *R*-octane-1,3-diol were determined by GC–IRMS (Table 4). Comparison of δ ^{13}C values ($^{13}C/^{12}C$ isotope ratios compared to the international standard) of the diol obtained from untreated apples (control) with those obtained after the administration of hydroperoxides and linoleic acid demonstrated the incorporation of ^{13}C isotopes into the target molecule. The most efficient precursor was linoleic acid followed by 10-HPOD and 9-HPOD. A common feature of these compounds was the *cis* double bond at carbon 13. Dilution of the isotopically labeled diol by the natural pool of octane-1,3-diol was similar in the individual experiments, as the apples contained similar amounts of the diol (Table 4). The lowest concentration of the diol was quantified in the experiment with 12-HPOD. Therefore, the actual δ ^{13}C value for octane-1,3-

TABLE 4
δ ^{13}C Values of *R*-Octane-1,3-diol Obtained from Apples (control) and After the Application of $^{13}C_{18}$ Linoleic Acid, Uniformly $^{13}C_{18}$-Labeled 9-, 10-, 12-, 13-, and 13(*E,E*)-HPOD to Apples (*R* = -$(CH_2)_4$-COOH)[a]

	Structure	δ ^{13}C value	mg/apple
Apples (control)		−43.39 to −46.87	
Linoleic acid	R	−16.90 ± 0.09	7.53
10-HPOD	OOH, R	−19.71 ± 0.28	7.15
9-HPOD	R, OOH	−25.41 ± 0.17	8.81
13-HPOD	OOH, R	−32.69 ± 0.29	7.82
13(*E,E*)-HPOD	OOH, R	−32.70 ± 0.11	7.99
12-HPOD	R, OOH	−33.33 ± 0.23	2.56

[a]HPOD, hydroperoxyoctadecadienoic acid.

diol might be even lower when corrected by the amount of the natural pool.

Application of $^{2}H_2$*-labeled octanol derivatives*. Deuterated octanol derivatives were prepared by chemical synthesis, and 5 mg of each compound was administered to apples. After 9 d, apples were worked up by solid-phase extraction, and the labeling pattern of formed *R*-octane-1,3-diol was determined by GC–CI–MS. We used CI–MS in order to gain sensitivity, as the intensities for *m/z* 128 $[M - H_2O]^+$ and *m/z* 110 $[M - 2H_2O]^+$ in the EI mass spectrum of *R*-octane-1,3-diol were less than 1% and a molecular ion $[M]^+$ was not detected. The CI–MS fragment ion *m/z* 129 $[M - 2H_2O + H]^+$ exhibited an intensity of 25% and was used for the determination of the labeling pattern of the formed diol. A ratio (heavier isotopomer is expressed as the percentage of the lighter isotopomer) of 0.6% was obtained for *R*-octane-1,3-diol formed after the application of unlabeled linoleic acid. This value did not significantly increase after the administration of 2,3-$^{2}H_2$-octenol (0.8%) and 2,3-$^{2}H_2$-octenal (0.8%). Analysis showed that these substances were completely degraded and predominantly reduced to 2,3-$^{2}H_2$-octanol. Labeling patterns of 1.8 and 2.8% were obtained after the administration of 5,6-$^{2}H_2$-ethyl-3-oxooctanoate and 5,6-$^{2}H_2$-ethyl-3-hydroxyoctanoate, respectively. Complete degradation of the esters was observed. Although appreciable amounts of 1,1-$^{2}H_2$-3-oxooctanol and 4,5-$^{2}H_2$-3-hydroxyoctanal were detected after the incubation period, they showed the highest isotope content with 4.0 and 5.6%, respectively.

DISCUSSION

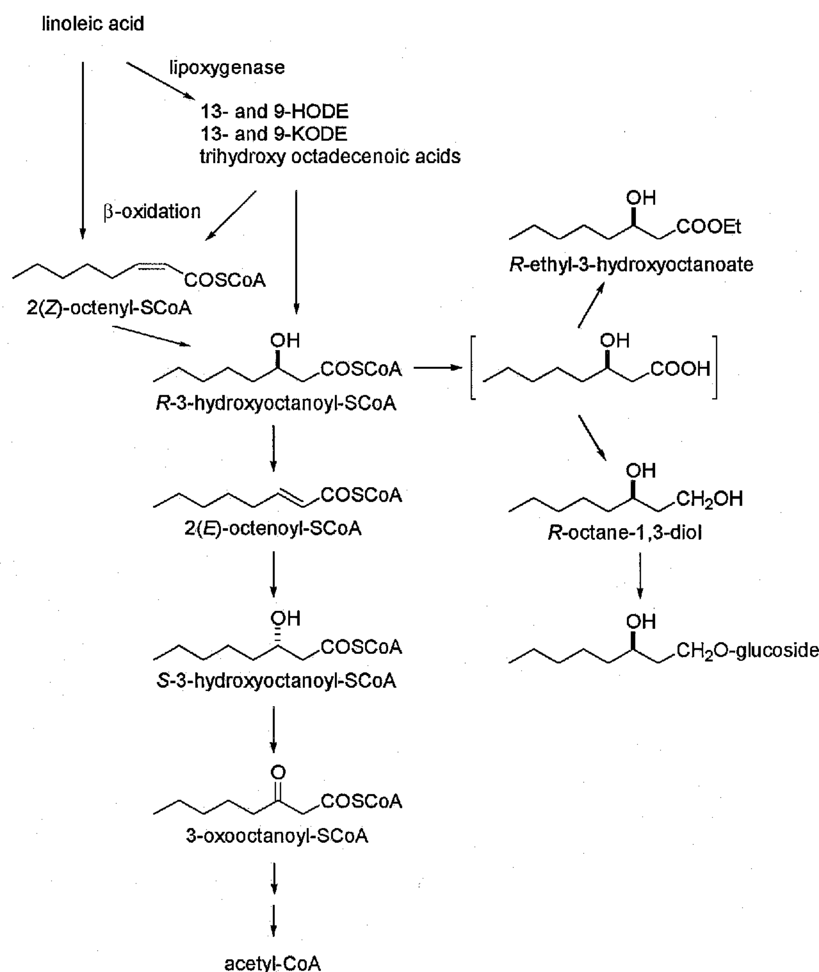
After harvesting, ripening of fruit is characterized by changes in color, texture, and aroma development that are related to physiological and metabolic processes. Apples are climacteric fruits, and carbon dioxide production as well as oxygen uptake increases by 50 to 100% during ripening, apparently without any change in respiratory quotient (16). Ethylene production increases about 1000-fold and at the same time as the respiratory rise (17). Softening, color change, formation of cuticular waxes, and synthesis of aroma compounds all seem to be associated with the climacteric.

Several apple cultivars such as Douce Moen, Peau de Chien and Rheinischer Bohnapfel accumulate high amounts of the enantiomerically pure *R*-octane-1,3-diol during this period. Early studies demonstrated that the diol is formed by degradation of linoleic acid either by β-oxidation or by a lipoxygenase-like reaction followed by the action of a lyase (8).

The time-course experiment with [1-^{14}C]- and [9,10,12,13-^{3}H]linoleic acid demonstrated rapid degradation of the unsaturated fatty acid. 13- and 9-HODE, 13- and 9-KODE, and the stereoisomers of trihydroxyoctadecenoic acid were identified as the major products after 12 h. They are probably artifacts formed by apple lipoxygenase during the injection of the substrate. However, radioactively labeled C_{10} compounds were not observed. In addition, a high portion of the fatty acid entered the β-oxidation pathway, as *ca*. 31% of the applied radioactivity was released as CO_2, and only even-carbon-numbered metabolites were formed. Degradation of HODE, KODE, and trihydroxy acids accounted for the additional release of $^{14}CO_2$ in the course of the experiment. Highest concentration of radioactively labeled *R*-octane-1,3-diol in the diethyl ether extract was obtained at days 4–9. After that period, the amount of the ^{3}H-labeled diol in the diethyl ether extract decreased, but in parallel the content of 3-hydroxyoctyl β-D-glucopyranoside in the methanol increased. However, diol derivatives accumulated constantly throughout the course of the study.

On the basis of the presented results we assume that *R*-octane-1,3-diol is formed by β-oxidation from linoleic acid in stored apples as shown in Scheme 1. Within the higher plant cell, fatty acid degradation is located in a distinct subcellular compartment, the peroxisome (18). Following activation by acyl-CoA synthase, the fatty acid is degraded by repetitive passages through the β-oxidation reaction sequence. As the *cis* double bonds form a barrier to degradation by continuous passages through the β-oxidation reaction sequence, additional enzymes (Δ^2, Δ^3-enoyl-CoA isomerase, 2,4-dienoyl-CoA reductase, D-3-hydroxyacyl-CoA dehydratase) are involved in the degradation of the activated unsaturated fatty acids (18). Common, straight-chain fatty acids are activated to their corresponding acyl-CoA by acyl-CoA synthase. The acyl-CoA synthase shows low activity toward short- and medium-chain acids, and it activates preferentially the C_{18} unsaturated fatty acids. However, low or no activity of acyl-CoA synthases toward oleic acid was reported. A single protein catalyzes the hydration of 2-*trans*-enoyl-CoA to 3-hydroxyacyl-CoA and the subsequent oxidation. In the course of peroxisomal β-oxidation the L(*S*)-isomer of 3-hydroxyacyl-CoA is a product of the enoyl-CoA hydratase activity as well as a substrate of the 3-hydroxyacyl-CoA dehydrogenase. However, D(*R*)-3-hydroxyacyl-CoA is formed by the 2-enoyl-CoA hydratase activity of the multifunctional protein when 2-*cis*-enoyl-CoA is the substrate instead of 2-*trans*-enoyl-CoA (18). Circumvention of the 12-*cis* double bond of linoleic acid can be achieved by involvement of D(*R*)-3-hydroxyacyl-CoA dehydratase at the C_8-intermediate level. By using purified enzymes in a reconstituted β-oxidation system, conversion of 4-*cis*-decenoyl-CoA (intermediate in linoleate degradation) to 2-*cis*-octenoyl-CoA was demonstrated (19), indicating that the plant multifunctional protein is able to perform this reaction. Hydration of 2-*cis*-octenyl-CoA yields D(*R*)-3-hydroxyoctanoyl-CoA. An epimerase converting D(*R*)-3-hydroxyacyl-CoA into L(*S*)-3-hydroxyacyl-CoA was thought to be involved in the β-oxidation pathway at this point. It was recently demonstrated that the epimerase activity results from two reactions: dehydration to 2-*trans*-enoyl-CoA and subsequent hydration to L(*S*)-3-hydroxyacyl-CoA.

We propose that during storage of the apples D(*R*)-3-hydroxyoctyl-CoA is formed from 2-*cis*-octenyl-CoA, and it accumulates due to an increased turnover of lipids and a limited capacity of the following enzyme(s) to convert the *R*-3-hydroxyoctyl-CoA to the *S*-enantiomer. Therefore, 3-hydroxy-



SCHEME 1

octanoic acid is released and subsequently reduced to *R*-octane-1,3-diol or transformed to *R*-ethyl-3-hydroxyoctanoate (2,7) in order to avoid feedback inhibition of the accumulated intermediate. In parallel, *R*-5(*Z*)-octene-1,3-diol is formed from the respective unsaturated intermediate *R*-3-hydroxy-5(*Z*)-octenyl-CoA.

Phospholipids and fatty acyl groups typical of cell membranes remain constant or increase slightly during apple ripening. However, [^{14}C]acetate incorporation by apple cortex into various phospholipids increases approximately tenfold from pre- to post-climacteric; this indicates that turnover of lipid increases during storage of apples, even though composition does not change (20,21). Apple alcohol dehydrogenase activity was demonstrated in apple tissues and whole apples (22,23). Apple tissue metabolizes fatty acyl precursors by β-oxidation to shorter-chain compounds and by reduction to alcohols (20). The activity decreases with carbon number of the substrate (24). Dehydrogenase activity is also responsible for the complete reduction of 2,3-$^2H_2$-octenol and 2,3-$^2H_2$-octenal to 2,3-$^2H_2$-octanol. Therefore, incorporation into *R*-octane-1,3-diol was negligible for these compounds. Our experiments demonstrated that the incorporation of labeled fatty acids into *R*-octane-1,3-diol increases from hexanoic acid, octanoic acid, oleic acid to linoleic acid, whereas linolenic acid was transformed to *R*-5(*Z*)-octene-1,3-diol. The 12-*cis* double bond was an important feature of fatty acid derivatives exhibiting a high incorporation (Table 4). The results showed that the diols are formed by β-oxidation of linoleic acid, and the point of regulation may be located at the C_8-intermediate level where the β-oxidation barrier caused by the 12-*cis* double bond of linoleate has to be surmounted. Comparison of the incorporation of [U-^{14}C]linoleic acid (20.6%) with [U-^{14}C]oleic acid (8.8%) demonstrated that the introduction of the *cis* double bond led to a significant increase of incorporation into *R*-octane-1,3-diol derivatives.

We expected a high labeling pattern of *R*-octane-1,3-diol after the application of 5,6-$^2H_2$-ethyl-3-hydroxyoctanoate and 5,6-$^2H_2$-ethyl-3-oxooctanoate. However, the values were smaller than those for 1,1,-$^2H_2$-3-oxooctanol and 4,5-$^2H_2$-3-hydroxyoctanal. The latter ones are probably reduced by a nonspecific reductase to *R*-octane-1,3-diol. In our opinion, the ketone and the aldehyde are not the natural precursors, as they are still detectable in the extracts. On the other hand, $^2H_2$-labeled ethyl-3-hydroxyoctanoate and ethyl-3-oxooctanoate are either degraded by β-oxidation in peroxisomes or used by the fatty acid *de novo* synthesis in the cytosol or plastids. The 3-

oxo-ester, a good substrate for both transformations, is converted to *R*-octane-1,3-diol only after its incorporation into linoleic acid and subsequent degradation. The low transformation of the 3-hydroxyester to the diol can be rationalized by the fact that the *R*-enantiomer is removed from the cytosol by *de novo* synthesis. Therefore, only small amounts of *R*-ethyl-3-hydroxyoctanoate enter the peroxisomes and act as precursor for the diol. The *S*-enantiomer, which is not used by *de novo* synthesis in the cytosol, is degraded in the peroxisomes without formation of *R*-octane-1,3-diol.

Summarizing, the biosynthesis of the C8-diols resembles the biogenesis of jasmonic acid, alkanolides, and the synthesis of mono-, di-, and triunsaturated esters identified in the aromas of quince and Bartlett pear (25). They can all be accounted for by β-oxidation of polyunsaturated linoleic and linolenic fatty acids as well as their oxygenated derivatives. The fatty acids are transformed to intermediates which cannot further be degraded by the multienzyme complex, and thus they are released and metabolized.

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Excess Vitamin E Decreases Canthaxanthin Absorption in the Rat

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ABSTRACT: The recent attention given to the possible role of α-tocopherol (α-Toc) and carotenoids in the prevention and treatment of a variety of illnesses resulted in segments of the population increasing their consumption of these nutrient/antioxidants. Once consumed, α-Toc and carotenoids are thought to follow the same absorptive pathway and may influence each other's absorption, particularly when taken in large doses. The purpose of this study was to determine if α-Toc and the carotenoid, canthaxanthin (CTX), interact during absorption. Rats were intraduodenally infused with corn oil emulsions containing combinations of α-Toc (0 or 300 μmol/L) and CTX (5, 10, 15, 20 μmol/L) in a 2 × 4 factorial arrangement. Absorption was determined by measuring recovery of CTX and α-Toc in the mesenteric lymph. The amount of CTX in the lymph increased significantly with the amount infused into the duodenum. The overall efficiency of CTX absorption from emulsions without α-Toc averaged 12% with individual animals having a range of 8 to 18%. Efficiency of absorption was not related to concentration of CTX infused. When α-Toc (300 μmol/L) was added to the oil emulsion, the absorption of CTX was decreased by at least 50%. Recovery of α-Toc in the lymph averaged *ca.* 10% and was not affected by CTX. These results suggest that concurrent consumption of a large dose of α-Toc may influence carotenoid bioavailability.

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A vast amount of epidemiological data supports a positive relationship between the consumption of foods high in carotenoids and vitamin E and a decrease in chronic illnesses such as cardiovascular disease, certain cancers, cataract formation, and macular degeneration (1–3). As a result many individuals increased consumption of vitamin E and carotenoids by altering their diet or consuming dietary supplements (4). However, consumption of large amounts of these nutrient/antioxidants may be of concern as studies suggest that carotenoids interact with vitamin E during absorption (see recent reviews 5–7). The purpose of the current study was to investigate the interaction between vitamin E and carotenoids during absorption by using the mesenteric lymph duct cannulated rat intraduodenally infused with lipid emulsions. This animal model was previously used in our laboratory to study the absorption of canthaxanthin (CTX) and lycopene (8).

In designing the current study, two factors were of primary consideration. One factor was the type of carotenoid used. Carotenoids are often classified as either nonpolar carotenes (hydrocarbon carotenoids containing no oxygen) or as polar oxycarotenoids (xanthophylls containing at least one alcohol or carbonyl group). The physical properties of the two groups of carotenoids differ, affecting how they are handled during the lumenal phase of absorption. Within the intestinal lumen the nonpolar carotenes, such as β-carotene, are thought to locate in the hydrophobic core of lipid emulsions and bile salt micelles, while the more polar xanthophlls are thought to locate at the surface (9). Vitamin E is also thought to locate on the surface of lipid emulsions and micelles (10). Because vitamin E and polar xanthophylls are both surface components, the potential for their interaction within the intestinal lumen is greater than interaction between vitamin E and a nonpolar carotene. To maximize the potential for interaction between vitamin E and carotenoids, a xanthophyll was used in the current study. The xanthophyll selected was CTX. While CTX (4,4′-diketo-β-carotene) is not a major dietary carotenoid, it is widely used as a model carotenoid because it is relatively stable, has physical properties similar to other xanthophylls, and is commercially available (5–7).

The second factor considered when designing this study was the relative amounts of α-tocopherol (α-Toc) and CTX. Recently, concurrent consumption of equal molar concentrations of α-Toc and β-carotene was reported to enhance absorption of β-carotene in the ferret (11). This was somewhat unexpected as previous work with rats fed an excess of β-carotene observed a decrease in plasma and tissue levels of β-carotene in response to vitamin E supplementation (12,13). Interestingly, as indicated in literature from more than 50 yr ago, small doses of vitamin E enhanced utilization of β-carotene in the rat while large doses inhibited utilization (14,15). These observations suggest that the nature of vitamin E interaction with carotenoids may depend on their relative concentrations. We, therefore, decided to study the effect of an excess of α-Toc on CTX absorption.

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Abbreviations: α-Toc, α-tocopherol; CTX, canthaxanthin; HPLC, high-pressure liquid chromatography.

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MATERIALS AND METHODS

Animals and surgical procedures. The animal protocol used in this study was approved by The University of Connecticut Institutional Animal Care and Use Committee. Male Holtzman albino rats obtained from Harlan Sprague-Dawley (Indianapolis, IN) weighing 300–350 g at the time of surgery were used. Prior to surgery, animals were housed individually in stainless-steel cages in the Department of Nutritional Sciences Animal Facility. The animals were fed a standard laboratory diet—Purina Rodent Chow 5001 (Purina Mills, St. Louis, MO) and water *ad libitum*.

Surgery was performed under methoxyflurane-pentobarbital as previously described (16). A right subcostal incision was made, and the major mesenteric lymph duct was located. A beveled heparin-filled vinyl tube (medical grade, 0.50 mm i.d., 0.80 mm o.d.; Dural Plastics and Engineering, Dural, Australia) was threaded into the lymph duct and secured using methylcyanoacrylate adhesive. All visible accessory lymph ducts were blocked. A feeding tube was placed in the duodenum by passing micro-renathane tubing (MRE 040; Braintree Scientific, Braintree, MA) in a caudal direction through a gastric puncture. The abdominal wall was closed by suturing the muscle layer and skin.

Immediately following surgery, the rats were placed in a warm, dark environment and allowed to recover for *ca.* 36 h. The rats had access to water and received a glucose/electrolyte solution (Pedialyte; Ross Laboratories, Columbus, OH) at 2.0 mL/h during the recovery period *via* the duodenal feeding tube.

Following their recovery, three rats per treatment were infused (2.0 mL/h) for 12 h through the feeding tube. After infusing the emulsion for 6 h, lymph was collected for analysis during the final 6 h of infusion. After collection, the lymph was stored at −70°C until analyzed.

Preparation of carotenoid and tocopherol stock solutions. A stock solution of CTX (Sigma Chemical, St. Louis, MO) was prepared with dichloromethane. A stock solution of α-Toc (all-*rac*-α-tocopherol, 95%; Sigma Chemical) was prepared in methanol. The concentration of each stock solution was determined by absorption spectrometry. The concentration of CTX was estimated using the molar extinction coefficient E at 466 nm ($E^{1\%}_{1\,cm}$ = 2200) and α-Toc was estimated at 292 nm ($E^{1\%}_{1\,cm}$ = 75.8).

Treatment emulsions. Eight treatment emulsions containing combinations of α-Toc and CTX were used in this study. The final emulsion consisted of a phosphate-buffered saline solution (6.8 mmol/L Na_2HPO_4, 16.5 mmol/L NaH_2PO_4, 115 mmol/L NaCl, and 5 mmol/L KCl), 10 mmol/L sodium taurocholate (Sigma Chemical) and 3% (wt/vol) tocopherol-stripped corn oil (ICN, Costa Mesa, CA) with two levels of α-Toc (0, 300 μmol/L) and four levels of CTX (5, 10, 15, 20 μmol/L) in a 2 × 4 factorial arrangement.

To prepare emulsions, corn oil was placed in a round-bottomed flask, and appropriate amounts of CTX and α-Toc were added to the oil and mixed. Solvent was removed with a stream of nitrogen. Sodium taurocholate and buffer were added to the flask. The contents of the flask then were emulsified using a probe sonicator producing *ca.* 40 watts output for 15 s, repeated four or five times until no lipid droplets were observed (Branson Sonifier, Model 185; Baranson Sonic Power, Danbury, CT). An aliquot of the emulsion was extracted three times with hexane and the final concentration of CTX and α-Toc determined by high-pressure liquid chromatography (HPLC) as described below for lymph analysis.

Analysis of carotenoid and vitamin E in lymph. To correct for loss of CTX during preparation for HPLC analysis, ethyl β-apo-8′-carotenate (Fluka, Ronkonkoma, NY) was used as an internal standard. To correct for recovery of α-Toc, α-Toc acetate (Sigma Chemical) was added to the lymph as an internal standard. Extraction of total lipid from the lymph was by the method of Folch *et al*. (17).

Canthaxanthin and α-Toc were analyzed by HPLC using a Waters C18 Resolve column (15 cm × 3.9 mm; Millipore, Milford, MA) protected with an Upchurch C18 guard column (Upchurch Scientific, Oak Harbor, WA) and an isocratic mobile phase consisting of acetonitrile/dichloromethane/methanol/*n*-butanol /ammonium acetate (90:15:10:0.1:0.1, by vol) (18). CTX and ethyl β-apo-8′-carotenate were identified and quantified at a wavelength of 450 nm. α-Toc and its internal standard, α-Toc acetate, were identified and quantified at a wavelength of 290 nm.

Statistical analysis. Values presented are means ± standard deviation. This study was a completely randomized design with treatments in a 2 × 4 factorial arrangement (19). The statistical analysis was a two-way analysis of variance with three animals per treatment. Because the amounts of CTX infused were equally spaced, the linear, quadratic, and cubic relationship between amount CTX infused and the amount absorbed was tested. Simple correlation analysis and linear regression also were used to determine the statistical relationship between the amount of CTX infused and the amount recovered in the lymph. Values were considered statistically significant if P values were less than or equal to 0.05.

RESULTS

The lymph flow for rats in the study ranged from 2.0 to 3.3 mL/h with an average of 2.4 mL/h. Lymph flow was not influenced by treatment. Rats with a lymph flow of less than 2.0 mL/h or rats with less than 90% recovery of total lipid in the lymph were excluded from the study.

The results of the study are presented in Table 1. The concentration of CTX infused into the duodenum significantly ($P < 0.01$) affected CTX absorption. As shown in Figure 1, there was a significant ($P < 0.05$) linear relationship between CTX infused and the amount absorbed. The quadratic and cubic relationships were not significant. The amount of CTX infused and lymphatic output of CTX were highly correlated, for the treatments without α-Toc ($r = 0.99$; $P < 0.01$) and for the treatments with α-Toc ($r = 0.98$; $P < 0.01$). There was a significant negative effect of α-Toc on CTX absorption. For each

TABLE 1
Recovery of Canthaxanthin and α-Tocopherol in the Mesenteric Lymph of Rats[a]

	Treatments			
CTX (μmol/L)	α-Toc (0 μmol/L)	α-Toc (300 μmol/L)	α-Toc (0 μmol/L)	α-Toc (300 μmol/L)
	Lymphatic CTX (nmol/h)		Lymphatic α-TOC (nmol/h)	
5	1.22 ± 0.15	0.79 ± 0.44	N.D.	55.48 ± 26.44
10	2.74 ± 0.95	1.39 ± 0.54	N.D.	65.54 ± 40.05
15	3.53 ± 0.33	1.71 ± 0.62	N.D.	62.33 ± 32.54
20	4.92 ± 0.85	2.48 ± 0.98	N.D.	58.42 ± 37.43

[a]Values are means of lymph collected from three rats ± SD. Abbreviations: CTX, canthaxanthin; α-Toc, α-tocopherol; N.D., not detected (<10 (mol/L).

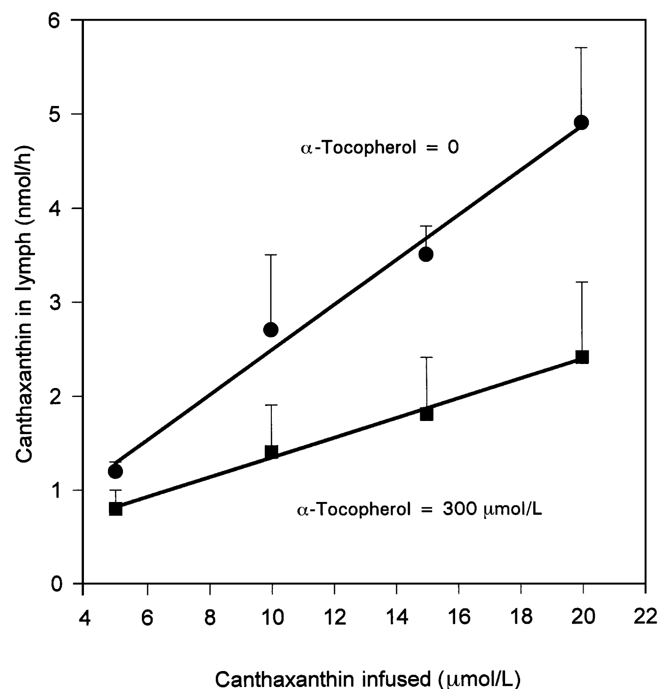

FIG. 1. Canthaxanthin absorption into the mesenteric lymph of rats intraduodenally infused with emulsions containing canthaxanthin and either no α-tocopherol or 300 μmol/L α-tocopherol. Values are means from three rats per treatment ± SD.

10 nmoles of CTX infused in treatments without α-Toc, 1.21 nmoles were recovered in the lymph (*ca.* 12% absorption). When CTX was infused with α-Toc, for each 10 nmoles of CTX infused only 0.53 nmoles was recovered in the lymph (*ca.* 5% absorption).

The interaction term in the analysis of variance for CTX and α-Toc was not significant at the .05 level. This is interpreted to mean that α-Toc decreased CTX absorption approximately the same for all treatment levels of CTX. This may be due to the large amount of α-Toc used in the study relative to CTX. The molar ratio of α-Toc/CTX at the lower treatment level of CTX was 60:1 and at the higher level of CTX the ratio was 15:1.

As shown in Table 1, the amount of α-Toc recovered in the lymph was not significantly affected at the .05 level by the amount of CTX infused. The average α-Toc recovered in the lymph from the treatments containing α-Toc was 60.4 nmoles/h (*ca.* 10% absorption). Comparing the standard deviations in Table 1, the variation associated with recovery of α-Toc in the lymph was greater than for CTX.

DISCUSSION

Reports of carotenoid and α-Toc interactions are often based on observations from animals fed very high levels of carotenoid, making the relevance of these observations to normal nutrition uncertain. The amounts of CTX and α-Toc infused per unit of body weight in the current study were comparable to those administered in human studies. Supplementation of β-carotene in human studies ranges from 20 to 300 mg/d (20). For an adult weighing 70 kg, the daily dose of β-carotene would be 0.29 to 4.3 mg/kg body weight. The CTX infused in the current study ranged from *ca.* 0.1 to 0.9 mg/kg body weight. The suggested therapeutic range for α-Toc starts at several hundred mg/d, with doses of up to *ca.* 10 mg/kg per day not expected to cause side effects (21). The amount of α-Toc provided to the rats in the current study was *ca.* 10 mg/kg body weight. This dose would be considered pharmacological.

The efficiencies of absorption of CTX and α-Toc in the current study were within the range of values reported in the literature. In a previous study we observed the efficiency of CTX absorption to range from 10–20% with an average of *ca.* 16% (8). The overall 12% efficiency of absorption of CTX in the current study is slightly less than we observed in our previous study. Actual measurements of carotenoid absorption are sparse. In studies using hospitalized patients with cannulated thoracic ducts, Goodman *et al.* (22) reported that 10% of an oral dose of radioactive β-carotene was absorbed into the lymph, while Blomstrand and Werner (23) presented data from a similar study reporting 23% absorption. Using a stable isotope of β-carotene, Novotny *et al.* (24) suggested that 22% of an oral dose of β-carotene in olive oil was absorbed in humans.

As presented in Table 1, the recovery of α-Toc in the mesenteric lymph averaged between 8 and 15% and was not influenced by CTX. These values are on the lower end of a range of values from animal studies. In a recent study of marginal zinc deficiency, Kim *et al.* (25) reported the efficiency of α-Toc absorption by control rats to be 13–17%. In a study comparing absorption of tocotrienols and α-Toc, the recovery of α-Toc in the thoracic lymph of rats was 10.5–24.5% (26). In an earlier study with rats, Bjorneboe *et al.* (27) reported 15% recovery of radioactive α-Toc in lymph, although this value may be low due to poor overall fat absorption in this study. A factor that may have influenced α-Toc absorption in the current study is the relatively large amount infused. Traber *et al.* (28) observed 65% recovery in the lymph when small amounts of α-Toc were infused into the duodenum and 18% when much greater amounts were infused.

Another difference between the current study and previous studies that may have decreased absorption of both CTX and α-Toc was the use of corn oil emulsions. Polyunsaturated fatty acids, such as those in corn oil, are reported to decrease the absorption of fat-soluble vitamins A, E, and K in rats (29). A similar study with rats by Gallo-Torres and colleagues (30) also reported a decrease in absorption of vitamin E with polyunsaturated fatty acids. They observed *ca.* 7% absorption of α-Toc when rats consumed α-Toc in emulsions with linoleic acid and 25% absorption of α-Toc when emulsions contained triolein. The mechanism by which polyunsaturated fatty acids decrease absorption of fat-soluble vitamins is unknown and deserves further study.

There are several animal studies reporting that carotenoids and vitamin E affect each other's bioavailability. Blakely *et al.* (31) fed rats β-carotene or CTX (at 0.048 or 0.2% of the diet) and observed a decrease in plasma α-Toc in response to feeding CTX, but not β-carotene. In a similar study Bendich and Shapiro (32) fed growing rats either CTX or β-carotene (2 g/kg body weight) and observed a reduction in plasma α-Toc in response to both carotenoids. Mayne and Parker (33) fed chicks CTX (0.5 g/kg body wt) for 6 wk and reported a decrease in plasma α-Toc. A similar study with chicks fed either astaxanthin or β-carotene resulted in a decrease in plasma α-Toc (34). Ferrets fed CTX (50 mg/kg body wt/d) for 2 yr also had a decreased plasma α-Toc (35). In these animal studies, it should be noted that high levels of carotenoids were fed to attain the effects on vitamin E.

As shown in Figure 1, addition of α-Toc (300 μmol/L) to the emulsion significantly ($P < 0.01$) decreased CTX absorption by *ca.* 50%. The effect of α-Toc on CTX absorption was similar at all treatment levels of CTX. While the mechanism by which α-Toc decreases the absorption of CTX is unclear, one possibility is that α-Toc and CTX compete for incorporation into bile salt micelles.

Normal absorption of carotenoids and α-Toc includes the transfer from lipid emulsion particles in the lumen of the small intestine into bile salt micelles. The incorporation of carotenoids into micelles is limited and was suggested as a controlling step in carotenoid absorption (36). As previously mentioned, CTX and α-Toc are thought to preferentially incorporate onto the surface of bile salt micelles while the less polar carotenes incorporate into the core of the micelle (9,10). If α-Toc and CTX compete for incorporation into the surface of the micelle, the presence of large amounts of α-Toc compared to CTX may decrease the incorporation of CTX into micelles and therefore depress absorption.

Recently, concurrent consumption of equal molar concentrations of α-Toc and β-carotene was reported to enhance absorption of β-carotene (11). This appears to be contrary to the observations in the current study. There are at least two major differences between the current study and the ferret study. One major difference is the relative amounts of carotenoid and α-Toc given to the animals in the two studies. The interaction observed when equal molar concentrations of α-Toc and carotenoid are given may be different from that observed when the amount of α-Toc consumed is 15 to 60 times greater than the amount of carotenoid consumed.

The second major difference between studies was the type of carotenoid used. In our study, α-Toc and CTX most likely compete for incorporation into the bile salt micelle surface. The β-carotene used in the ferret study would solubilize into the center of the micelle. Rather than compete for incorporation into micelles, α-Toc may actually enhance β-carotene inclusion into the micelle. This suggestion is based on observations made by El-Gorab and Underwood (37), who studied the solubility of retinol (polar) and β-carotene (nonpolar) into solutions containing bile salt micelles. Retinol was incorporated into the micelle surface, expanding the micelle and enhancing β-carotene solubilization into the micelle core. Possibly, α-Toc or CTX, being more polar than β-carotene, may function similarly to retinol and enhance solubilization of β-carotene into micelles.

In conclusion, concurrent consumption of a large amount of α-Toc with CTX was observed to decrease the absorption of CTX by roughly 50%. Additional research is needed to determine if the observed negative effect of α-Toc on CTX absorption varies with their relative concentrations. The observation that CTX did not influence α-Toc absorption should be viewed with caution as the study was not specifically designed to measure this. The large amount of α-Toc relative to CTX used in the current study and the large variation in measurement of lymphatic α-Toc made it difficult to detect any influence of CTX on α-Toc absorption.

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The Metabolism and Distribution of Sesame Lignans (sesamin and episesamin) in Rats

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ABSTRACT: In this study, we examined the distribution and metabolism of refined sesame oil lignans (sesamin and episesamin) in rat. For 8 wk rats were fed the diet including 0.5% (w/w) sesame lignans (sesamin and episesamin) with 5% (w/w) corn oil or eicosapentaenoic acid (EPA)-rich oil. The concentrations of sesamin and episesamin in rat liver after their administration for 8 wk were very low; both of them were less than 0.5 μg/g liver. These were observed in both oil groups although the fatty acid compositions of dietary oils were completely different. No significant difference existed in lymphatic absorption between sesamin and episesamin. To investigate the distribution of sesamin and episesamin in rats, the concentrations of sesamin and episesamin were determined in tissues and serum within 24 h after administration to rats. Sesamin and episesamin may be, at first, incorporated into the liver and then transported to the other tissues (lung, heart, kidney, and brain). They are lost from the body within 24 h after administration. There was no significant difference in lymphatic absorption between sesamin and episesamin, but the amount of sesamin was significantly lower than that of episesamin in all tissues and serum. These results suggest that sesamin is absorbed in lymph the same as episesamin, but that sesamin is subsequently metabolized faster by the liver.

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The sesame lignans (sesamin and episesamin) are compounds commonly found in refined sesame seed oil. Actually, episesamin is rarely found in unrefined sesame seed oil but is generated in equivalent amounts from sesamin by isomerization during the acid–clay bleaching of the oil (1). Sesame lignans (sesamin and episesamin) are known to have multiple physiological functions including antioxidant activity (2,3), anticarcinogenenicity (3), antihypertensive effects (4,5) in rats, and alleviation of hepatic injury caused by alcohol or carbon tetrachloride (6) in mice. In addition, sesame lignans also affect lipid metabolism by inhibiting cholesterol absorption from the small intestine, reducing 3-hydroxy-3-methylglutaryl CoA reductase activity in liver microsomes (7,8), and affecting the incorporation of linoleic acid into lipid subfractions (9) in rats. They also increase the content of dihomo-γ-linolenic acid by inhibition of Δ5 desaturase activity in the *Mortierella alpina* fungus and rat liver microsomes (10–13) and inhibit marked changes of the n-6/n-3 fatty acid ratio during dietary manipulation by reducing hepatic polyunsaturated fatty acid content in rat (14,15). Sesame lignans were also reported to have hypocholesterolemic effects in humans (16).

Almost all of the studies on the physiological effects of sesame lignans were performed using a mixture of sesamin and episesamin. The inhibitory effects of episesamin on the Δ5 desaturase in rat liver microsomes are almost the same as those of sesamin (17). However, the effect of sesamin and episesamin has been independently investigated only on Δ5 desaturase in rat microsomes. Since almost all of the sesame lignan studies have used a mixture of sesamin and episesamin, neither the functions of seamin and episesamin nor their distribution and metabolism *in vivo* have been studied independently.

The aim of this study was to investigate the distribution and metabolism of sesamin and episesamin and to compare them in rats. In this study, the following three experiments were done: (i) the investigation of the accumulation of sesamin and episesamin in rat liver after a long-term administion (8 wk), (ii) the comparison of lymphatic absorption of sesamin and episesamin, and (iii) the investigation of the distribution of sesamin and episesamin in rat tissues within 24 h after administration by measuring sesamin and episeamin *in vivo*.

MATERIALS AND METHODS

Materials. Sesame lignans (a mixture of sesamin and episesamin, 47:53,w/w) prepared from refined sesame seed oil and purified by the method of Fukuda *et al.* (1) and arachidonic acid (AA) ethyl esters (purity >99%) were kindly donated by Suntory Ltd. (Osaka, Japan). Eicosapentaenoic acid (EPA) ethyl esters (purity >97%) were kindly donated by Sagami Chemical Research Center (Kanagawa, Japan). Rapeseed oil was provided by Ajinomoto Co., Inc. (Tokyo, Japan), and corn oil was provided by Eisai Co. (Tokyo, Japan).

Experimental procedure. All experiments were approved by the Animal Experimentation Ethics Committee of Ochanomizu University (Tokyo, Japan).

Experiment 1. Administration of sesame lignans (sesamin

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Abbreviations: AA, arachidonic acid; AIN, American Institute of Nutrition; EPA, eicosapentaenoic acid.

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and episesamin) for 8 wk in rats. Three-week-old male Wistar rats were purchased from Nippon Clea Co. (Tokyo, Japan). The rats were initially fed on a commercial diet (CE-2; Nippon Clea Co.) for 1 wk. The animals, weighing an average of 98 g, were then divided into two groups of six animals each. They were housed individually in an air-conditioned room at 23 ± 1°C with a 12-h light–dark cycle and were given experimental diets (15 g/d, 1–7 d; 20 g/d, 8–17 d; 25 g/d, 18–28 d) and water *ad libitum* for 8 wk. The basal diet, prepared according to the standards given by the American Institute of Nutrition (AIN) (18), was provided by Eisai Co., and contained the following percentage of ingredients according to weight: casein, 20; glucose, 25; sucrose, 25; cornstarch, 15; filter paper, 5; AIN mineral mixture, 3.5; AIN vitamin mixture, 1; choline bitartrate, 0.2; and DL-methionine, 0.3. The test diets were prepared by mixing 0.5% (w/w) sesame lignans (a mixture of sesamin and episesamin, 47:53, w/w) and 5% (w/w) experimental oil mixtures with the basal diet. The oil mixtures used were: (i) the corn oil group, (ii) the EPA-rich oil group (EPA); EPA ethyl esters/rapeseed oil = 2:3. The fatty acid compositions of the corn oil and of the EPA mixture, similar to that in our previous study (13), are shown in Table 1. After having been fed the test diets for 8 wk, all rats were fasted overnight before they were sacrified and their livers were collected.

Experiment 2. Lymphatic absorption of sesame lignans (sesamin and episesamin). Seven-week-old male Wistar rats, weighing an average of 194 g, were purchased from Nippon Clea Co. and were maintained on a commercial diet (CE-2; Nippon Clea Co.) for 1 wk. Under Nembutal anesthesia, rats were subjected to cannulation of the left thoracic lymphatic channel, and an indwelling catheter was placed in the stomach by the method of Ikeda *et al.* (19,20). Normal osmotic solution containing 139 mM glucose and 85 mM NaCl was infused continuously until the end of lymph collection at a rate of 3 mL/h through the gastric tube. After surgery, animals were placed in restraining cages under lights to recover and allowed free access to drinking water containing 139 mM glucose and 85 mM NaCl. The next morning, after collection of lymph for 2 h (blank lymph), each animal was administered 3 mL of a test emulsion through the gastric tube, and the lymph was collected for 24 h. Lipid emulsions for intragastric administration contained 200 mg of sodium taurocholate, 50 mg bovine serum albumin (fatty acid-free, Sigma Chemical Co., St. Louis, MO), 100 mg EPA ethyl esters, 100 mg AA ethyl esters, and 50 mg of sesame lignans (sesamin and episesamin) per 3 mL of distilled water. In this experiment, EPA and AA ethyl esters were used because sesame lignans affected EPA and AA metabolism in rats in our previous studies (13–15). The mixture was emulsified by sonication just prior to use, and the lymph was collected periodically for 24 h.

TABLE 1
Major Fatty Acid Composition of Dietary Oils[a]

	16:0	18:0	18:1 (n-9)	18:2 (n-6)	18:3 (n-3)	20:5 (n-3)
Corn oil	11.2	2.3	31.0	55.6		
EPA	3.0	1.3	36.0	14.8	6.6	38.4

[a]Values are expressed as percentage of weight. EPA; eicosapentaenoic acid, acid-rich oil diet (EPA ethyl esters/rapeseed oil = 2:3).

Experiment 3. Incorporation of sesame lignans (sesamin and episesamin) into rat tissues. Seven-week-old male Wistar rats, weighing an average of 193 g, were purchased from Nippon Clea Co. and were maintained on a commercial diet (CE-2; Nippon Clea Co.) for 1 wk. They were then fasted overnight and given 1 g of an oil mixture, that is, EPA ethyl esters/rapeseed oil = 2:3, containing 100 mg sesame lignans (sesamin and episesamin). This amount corresponded to a day's dietary fat intake and sesame lignan intake in the previous experiment (13). The fatty acid composition of the oil mixture was the same as that of EPA group in Experiment 1 (Table 1). The liver, lung, heart, kidney, brain and serum were collected at 1, 3, 6, 9, and 24 h after the administration.

Determination of sesamin and episesamin in the tissues, lymph, and serum. The liver, lung, heart, kidney, and brain samples were homogenized in 3 vol of distilled water. Sesamin and episesamin were extracted from the homogenate solution, serum, and lymph by the Bligh-Dyer method (21). Sesamin and episesamin were analyzed using high-performance liquid chromatography (Waters LC Module I as an instrument and Waters 805 data station as a detector; Japan Millipore Ltd., Tokyo, Japan) on Unisil Pack 5C18-150A (4.6 × 150 mm) column. The mobile phase was methanol/water (70:30, vol/vol) at a flow rate of 1 mL/min. The ultraviolet detection wavelength for sesamin and episesamin was 290 nm.

Statistical analysis. All values are expressed as means ± SD. Significance of the difference in mean values was evaluated by Students' *t*-test.

RESULTS

Table 2 shows the concentrations of sesamin and episesamin in rat liver after administration of sesame lignans for 8 wk. Only small amounts of sesamin and episesamin were found in both groups; their concentrations were less than 0.5 µg/g liver.

Figure 1 shows the cumulative concentrations of sesamin and episesamin in lymph. There was no significant difference in lymphatic absorption between sesamin and episesamin, and their ratio of lymphatic absorption was almost the same.

To investigate the distribution of sesamin and episesamin

TABLE 2
Effect of Dietary Sesame Lignans on the Concentrations (µg/g liver) of Sesamin and Episesamin in Rat Liver After 8 wk Administration[a]

	Sesamin[b]	Episesamin
Corn oil	0.28 ± 0.11	0.49 ± 0.07***
EPA	<0.16	0.43 ± 0.07

[a]Results are means ± SD (n = 6). Value sharing asterisk is significantly different from sesamin of the same dietary oil group. ***$P < 0.001$.
[b]The detection limit of sesamin concentration is 0.09 µg/g liver, and two samples in the EPA group had undetectable amounts. See Table 1 for abbreviation.

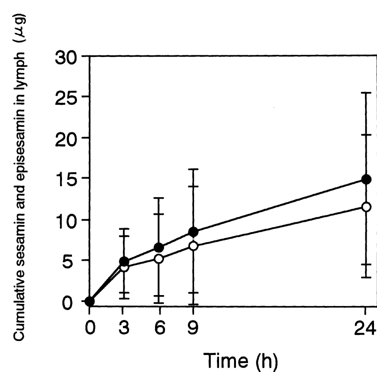

FIG. 1. Lymphatic absorption of sesamin (●) and episesamin (○). Lignans were extracted from the lymph collected at 3-h intervals during the first 9 h, and once at 24 h after their administration and analyzed by high-performance liquid chromatography (HPLC). The absorbed amounts are shown as cumulative values.

in rats, we administered sesamin and episesamin to rats and determined their concentrations in tissues within 24 h after administration. Time-dependent changes of orally administered sesamin and episesamin in the tissues and serum are shown in Figure 2. In the liver (Fig. 2A), the concentrations of sesamin and episesamin had reached a maximum at 3 h after administration, and were rarely found after 24 h. However, hepatic episesamin concentration was significantly greater than sesamin, despite the fact that equivalent amounts were administered. In the serum (Fig. 2B), episesamin concentration was also significantly greater than that of sesamin. The highest sesamin and episesamin concentrations were found between 3 and 6 h after administration. In the lung, heart, and kidney (Fig. 2C, D, E), episesamin concentration was significantly greater than sesamin, similar to liver and serum. The highest sesamin and episesamin concentrations were found at 6 h after administration. In the brain (Fig. 2F),

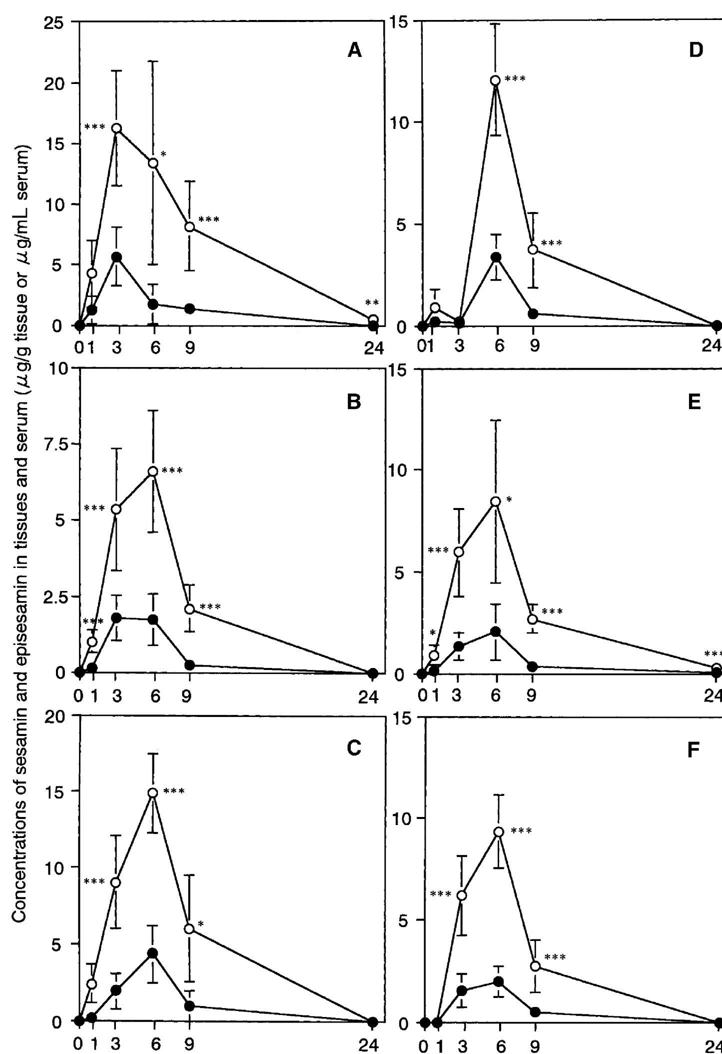

FIG. 2. Time-dependent changes of sesamin (●) and episesamin (○) concentrations in rat tissues and serum after oral administration. Lignans were extracted from the tissues and serum collected at 0,1,3,6,9, and 24 h after their administration and analyzed by HPLC. Differences at each time point are significant at ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$. A, liver; B, serum; C, lung; D, heart; E, kidney; F, brain. See Figure 1 for abbreviation.

sesamin and episesamin were first detected at 3 h after administration—comparatively later than the other tissues. As in other tissues, episesamin concentration was significantly greater than sesamin. The highest sesamin and episesamin concentrations were found at 6 h after administration. Sesamin and episesamin were rarely found after 24 h in any tissues or serum.

DISCUSSION

In this study, we measured the concentrations of sesamin and episesamin in rats to investigate: (i) the accumulation of sesamin and episesamin in rat liver after long-term administering (8 wk), (ii) the lymphatic absorption of sesamin and episesamin, and (iii) the distribution of sesamin and episesamin in rat tissues and serum within 24 h after administration.

The concentrations of sesamin and episesamin in rat liver after their administration for 8 wk were very low; both of them were less than 0.5 μg/g fresh weight (Table 2). These were observed in both oil groups, despite the fact that the fatty acid compositions of dietary oils corn oil and EPA-rich oil were completely different. These results suggest that sesamin and episesamin are not accumulated in rat liver regardless of dietary oil variety even if administrated for 8 wk.

The lymphatic absorption of sesamin and episesamin was also investigated. Hirose *et al.* (8) reported that sesamin was absorbed and found in the rat lymph, and the proportion of sesamin recovered during 24 h was 0.15 ± 0.01%. In this study, the recovery of sesamin and episesamin was 0.06 ± 0.04% and 0.06 ± 0.03%, respectively. The proportion of lymphatic absorption of sesamin was lower than in the report of Hirose *et al.* (8), but we found that there was no significant difference in lymphatic absorption between sesamin and episesamin (Fig. 1).

It was observed that sesamin and episesamin were incorporated into rat tissues and serum after administration (Fig. 2). The highest sesamin and episesamin concentrations were found at 3 h after administration in the liver, between 3 and 6 h after in the serum, and after 6 h in the other tissues (lung, heart, kidney, and brain). These results suggest that sesamin and episesamin may be, at first, incorporated into the liver and then transported to the other tissues. Sesamin and episesamin are lost from the body within 24 h after their administration; that is the reason why they do not accumulate in rat tissues, even if administered for 8 wk. Hirose *et al.* (8) also reported that fecal excretion of sesamin was 15–35%.

There was no significant difference in lymphatic absorption between sesamin and episesamin (Fig. 1), but the amount of sesamin was significantly lower than that of episesamin in all tissues and serum (Fig. 2). These results suggest that sesamin is absorbed in lymph the same as episesamin but that sesamin is subsequently metabolized more rapidly by the liver. The results of our present study also suggest that sesamin may be metabolized in the liver, and it is possible that the metabolites may have physiological effects. Further investigation is needed about metabolism of sesamin and its metabolites in the liver.

This study has yielded three important findings: (i) sesamin and episesamin may at first be incorporated into the liver, after which they are transported to peripheral tissues through the bloodstream; (ii) sesamin and episesamin are lost from the body within 24 h and are not accumulated, and (iii) there was no significant difference in lymphatic absorption between sesamin and episesamin, but the concentration of sesamin was significantly lower than episesamin in rat tissues and serum. In this study, we revealed a part of the metabolism and distribution of sesamin and episesamin in rat.

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COMMUNICATION

Seasonal Variation in the Fatty Acid Composition and Quality of Sardine Oil from *Sardinops sagax caeruleus* of the Gulf of California

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ABSTRACT: One of the few sources of long-chain n-3 polyunsaturated fatty acids is fish oil, but considerable variation may exist according to species and season. In this study, the fatty acid profiles of sardine oils from *Sardinops sagax caeruleus* of the Gulf of California, Mexico, were evaluated in three seasonal catch periods. Oil quality was also evaluated by peroxide and free acid values. The most abundant fatty acids found in the oils were palmitic acid (19.3%), oleic acid (14.3%), eicosapentaenoic acid (EPA, 20.4%), and docosahexaenoic acid (DHA, 12.2%). There was no significant difference in the composition and quality among the six reduction plants where the samples were obtained. However, a significant difference in the proportion of EPA and DHA in one of the catch seasons analyzed was observed.

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Hydrogenated fish oil was widely consumed in the United States during the period 1921–1951, but this ceased when the California sardine fishery failed in the late 1940s (1,2). In the Gulf of California, sardine capture began in 1967. The greatest annual landing was 322,000 tons during 1988–1989, made up of 91% *Sardinops sagax caeruleus* (3), the most important species supporting the commercial fish industry in northwest Mexico (Fig. 1). Most landings are used by the fish meal industry in providing protein for animal feeds. By-product sardine oil from these factories could be an inexpensive source of long-chain n-3 polyunsaturated fatty acids (PUFA) for human consumption.

The potential health role of marine oil n-3 fatty acids, principally eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), was demonstrated in the prevention and treatment of cardiovascular diseases (4,5). In spite of this knowledge, diets in Western countries do not fulfill the daily requirements of n-3 PUFA, and people need to eat more fish, take dietary supplements, or consume fortified processed foodstuffs to obtain the beneficial effects (6,7). Although the best source of long-chain n-3 PUFA is fish and fish products (7,8), the fat content and the fatty acid profiles are variable among fish species because of differences in season and type of feed (9,10).

The seasonal variation in the fatty acid compositions of the sardine oil (*Sardinops* sp.) from the Gulf of California is unknown. For this reason, our objective was to study the fatty acid composition and quality of the sardine oil (*S. sagax caeruleus)* as related to the season of catch in the Gulf of California.

EXPERIMENTAL PROCEDURES

Sample handling. Fresh crude oil from whole sardine (*S. sagax caeruleus*) was collected from six different fish meal factories, from the two main ports in the Gulf of California, three in Guaymas, Sonora, and three in Yavaros, Sonora, Mexico (Fig. 1). The fish meal factories were chosen according to their processing capacity. The catch sample date was selected in accordance with the seasons of February, April, and June. All sardine oils were received in sealed 4-L glass

FIG. 1. Fishery ports of the Gulf of California.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; PUFA, polyunsaturated fatty acids; PV, peroxide value.

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containers (dark color). The samples were kept on ice for transportation to the laboratory (≅5 h). Immediately upon arrival, the oil was stored at −40°C, and before use it was held at room temperature and allowed to reach 22ºC.

Methods. The fatty acid compositions of the sardine oils were determined after methyl esterification according to methods of the American Oil Chemists' Society (11), by procedure Ce 2-66, followed by gas–liquid chromatography on a Varian 6000 chromatograph (Varian de Mexico, Mexico City, Mexico), equipped with a 2-m long column, packed with 3% SP-2310/2% SP-2300 on Chromosorb WAW (Supelco Inc., Bellefonte, PA) (12). The oven program was 190°C (2 min), 190–220°C (2°C/min). The injector and flame-ionization detector were held at 250°C. The carrier gas was nitrogen at a flow of 20 mL/min. Retention times and peak areas were processed by a Varian CDS-401 computing integrator (Varian de Mexico). Identification and quantification were done by comparison with the retention times and peak areas of methyl ester standards of each fatty acid (Supelco Inc.). Menhaden oil fatty acid methyl esters (Sigma Chemical Co., St. Louis, MO) were also utilized as secondary standard. Three different commercial samples of fish oil capsules (A, B, and C) from the United States, labeled as high in n-3 fatty acids, were also analyzed for comparison.

Chemical analyses of the fresh crude oils were carried out according to official AOCS procedures (11). Saponification value was obtained by procedure Cd 3-25; conjugated dienes (Ti 1a-64) were measured using a spectrometer PE UV-vis Lambda 2S (Perkin-Elmer de Mexico, Mexico City, Mexico); refractive index (Cc 7-25) was measured using an Abbé refractometer (Bausch & Lomb, Rochester, NY). Peroxide value (PV) and free fatty acid (FFA) as percentage oleic acid were determined by titration (methods Cd 8-53 and Ca 5a-40, respectively). Reagents, including boron trifluoride (16% in methanol), and all solvents were of analytical grade and were purchased from Merck (Darmstadt, Germany).

Oil quality. A quality index (70–100 points) was applied to the fish oil samples according to the following scale: 90–100, good; 80–89, regular; 70–79, bad.

An oil with 10 meq/kg PV and 5% of FFA (as oleic acid) was indexed at 100 points according to suggested criteria (13–15). Five points were subtracted for each PV unit over 10 meq/kg as well as 5 points for each unit of FFA over 5%.

Statistical analysis. All values represent the mean of three analytical replications. Data obtained were subjected to one-way analysis of variance and Tukey's test analysis. Significance was declared at $P < 0.05$. Statgraphic computer software was used for statistical analysis (Statistical Graphics Corp., Rockville, MD).

RESULTS AND DISCUSSION

Fatty acid composition. There were no significant differences ($P < 0.05$) in the fatty acid composition between samples from Guaymas and Yavaros, probably because the vessels from each port fished in the same area of the Gulf of California. The most abundant fatty acids found in all the samples of sardine oil were palmitic acid (19.3%), oleic acid (14.3%), EPA (20.4%), and DHA (12.2%). The mean content of all saturated fatty acids was 31.9%, and PUFA totaled 37.6% (Table 1). Docosenoic acid (22:1) was very low in all samples and did not prejudice the measurement of EPA. The

TABLE 1
Fatty Acid Compositions of Oils from Whole Sardine *(Sardinops sagax caeruleus)* from the Gulf of California, Mexico[a]

Fatty acids	Total fatty acids (wt%)			
	February	April	June	Average
14:0	6.60 ± 0.48	6.06 ± 0.56	6.53 ± 0.37	6.39
16:0	17.12 ± 1.12	20.49 ± 1.06	20.46 ± 0.56	19.35
18:0	7.00 ± 0.29	5.80 ± 0.37	5.79 ± 0.44	6.19
Σ Saturates	30.72 ± 0.84[a]	32.34 ± 1.36[b]	32.78 ± 0.92[b]	31.93
16:1	9.43 ± 0.28	8.26 ± 0.53	8.20 ± 0.79	8.63
18:1	12.41 ± 1.78	15.21 ± 2.48	15.40 ± 0.99	14.34
20:1	0.98 ± 0.18	1.56 ± 0.33	1.86 ± 0.32	1.46
Σ Monounsaturates	22.82 ± 1.58[c]	25.32 ± 2.48[c]	24.78 ± 1.65[c]	24.43
18:2n-6	2.07 ± 0.38	1.16 ± 0.47	1.60 ± 0.11	1.61
18:3n-3	0.39 ± 0.10	0.48 ± 0.26	0.47 ± 0.08	0.44
18:4n-3	2.31 ± 0.10	2.00 ± 0.57	1.79 ± 0.21	2.03
20:4n-6	0.92 ± 0.39	0.91 ± 0.25	1.11 ± 0.14	0.98
20:5n-3	23.91 ± 0.98[d]	19.21 ± 0.95[e]	18.26 ± 1.30[e]	20.46
22:6n-3	9.61 ± 1.69[f]	13.70 ± 2.28[g]	13.43 ± 2.06[g]	12.24
Σ n-3	36.22 ± 1.81[h]	35.39 ± 3.20[h]	33.95 ± 1.34[h]	35.17
Σ Polyunsaturates	39.21 ± 2.03[i]	37.46 ± 3.39[i]	36.66 ± 1.25[i]	37.76

[a]Average of six fish meal factories. These values do not total 100% because minor fatty acids are not reported. Values in the rows with different superscripts (a–i) are significantly different ($P < 0.05$).

TABLE 2
Mean Fatty Acid Composition of Sardine Oil *(Sardinops sagax caeruleus)* from Principal Ports of the Gulf of California and of Three Commercial Encapsulated Oils[a]

	Total fatty acids (wt%)				
	Sardine oils		Encapsulated oils		
Fatty acids	Guaymas	Yavaros	A*	B*	C*
14:0	6.52 ± 0.65	6.48 ± 0.55	7.44 ± 0.28	6.45 ± 0.09	4.72 ± 0.08
16:0	19.13 ± 1.69	19.47 ± 2.08	18.58 ± 0.11	14.50 ± 0.13	13.10 ± 0.08
16:1	8.17 ± 0.80	8.85 ± 0.78	10.24 ± 0.16	8.47 ± 0.04	5.42 ± 0.10
18:0	6.04 ± 0.70	6.25 ± 0.58	4.86 ± 0.00	3.51 ± 0.02	2.76 ± 0.01
18:1	13.77 ± 2.16	14.88 ± 2.02	12.41 ± 0.01	13.09 ± 0.06	30.42 ± 0.35
18:2n-6	1.63 ± 0.44	1.68 ± 0.55	1.46 ± 0.11	1.41 ± 0.02	8.09 ± 0.07
18:3n-3	0.57 ± 0.21	0.38 ± 0.08	0.43 ± 0.08	0.65 ± 0.01	3.10 ± 0.01
18:4n-3	2.19 ± 0.37	1.83 ± 0.35	3.32 ± 0.10	3.26 ± 0.01	2.37 ± 0.01
20:0	1.62 ± 0.59	1.48 ± 0.48	1.82 ± 0.10	3.76 ± 0.04	3.76 ± 0.03
20:4n-6	1.01 ± 0.48	1.11 ± 0.29	0.79 ± 0.02	0.79 ± 0.08	0.58 ± 0.01
20:5n-3	20.66 ± 2.86[a]	20.25 ± 2.77[a,b]	20.17 ± 0.66[a,b]	24.32 ± 0.00[a]	14.30 ± 0.08[b]
22:6n-3	12.87 ± 3.03[c]	11.62 ± 2.34[c]	14.42 ± 0.04[c]	16.21 ± 0.03[c]	8.88 ± 0.17[c]
Total saturated	31.69 ± 0.97[d]	32.20 ± 1.15[d]	30.88 ± 0.39[d]	24.48 ± 0.02[e]	20.59 ± 0.14[e]
Total monounsaturated	23.39 ± 2.13[f]	25.21 ± 1.81[f]	24.48 ± 0.08[f]	25.33 ± 0.06[f]	39.60 ± 0.23[g]
Total polyunsaturated	38.63 ± 2.49[h]	36.92 ± 1.74[h]	40.59 ± 0.98[h,i]	46.65 ± 0.08[i]	37.33 ± 0.15[h]
Total n-3	36.26 ± 2.33[j,k]	34.10 ± 1.13[j,k]	38.34 ± 0.89[j,l]	44.44 ± 0.02[l]	28.66 ± 0.23[m]
PUFA/SFA	1.21	1.14	1.31	1.91	1.81

[a]Values are means of at least duplicates ± standard deviations. These values do not total 100% because minor fatty acids are not reported. Asterisk = encapsulated commercial oil labeled as "high n-3." Values in the rows with different superscripts (a–m) are significantly different ($P < 0.05$). PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

DHA and EPA accounted for 86.5% of total PUFA. The oil fatty acid profile is characteristic of filter-feeding fish such as menhaden and sardine (8,15).

The total n-3 content in the oil sardine samples was similar to that of one encapsulated fish oil labeled as "high n-3" (A) and significantly higher than another (C); this suggests that sardine oil could be an equally good source of n-3 PUFA for encapsulation (Table 2).

Effect of the catch season on EPA and DHA content. The EPA content in oil collected in February was significantly higher ($P < 0.05$) than that obtained at the other two sampling times, presenting a mean value of 23.9% (Table 1). In contrast, the DHA content of 9.6% was inversely decreased in the samples obtained in February. A similar observation was made for these species but in other areas of catch and in different months (16). This increase in EPA concentration could result from the sardine diet, because EPA is a principal fatty acid of phytoplankton subject to seasonal blooms. These changes may also be attributed to the spawning cycle (17), and the highest gonadosomatic index in the sardine *S. sagax*

TABLE 3
Characteristics of Sardine *(Sardinops sagax caeruleus)* Crude Oil Produced in the Two Main Ports of the Gulf of California[a]

	Sample month		
	February	April	June
Guaymas			
Saponification value	174.92 ± 10.68	179.12 ± 1.19	182.50 ± 3.05
Refractive index	1.4730 ± 0.00	1.4739 ± 0.00	1.4730 ± 0.00
Conjugated dienes (%)	0.73 ± 0.12	0.95 ± 0.34	1.21 ± 0.37
Peroxide value (meq/g)	9.13 ± 2.08	6.88 ± 4.52	5.98 ± 5.35
Free fatty acid (% oleic)	2.30 ± 0.85	1.08 ± 0.70	2.45 ± 0.74
Quality index	98.88 ± 1.10	96.67 ± 5.77	96.43 ± 6.18
Quality	Good	Good	Good
Yavaros			
Saponification value	180.81 ± 1.82	182.74 ± 1.89	181.60 ± 6.05
Refractive index	1.4738 ± 0.00	1.4726 ± 0.00	1.4729 ± 0.00
Conjugated dienes (%)	0.73 ± 0.17	0.60 ± 0.05	0.68 ± 0.2
Peroxide value (meq/g)	6.40 ± 2.99	4.44 ± 0.94	5.59 ± 3.28
Free fatty acid (% oleic)	3.32 ± 0.95	3.91 ± 3.24	3.05 ± 1.08
Quality index	100 ± 0.00	97.50 ± 3.70	100 ± 0.00
Quality	Good	Good	Good

[a]Values are means of triplicates ± standard deviation.

caeruleus from the Gulf of California was observed during February (17,18).

Oil quality. Table 3 shows the chemical and physical characterization of the oils examined. The low PV and FFA guarantee the good quality of the fresh crude oils collected over 5 mon, the quality index ranging from 96 to 100. Oil samples from the different fish meal plants did not show severe oxidation (PV below 10 meq/kg), presumably because the samples analyzed were freshly produced oils. This ensures that the proportions of DHA and EPA are not affected, according to Fritsche and Johnston (19), who examined the effects of oxidation on the EPA and DHA content.

The fatty acid composition of the sardine from the Gulf of California represents a good source of long-chain n-3 PUFA year-round and could be useful as a nutritional supplement. The fatty acid content is similar to that found in several commercial encapsulated fish oils.

The sardine oil could be used after refining for either encapsulation or inclusion in processed foodstuffs for human consumption. Recently the use of similar fish oil such as menhaden oil was approved for human consumption, with specific limitations by the regulatory agencies of different countries (20).

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ARTICLE

Cloning of Δ12- and Δ6-Desaturases from *Mortierella alpina* and Recombinant Production of γ-Linolenic Acid in *Saccharomyces cerevisiae*

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ABSTRACT: Two cDNA clones with homology to known desaturase genes were isolated from the fungus *Mortierella alpina*. The open reading frame in one clone encoded 399 amino acids and exhibited Δ12-desaturase activity when expressed in *Saccharomyces cerevisiae* in the presence of endogenous fatty acid substrate oleic acid. The insert in another clone contained an open reading frame encoding 457 amino acids and exhibited Δ6-desaturase activity in *S. cerevisiae* in the presence of exogenous fatty acid substrate linoleic acid. Expression of the Δ12-desaturase gene under appropriate media and temperature conditions led to the production of linoleic acid at levels up to 25% of the total fatty acids in yeast. When linoleic acid was provided as an exogenous substrate to the yeast cultures expressing the Δ6-desaturase activity, the level of γ-linolenic acid reached 10% of the total yeast fatty acids. Co-expression of both the Δ6- and Δ12-desaturase cDNA resulted in the endogenous production of γ-linolenic acid. The yields of γ-linolenic acid reached as high as 8% of total fatty acids in yeast.

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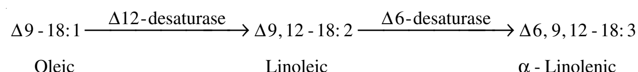
The primary products of fatty acid biosynthesis in most organisms are 16- and 18-carbon compounds. The relative ratio of chain lengths and degree of unsaturation of these fatty acids vary widely among species. Mammals, for example, produce primarily saturated and monounsaturated fatty acids, while most higher plants produce fatty acids with one, two, or three double bonds. Indeed, polyunsaturated fatty acids, such as linoleic acid (Δ9,12-18:2) and α-linolenic acid (Δ9,12,15-18:3), are regarded as essential fatty acids in the diet because mammals lack the ability to synthesize them. However, when ingested, mammals have the ability to metabolize linoleic and α-linolenic acids to form the n-6 and n-3 families of long-chain polyunsaturated fatty acids (LC-PUFA), respectively. These LC-PUFA are important cellular components conferring fluidity to membranes and functioning as precursors of biologically active eicosanoids such as prostaglandins, prostacyclins, and leukotrienes which regulate normal physiological functions (1).

In mammals, the formation of LC-PUFA is rate-limited by the step of Δ6-desaturation, which converts linoleic acid to γ-linolenic acid (GLA, Δ6,9,12-18:3) and α-linolenic acid to stearidonic acid (Δ6,9,12,15-18:4). Many physiological and pathological conditions have been shown to depress this metabolic step, and consequently, the production of LC-PUFA (2). However, bypassing the Δ6-desaturation *via* dietary supplementation with GLA can effectively alleviate many pathological diseases associated with low levels of PUFA (1). This beneficial effect prompted GLA-rich oil to become a much-demanded commodity. GLA is currently used in the treatment of eczema and mastalgia (1). At the present time, the predominant sources of GLA are oils from plants such as borage, evening primrose and black currant, and from microorganisms, such as *Mortierella* spp., *Mucor* spp. and cyanobacteria (3). However, these GLA sources are not ideal for dietary supplementation due to high fluctuations in availability, production/purification costs, unpleasant tastes and odors, and safety concerns. Thus, interest in developing more reliable and economical alternative sources of GLA and other LC-PUFA is growing.

The primary product of fatty acid biosynthesis in most plants and yeast is the monounsaturated, 18-carbon oleic acid. Two desaturation steps, at the Δ12 and Δ6 positions, necessary for the production of GLA from oleic acid, are shown below.



The cDNA clones encoding Δ12-desaturases were isolated from several species of cyanobacteria (4,5) and plants including *Arabidopsis* (6), soybean (7), and parsley (8). Δ6-Desaturase-encoding cDNA were isolated from cyanobacteria (9), borage (10), and nematode (11). These enzymes, as well as numerous examples of Δ15/n-3 desaturases (12,13), are all believed to be integral membrane proteins utilizing an acyl-

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Abbreviations: GC, gas chromatography; GLA, γ-linolenic acid; LC-PUFA, long-chain polyunsaturated fatty acid; MS, mass spectrometry; PCR, polymerase chain reaction; TPI, triose phosphate isomerase.

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lipid substrate, and with the exception of the cyanobacterial enzymes, requiring cytochrome *b5* for the electron transport. The deduced amino acid sequences of these desaturases show a good deal of similarity, most notably in the region of three histidine-rich motifs that are believed to be involved in iron binding (14).

In this study, we utilized the filamentous fungus, *Mortierella alpina*, as the source for desaturase genes. This approach was based on the fact that this fungus is rich in linoleic acid and its LC-PUFA n-6 metabolites, GLA, and arachidonic acid (Δ5,8,11,14-20:4). Using a strategy based on degenerate oligonucleotide primers designed to amplify sequences present at the second and third His boxes of known acyl lipid desaturases (14), we previously isolated a cDNA clone encoding the *M. alpina* Δ5-desaturase (15). A similar strategy utilizing different degenerate primers was also successful in amplifying the same Δ5-desaturase (16). Such polymerase chain reaction (PCR) approaches are limited, however, by the degree of homology of the target cDNA to the particular primers and conditions utilized. In order to achieve a more thorough examination of the fatty acid desaturases present in the fungus, an alternate approach of sequencing random cDNA clones was also employed. Since it was known that the previously characterized membrane-bound Δ12- and Δ15-desaturases, as well as the available cyanobacterial Δ6-desaturase sequences, showed significant amino acid sequence conservation, particularly in the histidine-rich regions, it was postulated that potential *Mortierella* desaturase cDNA could be recognized based on their deduced amino acid sequences. Indeed, this was the strategy that led to the identification of a borage Δ6-desaturase (11) and a castor oleate 12-hydroxylase (17). Because the first histidine-rich motif (His-box) region can occur from 80 to 160 amino acids (240–480 bp) from the N-terminus, and the third region can be roughly 250–300 amino acids (750–900 bp) into the desaturase sequence (14), 300–400 bp of DNA sequence information obtained from the 5′-end of full-length clones might not contain the regions of highest homology among desaturases. Since at the time this work was initiated, no desaturase sequence was identified from *M. alpina* and it was not known how much homology they might display to known sequences, we chose to obtain information from the internal sequences of cDNA clones instead of the 5′-end of full-length clones.

Expression of the *Mortierella* desaturase candidates was carried out in baker's yeast, *Saccharomyces cerevisiae*. This eukaryotic organism was previously shown to be a suitable host containing the necessary cofactors for functional expression of acyl-lipid desaturases. *Saccharomyces cerevisiae* contains a Δ9-desaturase capable of producing monounsaturated palmitoleic and oleic fatty acids, but does not carry out further desaturations. Expression of an *Arabidopsis FAD2* cDNA in *S. cerevisiae* resulted in the production of linoleic and Δ9,12-hexadecadienoic acids from the endogenous oleic acid and palmitoleic acid substrates, respectively (18,19). By culturing *S. cerevisiae* in the presence of exogenous fatty acid substrates, functional expression of a nematode Δ6-desaturase (11) and a fungal Δ5-desaturase (15,16) were demonstrated. In this study, we report the isolation of Δ12- and Δ6-desaturases from *M. alpina*. Simultaneous expression of these two genes in *S. cerevisiae* drives production of GLA at levels of up to 8% of the total fatty acids without the requirement for exogenous fatty acid substrates.

MATERIALS AND METHODS

cDNA library construction. Synthesis of *M. alpina* cDNA was described previously (15). Briefly, double-stranded cDNA were sized fractionated by column chromatography. The two fractions containing the largest cDNA were pooled and packaged to produce a "full-length" library (M7+8) containing *ca.* 6×10^6 clones with an average insert size of 1.77 kb. An additional library, (M11), to be used for random sequencing was constructed by packaging a fraction containing smaller cDNA, which would most likely contain less than full-length clones as well as full-length copies of shorter messages. The average insert size of this library was 1.1 kb; the titer was 240 pfu/µL. Library screening and plaque purification were carried out with the M7+8 library using standard protocols as described previously (20).

Random DNA sequencing. The cDNA-containing plasmids were excised from the λ-ZipLox clones following manufacturer's recommendations (Life Technologies, Gaithersburg, MD). Bacterial cells were plated on ECLB plates containing 50 µg/mL penicillin. DNA sequence was obtained from the 5′-end of the cDNA insert and compared to the National Center for Biotechnology Information nonredundant database using a BLAST server.

Plasmid construction. For expression in yeast, the *M. alpina* cDNA clones for Δ6- and Δ12-desaturase genes were first modified to create *Eco*RI and *Xho*I restriction sites adjacent to the start and stop codons, respectively. Each gene was amplified from the respective cDNA clone using PCR with a pair of primers which have homology to the 5′-end and 3′-end of the gene (restriction sites underlined):

RO-192 (5′-TAGGCTGAATTC**ATG**GCTGCTGCTCCCAGTGTGAGGACG-3′)

and

RO-193 (5′-AACTGCCTCGAG**TTA**CTGCGCCTTACCCATCTTGGAGGC-3′)

are forward and reverse primers with homology to the sequences around the initiation and termination codons of Δ6-desaturase (Ma524), respectively (shown in bold).

RO-194 (5′-TACCTCGAATTC**ATG**GCACCTCCCAACACTATCGATGCC-3″

and

RO-195 (5′-AACCGTCTCGAG**TTA**CTTCTTGAAAAAGACCACGTCTCC-3′)

are forward and reverse primers homologous to the 5′- and 3′-ends of the Δ12-desaturase (Ma648), respectively.

The *Eco*RI/*Xho*I putative desaturase gene fragments were cloned into the vector pYES2 (Invitrogen, San Diego, CA) for inducible expression under the control of GAL1 promoter

in yeast. This vector contains a selectable marker gene which confers uracil prototrophy in the host. The plasmids containing the putative Δ6-desaturase (Ma524) and Δ12-desaturase (Ma648) genes were designated as pCGR-5 and pCGR-7, respectively. To construct pCGR11 and pCGR12, the Δ6- and Δ12-desaturase coding regions were isolated from pCGR5 and pCGR7, respectively, as *Eco*R1-*Xho*I fragments and cloned into the pYX242 vector (Novagen, Madison, WI) digested with *Eco*R1-*Xho*I. The pYX242 vector contains a marker gene for selection of leucine prototrophy in the host and has the promoter of TPI (yeast triose phosphate isomerase gene), which allows constitutive expression. Co-expression of recombinant Δ6- and Δ12-desaturases can be achieved by simultaneous introduction of pCGR5 with pCGR12 or pCGR7 with pCGR11 in the appropriate host requiring both uracil and leucine for growth.

Yeast transformation and expression. Different combinations of pCGR5, pCGR7, pCGR11, and pCGR12 were introduced into a host strain of *S. cerevisiae*, SC334, which contains a mutation (*reg1-501*) that alleviates catabolite repression of GAL1 promoter (21). Transformation was done using the PEG/LiAc protocol as described previously (22). Transformants were selected by plating on synthetic medium plates with appropriate selection (21). Cells containing pCGR5 and pCGR7 were selected on media lacking uracil, whereas the pCGR11 and pCGR 12 constructs were selected on media lacking leucine.

Results from our preliminary studies showed that expression of genes (Δ6- and Δ12-desaturases) was enhanced when cultures were grown in synthetic medium at 15°C. In the present study, colonies of transformants were first grown overnight at 30°C in synthetic media. Overnight cultures (2–4 mL) were then used to inoculate 100 mL of minimal media for studying the activities of recombinant desaturases. Galactose was added at a final concentration of 2% to the medium for induction of GAL1 promoter in the strains containing pCGR5 and pCGR7. When the enzyme substrate was provided as the exogenous fatty acid, the fatty acid was supplemented at a concentration of 25 μM. The culture was grown for 48 h at 15°C, and subsequently harvested by centrifugation. Cell pellets were washed once with sterile *dd* H_2O to remove the media. The host strain transformed with vector alone was used as a negative control in all experiments.

Fatty acid analysis. The extraction of the yeast lipids followed the procedures described previously (15). Briefly, washed yeast pellets were extracted with 15 mL of methanol and 30 mL of chloroform containing 100 µg of tridecanoin. After extraction, the yeast lipids were first saponified, and the liberated fatty acids were methylated. The distribution of fatty acid methyl esters was then analyzed by gas chromatography (GC) using a Hewlett-Packard 5890 II Plus gas chromatograph (Hewlett-Packard, Palo Alto, CA) equipped with a flame-ionization detector and a fused-silica capillary column (Supelcomega; 50 m × 0.25 mm, i.d., Supelco, Bellefonte, PA). In the present study, the quantity of the product formed and the rate of conversion of substrate to product (conversion rate = product/(substrate + product)) were calculated to reflect the expression/activity of a given desaturase in this yeast cell assay system.

The identification of a given novel fatty acid was verified by gas chromatography–mass spectrometry (GC–MS) using a Hewlett-Packard mass selective detector (model 5920) operating at an ionization voltage of 70 eV with a scan range of 20–500 Da. The mass spectra of new peaks were compared with those of authentic standards (Nu-Chek-Prep, Elysian, MN) and those in the database NBS75K.L (National Bureau of Standards).

RESULTS

Isolation of a Δ6-desaturase-like cDNA clone from M. alpina. DNA sequence was obtained from the 5′-end of cDNA in randomly picked clones from the *M. alpina* M11 library. Sequence of one such clone, Ma524, exhibited limited homology to a known *Synechocystis* Δ6-desaturase (9) when compared to the databanks. Overall, the level of homology was low (BLAST score 114; P 4.7×10^{-7}). The partial cDNA was used as a probe to isolate a full-length clone, designated pCGN5532, from the M7+8 library. The cDNA insert in pCGN5532 (GenBank accession AF110510) was 1617 bp and contained an open reading frame encoding 457 amino acids flanked by 70 and 75 bp of 5′- and 3′-untranslated regions, respectively. The deduced amino acid sequence is aligned to that of borage Δ6-desaturase (10) in Figure 1. The three "His-boxes," known to be conserved among membrane-bound desaturases (6,14), were found to be present at amino-acid positions 172–176, 209–213, and 395–399 in this sequence. Similar to other membrane-bound Δ6- and Δ5-desaturases, the final "HXXHH" histidine box motif was found to be QXXHH (11,15,16). The predicted amino-acid sequence from this clone is similar to the Δ6-desaturases from the *Synechocystis* spp. and *Spirulina* spp. (9), the borage Δ6-desaturase (10), the nematode *Caenorhabditis elegans* (11), and a cytochrome *b5*/desaturase fusion protein from sunflower (23). As reported for other Δ5/Δ6 desaturases, the amino terminus of the protein encoded by pCGN5532 was also homologous to cytochrome *b5* proteins.

Isolation of a Δ12-desaturase-like cDNA clone from M. alpina. DNA sequence obtained from the 5′-end of another random clone, Ma648, showed homology to the soybean n-6 desaturase (7). The homology of the partial *M. alpina* sequence was again relatively weak (BLAST score 110, P 2.0×10^{-6}). Analysis of the open reading frames beginning at the 5′-end of Ma648 indicated that the first possible methionine was in frame +1 which was the frame that showed desaturase homology. Alignment of this open reading frame to 5′-sequence of other Δ12-desaturases indicated that the *M. alpina* Ma648 clone was full-length. This cDNA was designated pCGN5533, and no other corresponding clones were obtained by library screening. The 1488 bp cDNA insert in pCGN5533 (GenBank accession AF110509) contains 78 bp of 5′- and 113 bp of 3′-noncoding sequences flanking an open

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MaΔ6   1 M A A A P S V R T F T R A E V L N A E A L N E G K K D A E A P F L M I I D N K V Y D V R E F V P D H  50
BoΔ6   1 M A A Q - - I K K Y - - - - I T S D E L K N H - - - D K P G D L W I S I Q G K A Y D V S D W V K D H  41

MaΔ6  51 P G G S V I L T - H V G K D G T D V F D T F H P E A A W E T L A N F Y V G D I D E S D R D I K N D D  99
BoΔ6  42 P G G S F P L K S L A G Q E V T D A F V A F H P A S T W K N L D K F F T G - - - Y Y L K D Y S V S E  88

MaΔ6 100 F A A E V R K L R T L F Q S L G Y Y D S S K A Y Y A F K V S F N L C I W G - - L S T V I V A K W G Q 147
BoΔ6  89 V S K D Y R K L V F E F S K M G L Y D K - K G H I M F A T - - - L C F I A M L F A M S V Y G V L F C 134

MaΔ6 148 T S T L A N V L S A A L L G L F W Q Q C G W L A H D F L H H Q V F Q D R F W G D L F G A F L G G V C 197
BoΔ6 135 E G V L V H L F S G C L M G F L W I Q S G W I G H D A G H Y M V V S D S R L N K F M G I F A A N C L 184

MaΔ6 198 Q G F S S S W W K D K H N T H H A A P N V H G E D P D I D T H P L L T W S E H A L - E M F S D V P D 246
BoΔ6 185 S G I S I G W W K W N H N A H H I A C N S L E Y D P D L Q Y I P F L V V S S K F F G S L T S H F Y E 234

MaΔ6 247 E E L T R - M W S R F M V L N Q T W F Y F P I L S F A R L S W C L Q S I L F V L P N G Q A H K P S G 295
BoΔ6 235 K R L T F D S L S R F F V S Y Q H W T F Y P I M C A A R L N M Y V Q S L I M L L T K - - - - R N V S 280

MaΔ6 296 A R V P I S L V E Q L S L A M H W T W Y L A T M F L F I K D P V N M L V Y F L V S Q A V C G N L L A 345
BoΔ6 281 Y R A - H E L L G C L V F S I - - - W Y - P L L V S C L P N W G E R I M F V I A S L S V T G - M Q Q 324

MaΔ6 346 I V F S L N H N G M P V I S K E E A V D M D F F T K Q I I T G R D V H P G L F A N W F T G G L N Y Q 395
BoΔ6 325 V Q F S L N H F S S S V Y V G K P K G N - N W F E K Q T D G T L D I S C P P W M D W F H G G L Q F Q 373

MaΔ6 396 I E H H L F P S M P R H N F S K I Q P A V E T L C K K Y N V R Y H T T G M I E G T A E V F S R L N - 444
BoΔ6 374 I E H H L F P K M P R C N L R K I S P Y V I E L C K K H N L P Y N Y A S F S K A N E M T L R T L R N 423

MaΔ6 445 - - - - - - E V S K A A S K - - M G K A Q         457
BoΔ6 424 T A L Q A R D I T K P L P K N L V W E A L H T H G 448
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FIG. 1. Comparison of the deduced amino-acid sequences of *Mortierella alpina* and a prototype Δ6-desaturase from borage. Conserved amino acids are shaded light; identical residues are shaded dark. Ma Δ6, *M. alpina* Δ6-desaturase, pCGN5532; Bo 6, Δ6-desaturase from *Borago officinalis* (10). The three regions containing histidine residues (His-boxes) conserved among acyl-lipid desaturases and hydroxylases are overlined.

reading frame encoding 399 amino acids. Figure 2 shows the alignment of the deduced amino acid sequence of pCGN5533 to the *FAD2* (microsomal Δ12 desaturase) from *Arabidopsis* (6). The three His-boxes are again present at positions 111–115, 147–151, and 338–342. Unlike Ma524, no homology to cytochrome *b5* sequence is present on the N-terminus of this clone.

Functional expression of M. alpina *desaturase clone pCGN5533 (Ma648) in yeast*. In order to assess the functional specificity of the various *M. alpina* desaturase clones, the coding regions were expressed in *S. cerevisiae* using the inducible GAL1 promoter found in the commercial vector pYES2. As described previously (15), recombinant yeast cells were grown in the presence of various fatty acids in order to provide substrates for desaturases involved in LC-PUFA production. The deduced coding region of pCGN5533 (Ma648) was inserted into the yeast expression vector pYES2 to create pCGR7. Fatty acid profiles of lipid fractions from yeast grown in the absence of exogenous fatty acid substrate show that two novel fatty acids were produced in SC334(pCGR7) (Fig. 3A). The first fatty acid showed a mass peak $m/z = 266$ (the expected molecular ion of 16:2), a retention time of 13.48 min, and a fragmentation pattern identical to those of Δ9,12-16:2 (Fig. 3B). The second novel fatty acid exhibited a retention time (17.28 min) in GC (Fig. 3A), mass peak ($m/z =$ 294) and fragmentation pattern in GC–MS (data not shown) identical to those of the authentic linoleic acid (Δ9,12-18:2). These findings indicate that the endogenous oleic acid (Δ9-18:1) was converted to linoleic acid (Δ9,12-18:2) by a Δ12-desaturase activity expressed from the plasmid pCGR7. The rate of conversion was found to be 71.4% (Table 1).

Functional expression of M. alpina *desaturase clone pCGN5532 (Ma524) in yeast*. The recombinant yeast SC334(pCGR5), containing the Ma524 cDNA, was grown in the presence of exogenous linoleic acid (Δ9,12-18:2) which is the substrate for Δ6-desaturation. Analyses of the fatty acid profile in the yeast lipid fraction indicate that the exogenous linoleic acid was incorporated into lipids of both nontransformed and transformed yeast (Fig. 4). However, GC analysis revealed the presence of a novel fatty acid in the SC334(pCGR5) yeast that was not present in yeast transformed with vector alone. This novel fatty acid had a retention time of 17.96 min in GC (Fig. 4). Mass peak $m/z = 292$ and fragmentation pattern of this fatty acid in GC–MS were identical to those of the authentic GLA (Δ6,9,12-18:3) standard; however, the fragmentation pattern was different from that of the α-linolenic acid (Δ9,12,15-18:3) standard (data not shown). Thus, the Ma524 cDNA expressed from pCGR5 encodes a Δ6-desaturase. The expressed enzyme converted 29.4% of the incorporated linoleic acid to GLA (Table 1).

Since there were no traces of α-linolenic acid (Δ9,12,15-18:3) produced from the exogenous linoleic acid (Δ9,12-18:2) in the recombinant yeast strains, it is suggested that the enzyme produced by pCGR5 does not possess Δ15-desaturase activity. In addition, when exogenous α-linolenic acid was included in the growth medium, 3.9% of the incorporated α-linolenic acid (Δ9,12,15-18:3) was converted to stearidonic acid (Δ6,9,12,15-18:4). The identity of stearidonic acid was verified by both GC and GC–MS (data not shown). This finding further confirms the enzyme to be a Δ6-desaturase.

TABLE 1
Production of Linoleic Acid and GLA in Yeast Lipid Fraction

SC334 containing	Total fatty acids[a] (μg)	Oleic (wt%)	Linoleic (wt%)	GLA[c] (wt%)
pYES2	440.1	23.2	—	—
pCGR5[b]	497.1	10.2	25.1	10.3
pCGR7	460.9	10.0	24.8	—
pCGR11/pCGR7	340.8	10.2	10.1	7.9
pCGR5/pCGR12	367.9	6.7	7.0	6.6

[a]The volume of culture used for lipid extraction was 100 mL.
[b]Exogenous linoleic acid (25 μM) was added.
[c]No α-linolenic acid was detected. GLA, γ-linolenic acid.

In the absence of exogenous linoleic acid, the lipid fraction of the yeast strain expressing the Δ6-desaturase cDNA produced two novel fatty acids (Fig. 5A). The first novel fatty acid showed a mass peak $m/z = 266$, which is the expected molecular ion of 16:2. Although the GC–MS fragmentation patterns of this novel fatty acid and the authentic Δ9,12-16:2 were similar, they were different in intensity (Figs. 3B and 5B), and retention time (12.89 vs. 13.48 min) in GC (Figs. 3A and 5A). Since this novel fatty acid was produced in the presence of the Δ6-desaturase, it was most probably the Δ6,9-16:2. The second novel fatty acid produced in SC334(pCGR5) had an identical retention time (16.95 min) in GC (Fig. 5A), mass peak $m/z = 294$, and fragmentation pattern in GC–MS to that of the Δ6,9-18:2 standard (data not shown).

Production of GLA. As shown above, the recombinant Δ12- and Δ6-desaturases were effective in converting their substrates (endogenous oleic acid and exogenous linoleic acid) to their respective products, linoleic acid (Fig. 3) in SC334(pCGR7) and GLA (Fig. 4) in SC334(pCGR5). We were interested in determining the feasibility of producing GLA in a recombinant yeast strain in the absence of exogenously added fatty acid substrates. The biosynthesis of GLA from the endogenous oleic acid in *S. cerevisiae* would require the simultaneous expression of Δ12- and Δ6-desaturases. In order to allow co-expression of the Δ6- and Δ12-desaturase cDNA, they were cloned under the control of the constitutive TPI promoter into the leucine-selectable vector pYX242 to create pCGR11 and pCGR12. Both combinations of promoters GAL1 and TPI were assayed for production of GLA. The co-expression of pCGR11 (containing Δ6-desaturase gene under the control of TPI) and pCGR7 (containing Δ12-desaturase gene under the control of GAL1) resulted in *ca.* 7.9% of GLA in total fatty acids of SC334(pCGR7, pCGR11) (Table 1). The rates of conversion from oleic acid to linoleic acid and from linoleic acid to GLA were *ca.* 50 and 44%, respectively. In SC334 (pCGR12, pCGR5) containing the Δ6-desaturase gene behind GAL1 and the Δ12-desaturase gene behind TP1, a level of 6.6% of GLA was found in the total fatty acids (Table 1). In these recombinant yeast strains, the

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MaΔ12    1 M A P P N T I D A G L T Q R H I S T S A P N S A K P A F E R N - Y Q L P E F T I K E I R E C I P A H 49
Athfad2  1 M G - - - - - A G G - - R M P V P T S S K K S E T D T T K R V P C E K P P F S V G D L K K A I P P H 43

MaΔ12   50 C F E R S G L R G L C H V A I D L T W A S L L F L A A T - Q I D K F E N P L I R Y L A W P V Y W I M 98
Athfad2 44 C F K R S I P R S F S Y L I S D I I I A S C F Y Y V A T N Y F S L L P Q P L - S Y L A W P L Y W A C 92

MaΔ12   99 Q G I V C T G V W V L A H E C G H Q S F S T S K T L N N T V G W I L H S M L L V P Y H S W R I S H S 148
Athfad2 93 Q G C V L T G I W V I A H E C G H H A F S D Y Q W L D D T V G L I F H S F L L V P Y F S W K Y S H R 142

MaΔ12   149 K H H K A T G H M T K D Q V F V P K T R S Q V G L P P K E N A A A A V Q E E D M S V H L D E E A P I 198
Athfad2 143 R H H S N T G S L E R D E V F V P K Q K S A I K W Y G K - - - - - - - - - - - - - - - Y L N N - - P L 176

MaΔ12   199 V T L F W M V I Q F L F G W P A Y L I M N A S G Q D Y G R W T S H F H T Y S P I F E P R N F F D I I 248
Athfad2 177 G R I M M L T V Q F V L G W P L Y L A F N V S G R P Y D G F A C H F F P N A P I Y N D R E R L Q I Y 226

MaΔ12   249 I S D L G V L A A - L G A L I Y A S M Q L S L L T V T K Y Y I V P Y L F V N F W L V L I T F L Q H T 297
Athfad2 227 L S D A G I L A V C F G L Y R Y A A A Q - G M A S M I C L Y G V P L L I V N A F L V L I T Y L Q H T 275

MaΔ12   298 D P K L P H Y R E G A W N F Q R G A L C T V D R S F G K F L D H M F H G I V H T H V A H H L F S Q M 347
Athfad2 276 H P S L P H Y D S S E W D W L R G A L A T V D R D Y G - I L N K V F H N I T D T H V A H H L F S T M 324

MaΔ12   348 P F Y H A E E A T Y H L K K L L G E Y Y V Y D P S P I V V A V W R S F R E C R F V E - D - Q G D - - 393
Athfad2 325 P H Y N A M E A T K A I K P I L G D Y Y Q F D G T P W Y V A M Y R E A K E C I Y V E P D R E G D K K 374

MaΔ12   394 - V V F F K K     399
Athfad2 375 G V Y W Y N N K L 383
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FIG. 2. Comparison of the deduced amino-acid sequences of *M. alpina* and a prototype Δ12-desaturase from *Arabidopsis*. Conserved amino acids are shaded light; identical residues are shaded dark. Ma Δ12, *M. alpina* Δ12-desaturase, pCGN5533; Athfad2, Δ12-desaturase from *A. thaliana* (6). The three regions containing histidine residues (His-boxes) conserved among acyl-lipid desaturases and hydroxylases are overlined. See Figure 1 for abbreviation.

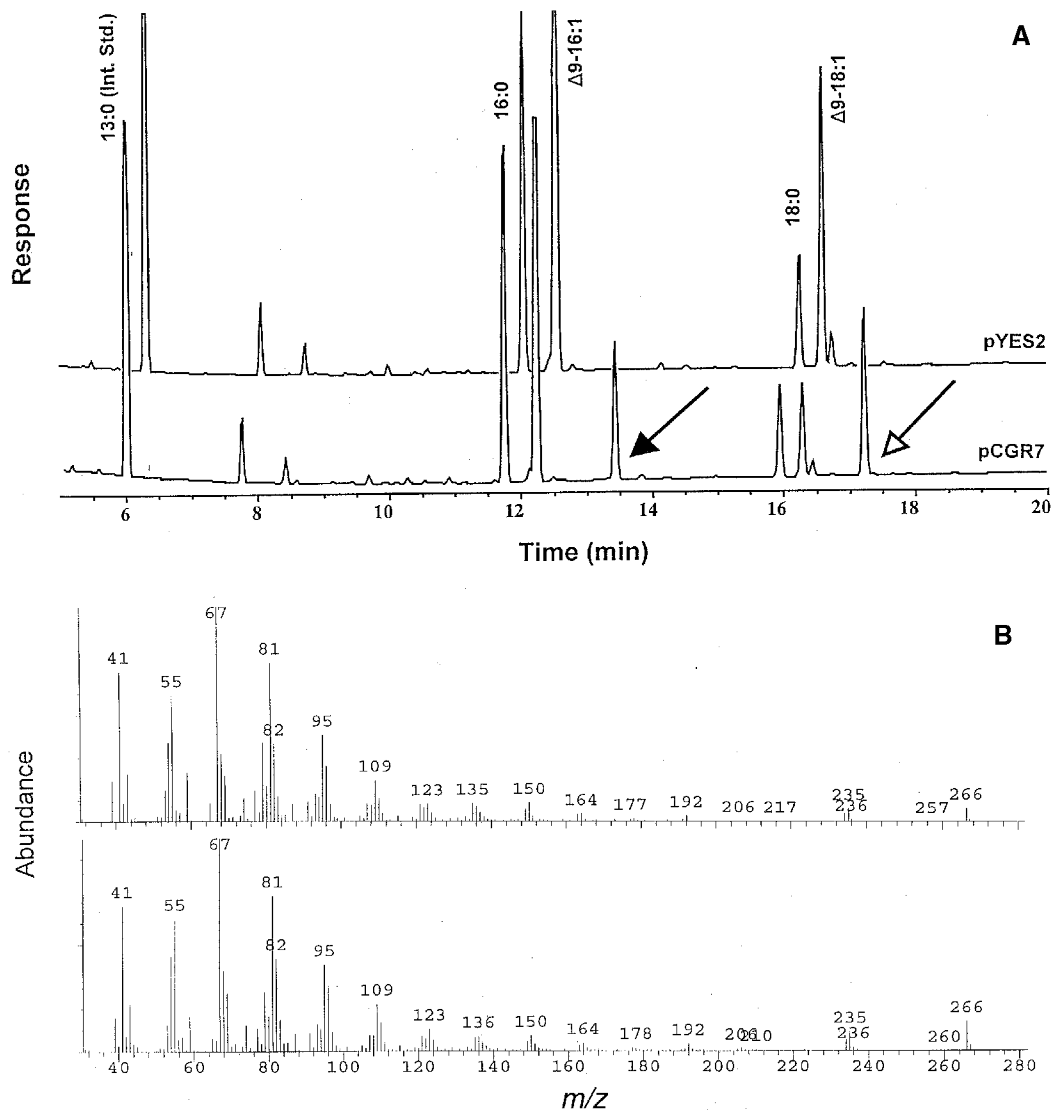

FIG. 3. (A) Gas chromatographic analysis of fatty acid methyl esters (FAME) from the lipid fraction in yeast containing pYES2 or pCGR7. Solid and open arrows indicate the fatty acids Δ9,12-16:2 and Δ9,12-18:2, respectively, present in SC334(pCGR7) cultures. (B) Gas chromatography–mass spectrometry (GC–MS) analysis of the novel peak (identified by the solid arrow in Fig. 3A) in yeast carrying pCGR7. The fragmentation pattern of the first novel peak (top) was compared with that of the authentic Δ9,12-16:2 standard (bottom). pYES2 contained only vector whereas pCGR7 contained the coding region of the *M. alpina* Δ12-desaturase cDNA clone, pCGN5533. All yeast strains were grown in the minimal medium. See Figure 1 for other abbreviations.

conversion rate for both oleic acid to linoleic acid and linoleic acid to GLA was about 50%. Among them, SC334(pCGR11, pCGR7) produced a higher level of GLA, and the GLA accumulated predominantly in the phospholipid fraction (data not shown). Hence, co-expression of *M. alpina* Δ6- and Δ12-desaturase genes under the control of independent promoters in yeast resulted in *de novo* synthesis of GLA.

Comparison of desaturase amino acid sequences. The availability of three desaturase sequences from *M. alpina* was used to examine the interspecies and interclass relationships of these sequences. The amino-acid sequences between the first and third His-boxes of representative desaturases were used to construct a similarity dendrogram (Fig. 6). Two major classes of desaturases can be discerned in this dendrogram. One class contains the Δ12/n-6 and Δ15/n-3 desaturases, while all known examples of Δ5- or Δ6-desaturases fall into a separate class. Although only the central amino-acid core sequence was used in the alignments, all the desaturase sequences with an N-terminal cytochrome *b5*-like sequence cluster into the latter sequence group. The presence of the cytochrome *b5* extension appears to be related to the functionality of the desaturase and not the source of the gene; of the three desaturases from *M. alpina*, only the Δ5- and Δ6-desaturases have the fused cytochrome sequence. In addition, all of the members of this class contain the H-Q substitution in the third His box.

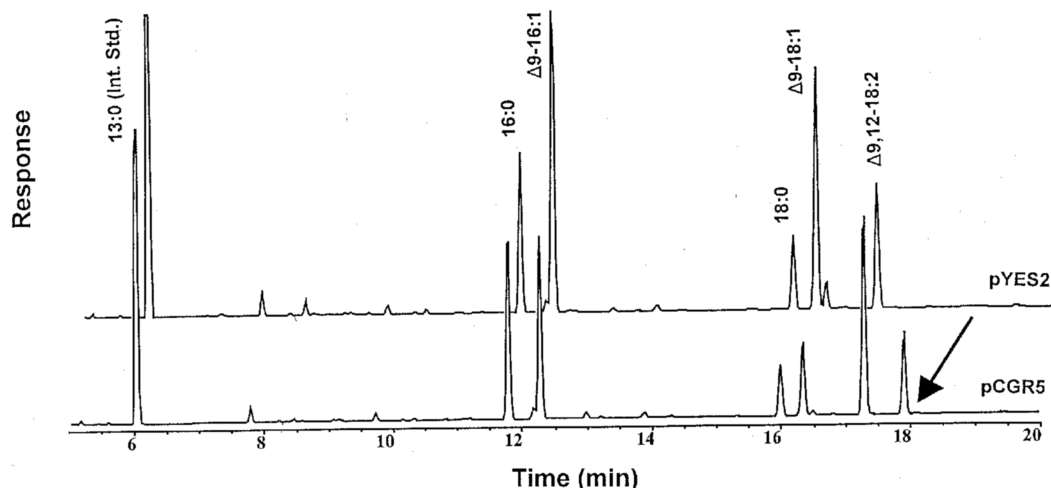

FIG. 4. Gas chromatographic analysis of FAME from the lipid fraction in yeast containing pYES2 or pCGR5. The arrow indicates a novel fatty acid (Δ6,9,12-18:3) present in SC334(pCGR5) cultures. pYES2 contained only vector, whereas pCGR5 contained the coding region of the *M. alpina* Δ6-desaturase cDNA clone, pCGN5532. All yeast strains were grown in minimal medium supplemented with the exogenous linoleic acid (Δ9,12-18:2). See Figures 1 and 3 for abbreviations.

DISCUSSION

We utilized a random sequencing approach to identify cDNA clones encoding fatty acid desaturases from the fungus, *M. alpina*. Partial sequence obtained from the 5′-end of randomly-selected clones was compared to the databanks, and homologies to known acyl-lipid desaturases were noted. This report describes the isolation of two different desaturase-like cDNA clones encoding Δ6- and Δ12-desaturases. These clones were identified in a first-phase sequencing of ~1200 cDNA. In addition to the two clones described in this work, the first phase of sequencing also revealed clones corresponding to the Δ5-desaturase originally obtained by heterologous PCR (15,16) and clones homologous to the yeast stearoyl-CoA desaturase (24) (data not shown). A more thorough sequencing effort of 5400 additional cDNA resulted in the identification of 13 sequences with homology to stearoyl-CoA desaturases, 8 Δ6-desaturases, 9 Δ5-desaturases, and 5 Δ12-desaturases. It should be noted that several of the random clones encoding Δ5- and Δ6-desaturases actually showed cytochrome *b5* matches in the BLAST results, due to the highly homologous cytochrome domain at the N-terminus of these desaturases. Had this domain not been previously identified, several of these cDNA might not have been recognized as desaturases in such a mass sequencing effort. This is an important point to keep in mind when interpreting BLAST results of all sequences; the presence of one highly conserved domain may lead to mis-annotation of the sequence.

The comparison of the desaturase amino-acid sequences shown in Figure 6 indicates that the *M. alpina* Δ5-desaturase is more closely related to the cyanobacterial Δ6-desaturases than to the plant and animal Δ6-desaturase sequences. The ultimate significance of this is hard to evaluate, due to the lack of other Δ5-desaturases for comparison. It should, however, be noted that the *C. elegans* ORF on cosmid T13F2 (GenBank accession number Z81122) that was proposed to be a possible Δ5-desaturase (16) shows more similarity to the *M. alpina* Δ6-desaturase sequence than to the *M. alpina* Δ5-desaturase sequence (data not shown).

In the present study, we showed that the recombinant enzyme expressed by a *M. alpina* desaturase-like gene (Ma648) in pCGR7 converted Δ9-16:1 to Δ9,12-16:2 and oleic acid (Δ9-18:1) to linoleic acid (Δ9,12-18:2) (Fig. 3A). These findings clearly demonstrated that this gene encodes the Δ12-desaturase. We also showed that the recombinant enzyme expressed by another *M. alpina* gene (Ma524) in pCGR5 converted n-6 fatty acid linoleic acid (Δ9,12-18:2) to GLA (Δ6,9,12-18:3) (Fig. 4). When an n-3 fatty acid, α-linolenic acid (Δ9,12,15-18:3), was used as the substrate, the SC334(pCGR5) produced the expected product, stearidonic acid (Δ6,9,12,15-18:4) (data not shown). In the absence of linoleic acid as substrate, this recombinant enzyme could convert the endogenous Δ9-16:1 to Δ6,9-16:2, and oleic acid (Δ9-18:1) to Δ6,9-18:2 (Fig. 5A). These findings demonstrate that this gene encodes the Δ6-desaturase.

In order to evaluate the feasibility of producing GLA—a high-value PUFA in this microorganism, we co-expressed the genes encoding Δ6- and Δ12-desaturases in yeast. When both genes were presented in a single construct in yeast, and expressed from a single promoter, GAL1, none of these transformed yeast strains produced a significant amount of GLA (data not shown). Therefore, it seemed likely that two independent promoters would be needed for the concurrent expression of these two desaturases. Indeed, when these desaturases were co-expressed *in trans* from two independent promoters, GAL1 and TPI, the production of GLA reached as high as 8% of the total lipids in yeast grown without exogenous substrates (Table 1). By the action of two separate pro-

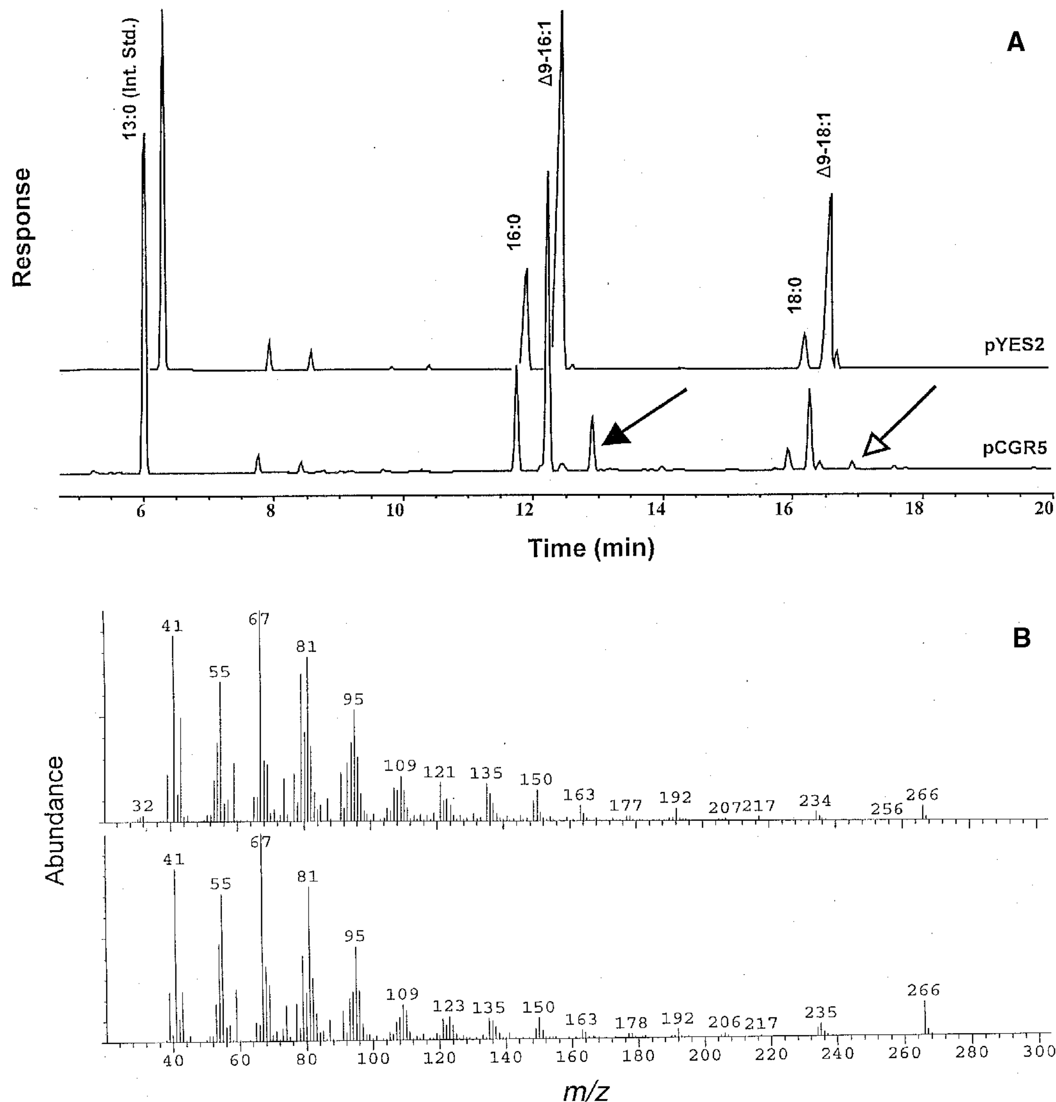

FIG. 5. (A) Gas chromatographic analysis of FAME from the lipid fraction in yeast containing pYES2 or pCGR5 grown without exogenous substrate. Solid and open arrows indicate novel fatty acids Δ6,9-16:2 and Δ6,9-18:2, respectively. (B) GC–MS analysis of the novel peak (identified by the solid arrow in panel A) in yeast carrying pCGR5. The fragmentation pattern of the first novel peak was compared with that of the authentic Δ6,9-16:2 standard pYES2 contained only vector, whereas pCGR5 contained *M. alpina* cDNA clone encoded with Δ6-desaturase. See Figures 1 and 3 for abbreviations.

moters, these enzymes were able to effectively convert (*ca.* 50%) their respective substrates to products.

In summary, we isolated two cDNA from *M. alpina* encoding the Δ6- and Δ12-desaturase genes using a random sequencing-based strategy. The identities of the two cDNA confirmed by functional expression and analysis in a widely used microorganism, baker's yeast. By introducing the two required desaturases (Δ6- and Δ12-) under the control of independent promoters in yeast, we developed a novel approach to synthesize GLA.

ACKNOWLEDGMENTS

The nucleotide sequences reported in this paper were submitted to the GenBank/EBI Data Bank with accession numbers AF110509 and AF110510.

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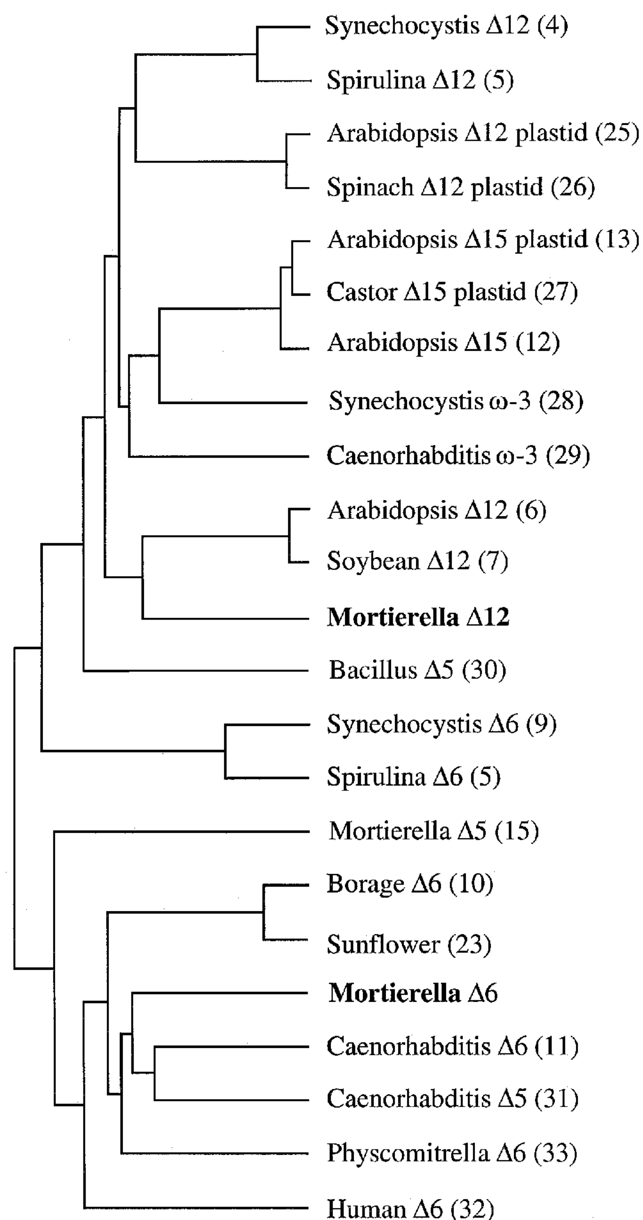

FIG. 6. Similarities of representative membrane-bound desaturases. Dendrogram was constructed using the CLUSTAL program to align deduced amino-acid sequences between the first and third His boxes. Numbers in parentheses indicate the references for the sequences; *Mortierella* Δ6- and Δ12-desaturase sequences are described in this work.

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Intermediates and Products Formed During Fatty Acid α-Oxidation in Cucumber *(Cucumis sativus)*

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ABSTRACT: Fatty acid α-oxidation is an essential metabolic pathway both in plants and in mammals which is still not completely understood. We previously described and purified an α-oxidation enzyme in cucumber which has been used in the present investigation of the α-oxidation reaction mechanism. Free fatty acids, and not the CoA thioesters, were found to undergo α-oxidation in cucumber. 2-Hydroxy- and 2-oxopalmitic acids were identified as palmitic acid α-oxidation intermediates by high-performance liquid chromatography and gas chromatography–mass spectrometry analysis in cucumber subcellular 150,000 × g_{max} pellets obtained by differential centrifugation. Incubation of purified α-oxidation enzyme with [1-^{14}C]palmitic acid resulted in the formation of both the above-described intermediates and the C_{n-1} product, pentadecanal, and $^{14}CO_2$. Besides $^{14}CO_2$, ^{14}C-formate was identified as an α-oxidation product from [1-^{14}C]palmitic acid in cucumber subcellular fractions. Fe^{2+} stimulated the $^{14}CO_2$ and ^{14}C-formate production, and the addition of ascorbate and 2-oxoglutarate together with Fe^{2+} resulted in optimal α-oxidation activities, suggesting a dioxygenase reaction mechanism, as previously shown in mammals. NADPH and, to a lesser extent, NADH stimulated the total ^{14}C-formate plus $^{14}CO_2$ production but had only slight or no effects on $^{14}CO_2$ production. H_2O_2 showed concentration-dependent inhibitory effects, while FAD had neither effect on $^{14}CO_2$ nor $^{14}CO_2$ plus ^{14}C-formate production. The results in the present study demonstrate that an α-oxidation enzyme in cucumber is capable of oxidizing palmitic acid *via* 2-hydroxy- and 2-oxopalmitic acid to produce pentadecanal and CO_2. In contrast to the subcellular 150,000 × g_{max} fraction, the purified α-oxidation enzyme could neither produce formate nor convert ^{14}C-formate into $^{14}CO_2$, indicating two possible α-oxidation routes in cucumber.

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When intact plant tissues are subjected to stress factors such as physical wounding or infection, their membrane lipids are susceptible to enzymatic degradation (1–4). In plants, the free fatty acids formed by lipolytic enzyme action have several pathways for partial or complete oxidation of the hydrocarbon chain. Saturated long-chain fatty acids (C_{12}–C_{18}) and unsaturated C_{18} fatty acids can readily undergo α-oxidation to yield a hydrocarbon with one less carbon atom and CO_2 (4,5), catalyzed by an α-oxidizing enzyme previously purified from cucumber (6).

The metabolic role of fatty acid α-oxidation in plants is still uncertain, although the α-oxidation pathway was implicated in initial wounding and cell damage respiration. Long-chain fatty aldehydes produced in the α-oxidative pathway seem connected to characteristic flavors which have different functions in plants (for review see Refs. 4,7). The α-oxidation reaction has been known since 1952 (8), and the reaction mechanism and subcellular localization of the enzyme(s) in plants, and later, also in mammals, algae, bacteria, and yeast have been regularly discussed since. The earliest proposed mechanism of fatty acid α-oxidation in higher plants, based on a series of studies on fat metabolism in peanut seeds (9) and pea leaves (10–12), involves the proposed intermediates 2-hydroxy and 2-oxo fatty acids and the products CO_2 and a C_{n-1} fatty aldehyde. Later, a reaction sequence involving the 2-hydroperoxyl-C_n fatty acid as a transitory intermediate was proposed (13). The aldehyde is finally oxidized to the C_{n-1} fatty acid by an NAD-dependent aldehyde dehydrogenase .

Fatty acid α-oxidation was studied in cucumbers (*Cucumis sativus*) because of the very active α-oxidation system in this tissue (5,6,14). Pentadecanal was identified as an α-oxidation product from palmitic acid (hexadecanoic acid) in cucumber homogenates (14) and subcellular fractions (6). In edible seaweeds, cultivated on the Japanese coast, long-chain fatty aldehydes were identified as characteristic flavor compounds, originating from fatty acid α-oxidation (15,16). Matsunaga *et al.* (17) reported bacterial α-hydroxylation activity with the formation of 2-hydroxymyristic acid from myristic acid in the presence of H_2O_2. Recently, the bacterial fatty acid α-hydroxylase was cloned and identified as a member of the cytochrome P450 superfamily (18).

In mammals, α-oxidation is essential for the metabolism of 3-methyl-substituted fatty acids such as the naturally occurring phytanic acid (3,7,11,15-tetramethylhexadecanoic acid) which cannot initially undergo β-oxidation (for review see Ref. 19). Recently, it was demonstrated that 3-methyl-substituted fatty acid α-oxidation consists of an activation step leading to the fatty acyl-CoA ester (20), followed by a 2-

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Abbreviations: GC, gas chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry.

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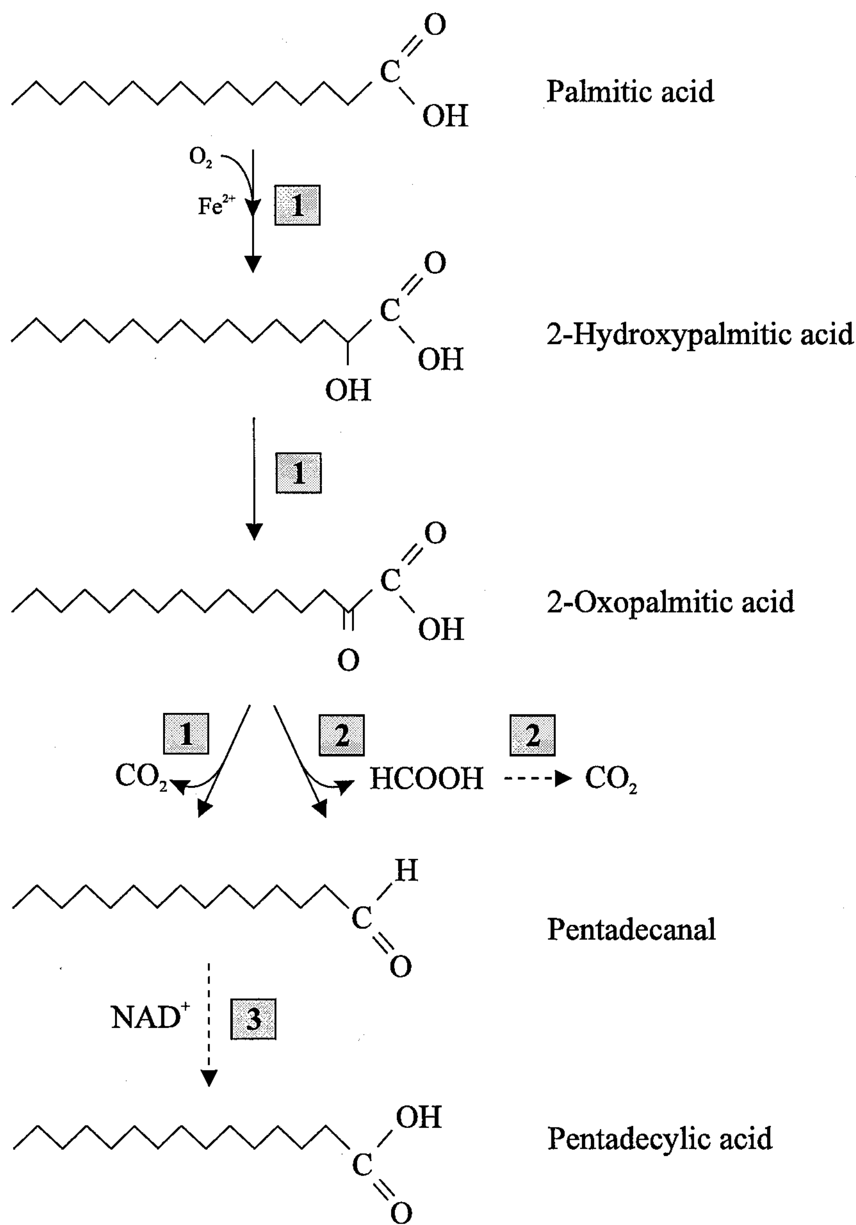

SCHEME 1

hydroxylation, yielding a 2-hydroxy-3-methylacyl-CoA intermediate in rat (21,22) and human (23,24) liver peroxisomes. The gene encoding phytanoyl-CoA hydroxylase was cloned and identified in humans (25,26). Formic acid was demonstrated to be the nonlipid α-oxidation product in human fibroblasts (27) and in rat liver (28). Recently, formyl-CoA, rather than formate, was shown to be the end product of α-oxidation of 3-methyl palmitic acid in rat liver peroxisomes (29). The fatty end product generated in mammalian α-oxidation was very recently shown to be an aldehyde (30,31) as in plants (4).

The aim of the present study was to further investigate the formation of α-oxidation intermediates and products in cucumber subcellular fractions and in purified α-oxidation enzyme fraction. The results presented show that palmitic acid, and not palmitoyl-CoA, is the preferred α-oxidation substrate, which can be converted to pentadecanal with 2-hydroxy and 2-oxopalmitic acid as intermediates by the purified α-oxidation enzyme. The only nonlipid long-chain fatty acid α-oxidation end product identified in plants is the C_1- unit in the form of CO_2. In the present study we investigated whether ^{14}C-formate was produced from [1-^{14}C]palmitic acid during fatty acid α-oxidation. Based on the results obtained, a fatty acid α-oxidation pathway in cucumber was proposed (Scheme 1, where the numbered boxes indicate the proposed enzymes involved: 1 is α-oxidation enzyme; 2 is unknown; 3 is aldehyde dehydrogenase). Several cofactors were examined in earlier plant studies; however, none has been reported to give markedly positive effects on fatty acid α-oxidation activity measured as $^{14}CO_2$ production (3,5,9,10,14). Finally,

the effects of possible required cofactors on α-oxidation activity were examined in cucumber.

MATERIALS AND METHODS

Materials. Cucumber fruits (*C. sativus*) were purchased from the local markets. [1-^{14}C]Palmitic acid (55.0 mCi/mmol), [1-^{14}C]palmitoyl-CoA (47.8 mCi/mmol), and sodium [^{14}C]-formate (56.0 mCi/mmol) were provided by Amersham Pharmacia Biotech (Buckinghamshire, United Kingdom). Pentadecanal was synthesized by oxidation of 1-pentadecanol using pyridinium chlorochromate as described (32). Tridecanal was provided by Chem Service Inc. (West Chester, PA). Unlabeled palmitic acid, palmitoyl-CoA, 2-hydroxypalmitic acid, sodium formate, and Triton X-100 were obtained from Sigma Chemical Co. (St. Louis, MO). Mercuric acetate and ethyl acetate were from Fluka Chemie AG (Buchs, Switzerland), methanolic-HCl (3 M) was from Supelco, Inc. (Bellefonte, PA), and BCA Protein Assay Reagent was from Pierce (Rockford, IL).

Preparation of subcellular fractions and purification of α-oxidation enzyme. Fresh cucumbers were washed, peeled, cut, and homogenized in 50 mM HEPES, 0.25 M sucrose, and 1 mM EDTA, pH 7.4 at 4°C, before filtration and differential centrifugation as described previously (6) (see Fig. 1). All organellar fractions were assayed for $^{14}CO_2$ liberation and ^{14}C-formate formation, and the pellet obtained after centrifuging a 37,500 × g_{max} supernatant at 150,000 × g_{max} for 70 min re-

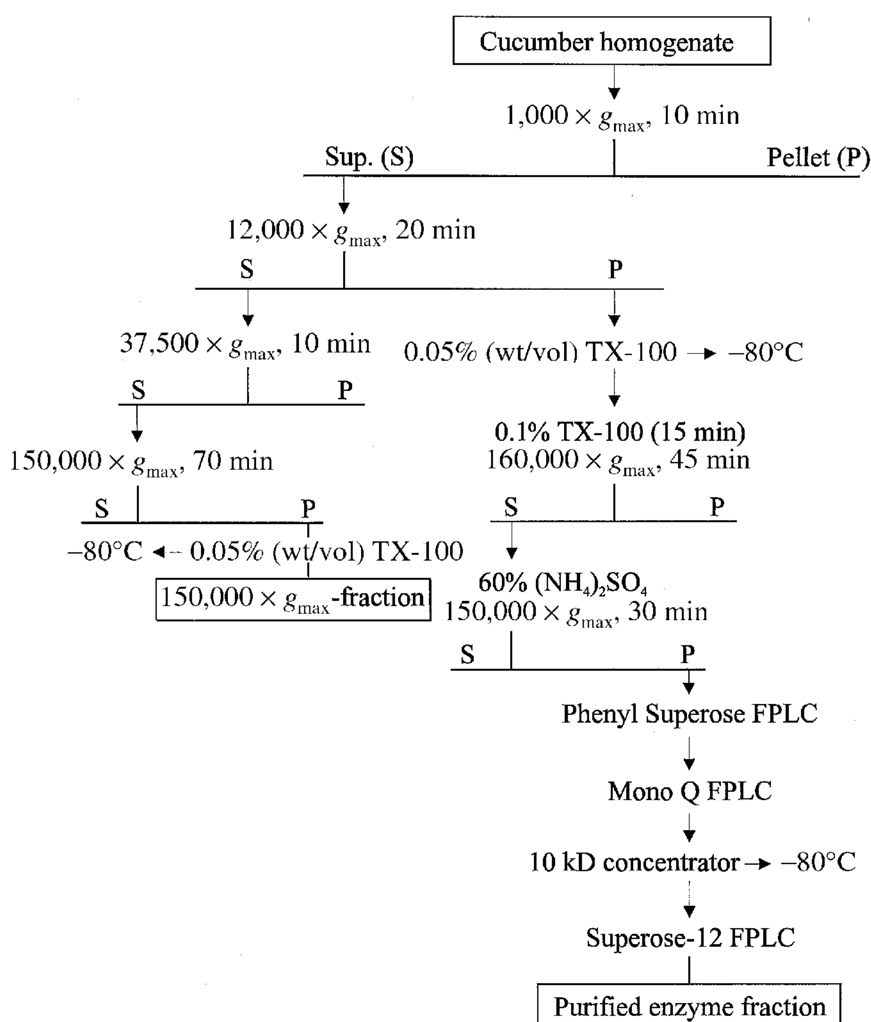

FIG. 1. Purification scheme for α-oxidation enzyme activity from cucumber. Cucumbers were peeled, coarsely cut, and homogenized in 50 mM HEPES, 0.25 M sucrose, 1 mM EDTA, pH 7.4 with a vertical cutter. After filtration the cucumber homogenate was subcellular fractionated by differential centrifugation as described before (6) and shown in this scheme. The 150,000 × g_{max} fraction was used as the crude α-oxidation enzyme source in our studies. The purification of the α-oxidation activity started with the 12,000 × g_{max} pellet and resulted in a near homogeneity purified α-oxidation enzyme with the molecular mass 240,000 (6). The α-oxidation activity in Superose-12 purified enzyme fraction, measured as $^{14}CO_2$ production, was 550 nmol × min^{-1} × (mg protein)$^{-1}$.

vealed the highest rates of $^{14}CO_2$ and ^{14}C-formate plus $^{14}CO_2$ formation per mg protein. The 150,000 × g_{max} pellets were resuspended in homogenization buffer with 0.05% (wt/vol) Triton X-100, and the 150,000 × g_{max} fraction was stored in small aliquots at −80°C and used in the further incubations.

The 12,000 × g_{max} pellets were subjected to further purification (6), and the purification steps are shown in Figure 1. The fractions with α-oxidation activity collected from fast protein liquid chromatography on Mono Q column (Amersham Pharmacia Biotech, Uppsala, Sweden) were subjected to concentration in 10 kDa Centriplus concentrators (Amicon, Bedford, MA). This enzyme material was stored in aliquots at −80°C over a period of several weeks without losing enzyme activity. When used for incubations, the enzyme samples were thawed and gel-filtered on a Superose-12 column (Amersham Pharmacia Biotech, Uppsala). The α-oxidation activity in the Superose-12 fractions was measured and the peak fractions were combined. The purity of the α-oxidation enzyme fraction was checked using native 4–15% gradient polyacrylamide gel electrophoresis. This enzyme fraction is described as "purified enzyme fraction" in the following.

Protein concentrations were determined with bicinchoninic acid (33) using bovine serum albumin as the standard protein.

Substrate preparation, incubation, and measurement of α-oxidation C_1-products. Fatty acid substrate solution was made of 10.0 mg unlabeled palmitic acid and [1-^{14}C]palmitic acid dissolved together in 0.2 mL toluene. Substrate buffer [50 mM HEPES, pH 7.4, containing 0.1% (wt/vol) Triton X-100 and 1 mM EDTA] was added dropwise to the toluene solution under mixing and taken up to the stock concentrations 0.1–2.0 mM (0.08 μCi). Unlabeled 2-hydroxypalmitic acid substrate solution was prepared as the palmitic acid substrate solution. [1-^{14}C]Palmitoyl-CoA was dissolved in 50 mM HEPES, pH 7.4 to the stock concentration 0.1 mM. The purity of the substrates was confirmed by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry (GC–MS) before incubation.

Incubations and measurement of liberated $^{14}CO_2$ from [1-^{14}C]fatty acids were performed essentially as described previously (6) using a variation of the method described by Galliard and Matthew (14). Samples were incubated in 25-mL conical flasks sealed with rubber caps (Kontes Glass Co., Vineland, NJ). The incubation mixture (final volume 1.0 or 2.0 mL) contained 50 mM HEPES buffer (pH 7.4), 1 mM EDTA, 150,000 × g_{max} fraction (0.09–0.2 mg protein) or purified enzyme fraction (2.5 μg protein), and the reaction was started by the addition of 0.05 mM [1-^{14}C]palmitic acid (0.04 μCi) in substrate buffer. The control samples consisted of all assay components together with protein boiled for 5 min. The reaction was performed at 25°C with shaking for 20 min (or other indicated periods of time), after which termination was achieved by adding 0.2 mL 1.0 M HCl, except when otherwise mentioned. $^{14}CO_2$ was trapped in hanging wells (Kontes) with 0.2 mL β-phenylethylamine in methanol (1:1, vol/vol). The radioactivity was measured by liquid scintillation counting (Beckman LS6000IC, Beckman Coulter, Inc., Fullerton, CA) after adding 5 mL Insta Gel II (Packard Instruments, Groningen, The Netherlands).

^{14}C-Formic acid was measured as described by Yang (34) with minor modifications of the method described by others (27,28). The incubations were performed as described above, and the reactions were terminated after 10 min (or as otherwise described) with 0.2 mL 1 M potassium phosphate, pH 2. Mercuric acetate (0.25 g in 1 mL H_2O) was added using a syringe through the rubber caps. After 40 min at 85°C, liberated $^{14}CO_2$ was trapped and measured as described above. The recovery was based on $^{14}CO_2$ production from ^{14}C-formate (0.05 mM).

HPLC analysis of palmitic acid α-oxidation intermediates. After termination of the enzyme reaction, the flasks with the incubated 150,000 × g_{max} fraction or purified enzyme fraction were kept on ice until, as soon as possible, they were transferred to an extraction tube and extracted twice with 3 vol of ethyl acetate. The organic phase was washed with 1 mL water, and the ethyl acetate was evaporated to dryness under N_2 after which 0.1 mL methanol was added. The samples were analyzed by reversed-phase HPLC essentially as described by Huang *et al.* (35) on a Spectra-Physics HPLC equipped with Spectra System P4000 Quat Gradient pump and a Nova-Pak C_{18} column (150 × 3.9 mm) (Waters, Milford, MA). The [1-^{14}C]metabolites were separated with a gradient of methanol and water, both with 0.1% (vol/vol) acetic acid, starting with 70% methanol, isocratic for 15 min, and then increasing to 100% methanol in 20 min, at a flow rate of 0.8 mL/min. Column effluents were monitored on-line using a radioactivity detector (Packard Radiomatic Flo-One/β, series A-100; Packard Instrument, Palo Alto, CA), with liquid scintillant (Pico Aqua from Packard Instrument) mixed with the eluate in a ratio of 3:1 (vol/vol). When fractions were collected for further analysis by GC–MS, aliquots of each column fraction were counted for radioactivity, and the radioactive fractions in each peak were combined. Unlabeled fatty acids and fatty acid derivatives were monitored with a DDL21 light-scattering detector (Eurosep Instruments, Cergy-Pontoise Cedex, France).

HPLC analysis of water-soluble products. The α-oxidation incubations were performed with a total volume of 1.0 mL, and the reactions were terminated with 0.2 mL of 6% (wt/vol) perchloric acid. The samples were then transferred to Eppendorf tubes and centrifuged at 13,000 × g for 10 min at 4°C. The pellet was resuspended in 0.1 mL 6% perchloric acid and centrifuged again. The total supernatant was frozen at −20°C and thawed just prior to the injection of 0.25 mL on an Aminex HPX-87H column (300 × 7.8 mm, 9 μm) (Bio-Rad Laboratories, Richmond, CA). The column was eluted with 6.0 mM sulfuric acid at a flow rate of 0.4 mL/min, isocratic for 40 min, and with 40% acetonitrile in 6.0 mM sulfuric acid for an additional 30 min. The eluate was monitored on-line at 210 nm using a Spectra Focus scanning detector (Spectra-Physics Inc., San Jose, CA), and 0.8 mL fractions were collected and counted for radioactivity. ^{14}C-Labeled formate was used as a standard and for determination of method recovery. Other organic acid standards were unlabeled.

GC–MS of α-oxidation intermediates and products. Incubations of the 150,000 × g_{max} fraction or purified enzyme fraction with the fatty acid substrate solution were performed as described above. The collected and combined radioactive fractions from reversed-phase HPLC were dried under N_2. The fatty acid moieties were derivatized using 3 M methanolic-HCl and extracted into 50 µL hexane. The identification of the α-oxidation intermediates was performed by a Hewlett-Packard 5890 series II gas chromatograph equipped with a split/splitless injector and an electron impact mass selective detector HP 5970 MSD (Hewlett-Packard Co.). The injection port and GC–MS interface were kept at 250°C. After splitless injection of 2 µL sample, the methyl esters were separated on a DB-23 MS column (30 m, 0.25 mm i.d., 0.25 µm film thickness) (J&W Scientific, Folsom, CA) with helium as carrier gas. The column oven temperature program was 100°C (4 min); 20°C/min; 160°C (2 min); 3°C/min; 220°C; 15°C/min; 240°C (10 min).

For analysis of fatty aldehydes in incubations of purified enzyme fraction (40 µg protein) with unlabeled palmitic acid (1.0 mM), tridecanal was added as internal standard (50 nmol) after termination of the reactions. The acidified samples were extracted twice with 3 vol of ethyl acetate. The ethyl acetate phase was washed with water and evaporated to dryness under N_2. The residue was dissolved in 50 µL hexane; 2 µL of the sample were subjected to GC–MS analysis, and the components were separated on a DB-23 MS column with the temperature program 60°C (1 min); 10°C/min, 220°C (2 min); 15°C/min, 240°C (20 min).

Aqueous fractions (50 µL) eluted from the Aminex HPLC column were applied into a glass tube packed with silanized glass wool. Volatiles from the glass wool were desorbed in a Perkin-Elmer Automatic Thermal Desorber ATD 400 (Perkin-Elmer Co., Norwalk, CT) and concentrated on a Tenax GR-packed coldtrap before injection on the gas chromatograph. The injected volatile compounds were separated on a DB-5 MS column (30 m, 0.25 mm i.d., 1 µm film thickness) (J&W Scientific) in an HP5890 series II GC. The volatiles were detected with an HP 5970 mass-selective detector in the scanning mode from molecular mass 29 to 350. Analyses of the mass spectra were based on the reference spectra of authentic standards.

Statistical analysis. The enzyme activities are expressed as mean values ± SEM of the indicated number of experiments. The significance of the differences within the cofactor experiments was tested by analysis of variance (ANOVA function in Minitab, Release 11.12, 32 bit, Minitab Inc., State College, PA) and two sample *t*-tests (Minitab).

RESULTS

Incubation of free fatty acid and acyl-CoA. To investigate whether an acyl-CoA thioester could act as a substrate for the cucumber α-oxidation system, we incubated cucumber homogenate, 150,000 × g_{max} fraction, or purified enzyme fraction with [1-^{14}C]palmitoyl-CoA. Incubation of the crude fraction for 60 min or more revealed very small amounts of $^{14}CO_2$, most likely because the palmitoyl-CoA solution contained small quantities of free palmitic acid due to thioester hydrolysis. No ^{14}C-formate production was detected in the [1-^{14}C]-palmitoyl-CoA incubations. Purified enzyme fraction incubated with [1-^{14}C]palmitoyl-CoA did not result in any production of $^{14}CO_2$ or ^{14}C-formate. HPLC and GC analysis of the ethyl acetate phase revealed no production of any ^{14}C-labeled intermediates, or pentadecanal as product with [1-^{14}C]palmitoyl-CoA as substrate (data not shown). These results establish the fact that the free fatty acids, and not CoA thioesters, are substrates for α-oxidation enzymes in cucumber.

Studies of palmitic acid α-oxidation intermediates. In our earlier studies (6), purified α-oxidation enzyme fraction from cucumber formed $^{14}CO_2$ from [1-^{14}C]palmitic acid without added cofactors. This finding introduced the possibility of an α-oxidative multifunctional enzyme with a possible hydroxylation and decarboxylation activity rather than two or more single enzymes catalyzing the α-oxidation reaction steps.

In order to identify putative palmitic acid α-oxidation intermediates, the subcellular 150,000 × g_{max} fraction was incubated with [1-^{14}C]palmitic acid, extracted, and analyzed by reversed-phase HPLC and GC–MS. The HPLC profile of the ethyl acetate extracts showed three radioactive peaks, void peak I, peaks II and III, in addition to the peak S corresponding to the unreacted palmitic acid (Fig. 2A). When heat-inactivated enzyme fraction was incubated, only the substrate peak S appeared. The small radioactive peak I in the void volume was further studied under HPLC conditions described for water-soluble products described in the Materials and Methods section. In a typical experiment, where 150,000 × g_{max} fraction (0.1 mg protein) was incubated with 0.05 mM [1-^{14}C]palmitic acid for 20 min, the percentage distribution of radioactivity was *ca*. 1–2% in void peak I, 10% in peak II, 30% in peak III, and the rest in the unreacted substrate peak S (Fig. 2A). The unknown peak III co-eluted with an unlabeled authentic 2-hydroxy palmitic acid standard on HPLC, measured with a light-scattering detector (data not shown). To verify the identity of this ^{14}C-labeled intermediate, the HPLC peak III fractions were collected, and the material was methylated before being subjected to combined GC–MS. One major peak at 24.5 min, whose elution time and mass spectra corresponded to the methyl ester of the authentic 2-hydroxy palmitic acid standard, was observed in the GC chromatogram (GC profile not shown). Figure 2B shows the electron impact mass spectrum of peak III, which corresponded to that of 2-hydroxy palmitic acid. The most characteristic ion at *m/z* 227 is caused by fragmentation alpha to the 2-hydroxyl group, and the elimination of methanol gives the ion at *m/z* 195. The expected molecular ion is present at *m/z* 286 and a typical McLafferty rearrangement at *m/z* 90. The second unknown ^{14}C-labeled peak in the HPLC profile, (peak II in Fig. 2A), was collected, methylated, and analyzed by GC–MS. This compound was identified as 2-oxopalmitic acid methyl ester using Wiley mass spectral data base (John Wiley & Sons Inc., licensed to Hewlett-Packard, Rev. 00.00.1989). The mass spectrum is shown in Figure 2C, and the fragmen-

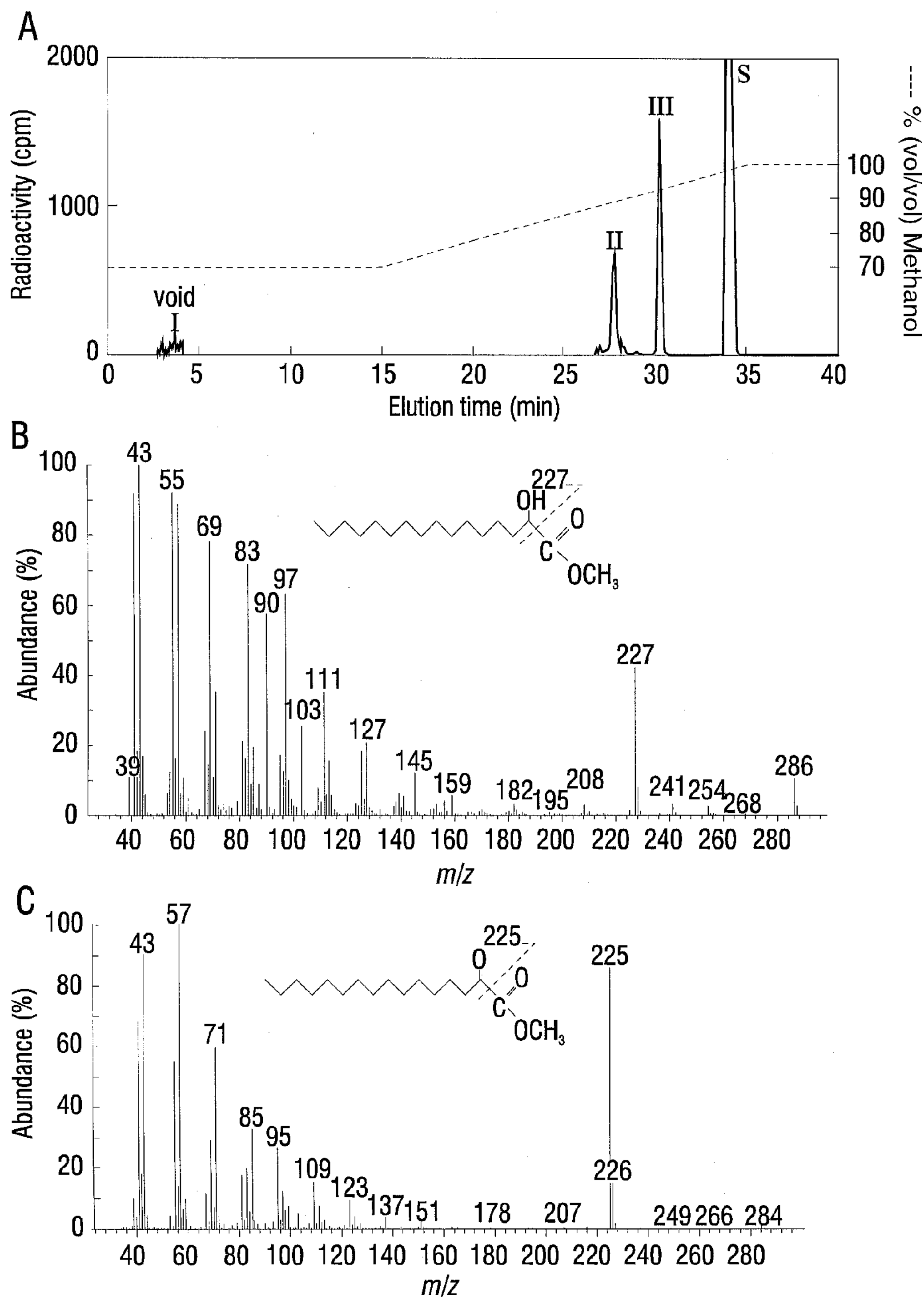

FIG. 2. Reversed-phase high-performance liquid chromatography (HPLC) analysis of palmitic acid α-oxidation products and identification of reaction intermediates by gas chromatography–mass spectrometry (GC–MS). Subcellular 150,000 × g_{max} pellets (0.2 mg protein), resuspended in 50 mM HEPES, pH 7.4, 0.05% Triton X-100 and 1 mM EDTA, were incubated with 0.05 mM [1-^{14}C]palmitic acid in 25-mL flasks sealed with rubber caps at 25°C. Incubations (total volume 2.0 mL) were terminated after 20 min by the addition of 0.2 mL 1 M HCl, and the reaction media were extracted with ethyl acetate. After evaporating the solvent, the organic residues were dissolved in 0.1 mL methanol and analyzed by reversed-phase HPLC on a C_{18}-column eluted with methanol and water both with 0.1% (vol/vol) acetic acid at 0.8 mL/min. The palmitic acid and the metabolites were separated with a gradient starting with 70% (vol/vol) methanol increasing to 100% in 20 min as shown in A (dashed line). The radioactivity in the column eluate was monitored on-line (solid line). For identification of peaks II and III, fractions from HPLC were collected, and the methylated moieties were analyzed by GC–MS. The mass spectrum of the methylated compound in peak III was identified as 2-hydroxypalmitic acid (B) and peak II was identified as 2-oxopalmitic acid (C), with molecular peaks at *m/z* 286 and 284, respectively.

tation patterns include the intense *m/z* 225 ion formed by a characteristic α-cleavage to the 2-oxo group and the molecular ion present at *m/z* 284.

When purified enzyme fraction was incubated with [1-^{14}C]palmitic acid, the HPLC elution patterns showed the same two ^{14}C-labeled peaks described above for the subcellular 150,000 × g_{max} fraction, identified as the 2-hydroxy and 2-oxopalmitic acids (data not shown).

Palmitic acid α-oxidation products in the purified enzyme fraction. In this work the formation of pentadecanal, which is the [1-^{14}C]palmitic acid α-oxidation product beside $^{14}CO_2$, was investigated. Since purified enzyme was shown to convert [1-^{14}C]palmitic acid into $^{14}CO_2$, a fatty product with one carbon less than the substrate was expected to be found. Figure 3A shows the GC profile of the aldehyde standards, and Figure 3B shows that the α-oxidation products in the purified enzyme fraction co-eluted with the synthesized pentadecanal standard. The peak with retention time 14.1 min is consistent with pentadecanal, and the mass spectrum of this peak confirmed this identity. A linear increase of pentadecanal, as quantified relative to the internal standard tridecanal (ret. time 12.0 min), was observed during the first 30 min of reaction (data not shown). The estimated specific activity of pentadecanal formed was 340 ± 26 nmol × min^{-1} × (mg protein)$^{-1}$ (±SEM), calculated by dividing the peak area and ion abundances of four different ions of pentadecanal by the same peak area and ion abundances of the internal standard tridecanal.

Incubations of crude enzyme fraction or purified enzyme fraction with unlabeled 2-hydroxypalmitic acid, one of the proposed intermediates, resulted in formation of pentadecanal. Figure 4 shows a time-dependent increase of the C_{15}-aldehyde from 2-hydroxypalmitic acid in purified enzyme fraction. The estimated amount of pentadecanal formed was *ca*. 20-fold less with 2-hydroxypalmitic acid than with palmitic acid as the α-oxidation substrate. When pentadecanal was added to the incubations, a concentration-dependent inhibitory effect on the α-oxidation activity was observed (data not shown).

Identification of formate as an α-oxidation product in the crude 150,000 × g_{max} fraction. Since the existence of formate as an α-oxidation end product besides CO_2 was described in mammals (24,25), but not in plants, we analyzed the incubation mixtures for water-soluble ^{14}C-labeled products by ion-exchange HPLC, to investigate whether or not formate was produced in the 150,000 × g_{max} fraction. The HPLC chromatograms of the samples incubated for 5, 10, and 20 min showed two major ^{14}C-labeled peaks eluted with 6 mM sulfuric acid (Fig. 5A–C). The first peak appeared after 22 min (elution volume 8.8 mL) and the second peak after 27 min (10.8 mL), and both peaks showed a time-dependent increase. The first peak co-eluted with an authentic formate standard (Fig. 5D), and GC analysis confirmed this identification. The second radioactive peak corresponded to propionic acid from the HPLC analysis of an authentic standard (Fig. 5D); however, GC–MS identification was not successful.

When cucumber 150,000 × g_{max} fraction was incubated with [1-^{14}C]palmitic acid, a significant amount of formate was produced, measured as $^{14}CO_2$ after oxidizing the ^{14}C-formate with mercuric acetate. In Figure 6, the time-dependent formate and CO_2 production in 150,000 × g_{max} subcellular fraction shows that the CO_2 formation exceeded the formate production already after 10 min reaction time, and after 20 min twice as much CO_2 as formate was measured. Under standard incubation conditions, $^{14}CO_2$ seemed to be the major α-oxidation product in cucumber subcellular 150,000 × g_{max} (Fig. 6).

Surprisingly, no ^{14}C-formate production could be detected in the incubations of purified α-oxidation enzyme. Furthermore, incubation of purified enzyme fraction with ^{14}C-formate (0.05 mM) for 10 or 20 min did not result in significant $^{14}CO_2$ production. In contrast, a small but significant amount of $^{14}CO_2$ was formed from ^{14}C-formate in subcellular 150,000 × g_{max} fraction (5.3 ± 0.15 nmol × min^{-1} × (mg protein)$^{-1}$, n = 3). The addition of a cytosolic fraction (150,000 × g_{max} supernatant) did not increase the rate of $^{14}CO_2$ production from ^{14}C-formate significantly (data not shown). However, no $^{14}CO_2$ formation from ^{14}C-formate was observed in boiled 150,000 × g_{max} fraction.

Oxygenase cofactor requirements. Oxygen requirements were investigated in the cucumber 150,000 × g_{max} fraction. Oxygen depletion by nitrogen flushing of the solutions prior to the incubations inhibited $^{14}CO_2$ formation by 84 ± 12% (n = 6), and air supply to the incubation mixture restored the α-oxidation activity.

The mammalian α-oxidation involves a dioxygenase type of enzyme using 2-oxoglutarate as cosubstrate and Fe^{2+} and ascorbate as cofactors (21–23). Whether similar positive effects by these dioxygenase cofactors on α-oxidation activity could be obtained in plant material was investigated. Table 1 shows the effect of Fe^{2+}, 2-oxoglutarate, and ascorbate on the production of $^{14}CO_2$ and ^{14}C-formate plus $^{14}CO_2$ in cucumber 150,000 × g_{max} fraction with [1-^{14}C]palmitic acid as substrate. Fe^{2+} (0.5 mM) showed a significant stimulating effect on the formation of $^{14}CO_2$ and ^{14}C-formate plus $^{14}CO_2$. However, the increase in the rate of ^{14}C-formate plus $^{14}CO_2$ formation was not higher than the increase of $^{14}CO_2$, i.e., Fe^{2+} had no effect on the formate production. The effects of adding 2-oxoglutarate (0.75 mM) and/or ascorbate (1.0 mM) to the incubation media were less clear to interpret according to the analysis of variance. The rate of $^{14}CO_2$ production was further increased from 26.80 ± 2.90 nmol × mg protein^{-1} × min^{-1} with Fe^{2+} present alone to 34.46 ± 2.83 nmol × mg protein^{-1} × min^{-1} with ascorbate and 2-oxoglutarate in addition to Fe^{2+} (P = 0.035). The effect on the total rate of production of $^{14}CO_2$ plus ^{14}C-formate was even higher, increasing from 46.99 ± 0.38 to 62.56 ± 0.24 nmol × min^{-1} × (mg protein)$^{-1}$ with Fe^{2+} and Fe^{2+}, ascorbate, and 2-oxoglutarate present, respectively (P < 0.001).

Monooxygenases require a second oxidizable substrate, e.g., NADPH or NADH. Table 2 shows that the total α-oxidation rate, ^{14}C-formate plus $^{14}CO_2$ production, was stimu-

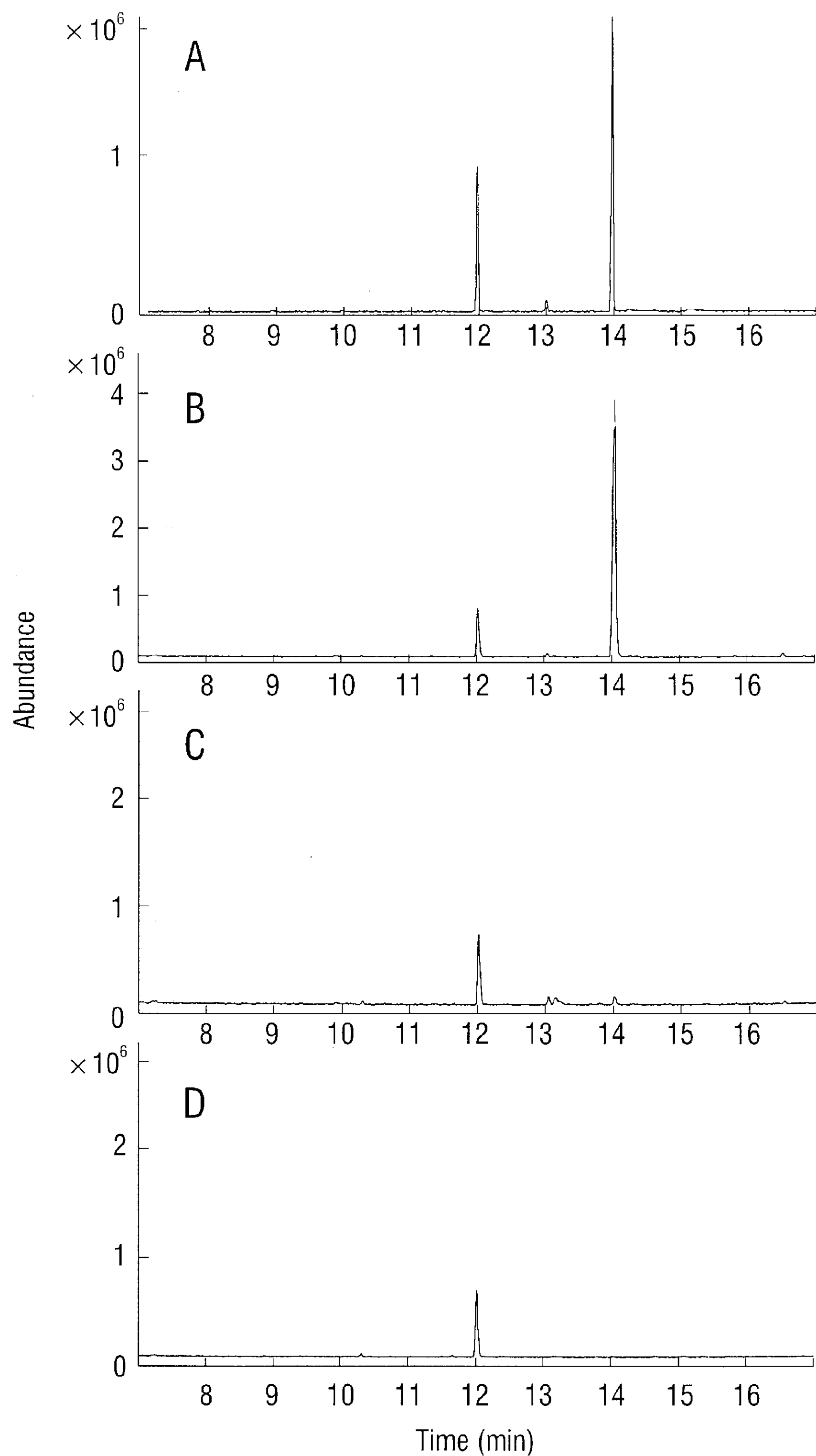

FIG. 3. Formation of pentadecanal from palmitic acid in purified α-oxidation enzyme fraction analyzed by GC. Incubations were performed with purified enzyme fraction (40 μg protein) and 1.0 mM palmitic acid, and the reactions were terminated after 20 min with acetic acid. The reaction mixtures received 50 nmol tridecanal as internal standard and were extracted with ethyl acetate. (A) The total ion chromatogram of a standard solution containing tridecanal (retention time 12.0 min) and pentadecanal (retention time 14.1 min). (B) The extracts from incubation medium with active enzyme, (C) boiled enzyme, and (D) without enzyme were analyzed by GC–MS for aldehydes with the temperature program described in the Materials and Methods section. See Figure 2 for abbreviations.

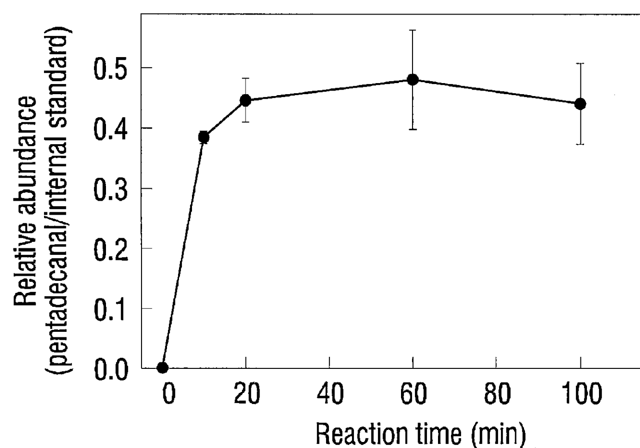

FIG. 4. Production of pentadecanal from 2-hydroxypalmitic acid in purified enzyme fraction. Purified enzyme fraction (40 μg protein) was incubated with 1.0 mM 2-hydroxypalmitic acid for 0, 10, 20, 60, and 100 min at 25°C. The reaction mixtures received 50 nmol tridecanal as internal standard and were extracted with ethyl acetate. The samples were analyzed by GC–MS and the amount of pentadecanal was calculated relative to the internal standard tridecanal. The results presented are mean ± SEM of two experiments. See Figure 2 for abbreviations.

lated with 0.5 mM NADPH or 0.5 mM NADH, 1.5- and 1.3-fold, respectively, compared to the controls. In contrast, NADPH and NADH had marginal or no effect on the $^{14}CO_2$ production. The analysis of variance showed significant effects of both NADPH and NADH, depending on the presence of Fe^{2+}, and both reductants decreased the positive effect obtained by Fe^{2+} alone ($P < 0.001$).

Involvement of H_2O_2 in fatty acid α-oxidation was discussed in several earlier studies (5,9,17,35,36). Consistent with the previous observations in cucumber (5), H_2O_2 was shown to be a potent inhibitor of the palmitic acid α-oxidation activity (Table 3). Micromolar concentrations decreased the rate of $^{14}CO_2$ and ^{14}C-formate plus $^{14}CO_2$ production by 23 and 32%, respectively, relative to the incubations without cofactors. Neither low (20 μM) nor high (0.5 mM) concentrations of FAD increased the $^{14}CO_2$ or the ^{14}C-formate plus $^{14}CO_2$ production. The stimulatory effect of Fe^{2+} (0.5 mM) was maintained in the presence of FAD, and no additional increase in the α-oxidation activity was observed (Table 3).

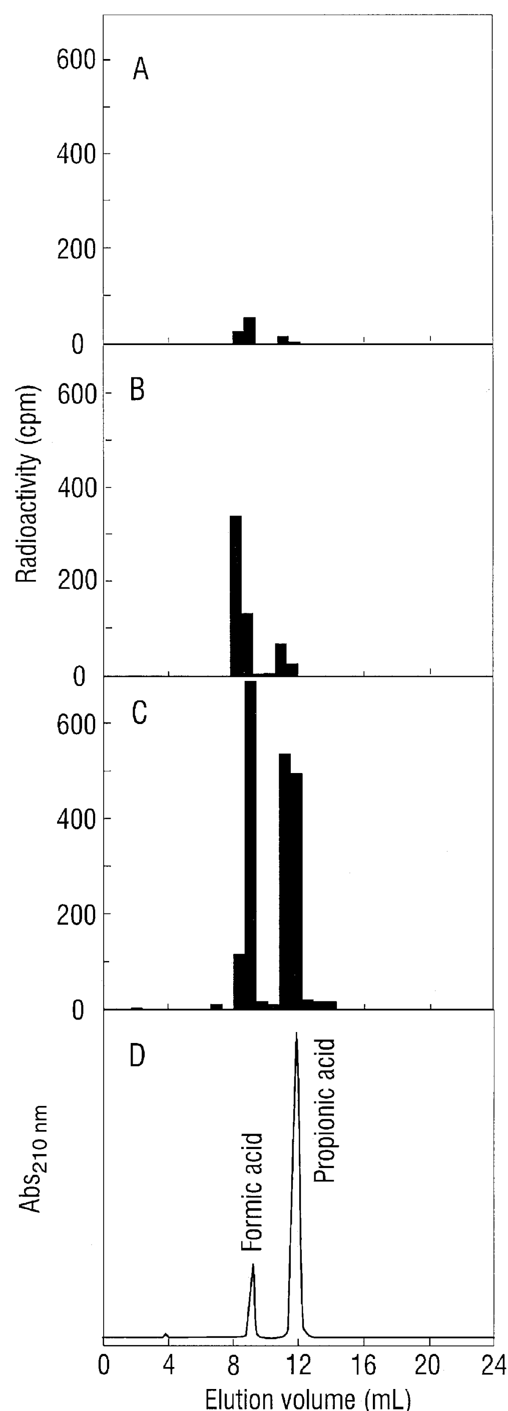

FIG. 5. Ion-exclusion HPLC analysis of the incubation mixture after α-oxidation of [1-^{14}C]palmitic acid. Cucumber 150,000 × g_{max} subcellular fraction (0.1 mg protein) was incubated with 0.05 mM [1-^{14}C]palmitic acid, and the reactions were terminated after 5, 10, or 20 min with $HClO_4$. The samples were centrifuged, and an aliquot of the supernatant was injected on a cation-exchange column eluted with 6 mM H_2SO_4 at a flow rate of 0.4 mL/min. HPLC effluents were monitored using an ultraviolet detector on-line, and fractions were collected for radioactivity counting. The radioactive peaks absorbed light at 210 nm and co-eluted with the unlabeled standards shown in the bottom panel. (A) 5 min; (B) 10 min; (C) 20 min reaction time; (D) standard mixture of unlabeled formate and propionic acid. The results are representative for three separate experiments. See Figure 2 for abbreviation.

DISCUSSION

The main purpose of this study was to identify the α-oxidation reaction intermediates in cucumber and to study the products formed by the α-oxidation enzyme purified from cucumber as previously described (6). The use of purified enzyme allowed us to study the α-oxidation reaction pathway in a more direct manner.

Different reaction mechanisms and intermediates were proposed in earlier studies of fatty acid α-oxidation in crude fractions from higher plants (9,13,14). In agreement with previous α-oxidation studies in higher plants (5,9,10,14), we found no evidence for any requirement for activated fatty

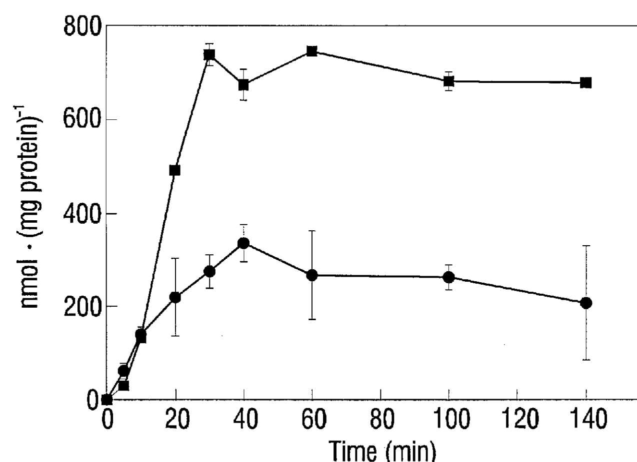

FIG. 6. Time course of ^{14}C-labeled formate and CO_2 formation in 150,000 × g_{max} subcellular fraction from cucumber. Cucumber 150,000 × g_{max} subcellular fraction (0.1 mg protein) was incubated with 0.05 mM [1-^{14}C]palmitic acid, and the production of $^{14}CO_2$ (■) and ^{14}C-formate (●) was measured. ^{14}C-Formate was determined as the total $^{14}CO_2$ released after mercuric acetate treatment of the incubation mixture minus release of $^{14}CO_2$ in the parallel sample. Each value represents the means ± SEM (n = 3).

acids in α-oxidation, either in cucumber crude fractions or in purified enzyme fraction. [1-^{14}C]Palmitoyl-CoA could not be oxidized to $^{14}CO_2$ in a crude fraction containing the entire α-oxidation system, and this result rules out the presence of a cucumber α-oxidation system dependent on CoA derivatives. In contrast, recent studies of mammalian α-oxidation showed the necessity of an activating step leading to the fatty acyl-CoA esters, before α-oxidation of methyl-substituted fatty acids occurs optimally (20).

In the present study, purified enzyme α-oxidized palmitic acid to yield the end products CO_2 and pentadecanal, which suggests that α-oxidation of fatty acids in cucumber involves an enzyme complex with more than one catalytic activity, most likely with at least an α-hydroxylase and a decarboxylase activity. 2-Hydroxy and 2-oxopalmitic acids were identified as palmitic acid α-oxidation intermediates in 150,000 × g_{max} subcellular cucumber fraction and were also shown to be formed by purified enzyme fraction as well. The intermediate and final product formation by the purified α-oxidation enzyme confirmed our proposals of a multifunctional nature of the α-oxidation enzyme in cucumber. Galliard and Matthew (14) earlier described an α-oxidation enzyme system in cucumber acetone powder which converted [U-^{14}C]palmitic acid to $^{14}CO_2$ and a mixture of products which involved aldehydes, alcohols, and small amounts of 2-hydroxypalmitic acid. An even earlier study proposed a 2-hydroxy fatty acid intermediate which first has to be oxidized to the 2-oxo fatty acid before decarboxylation (37). Our findings support an α-oxidation pathway such as 16:0 → 16OH → 16=O → 15al (Scheme 1). Incubation of cucumber subcellular 150,000 × g_{max} fraction or purified enzyme fraction with the proposed reaction intermediate, 2-hydroxypalmitic acid, resulted in a time-dependent production of pentadecanal, however, in lower amounts than with palmitic acid as substrate.

TABLE 1
Effect of $FeCl_2$, 2-Oxoglutarate, and Ascorbate on the Production of ^{14}C-Labeled CO_2 and Formate Plus CO_2 from [1-^{14}C]Palmitic Acid[a] [nmol × min^{-1} × (mg protein)$^{-1}$]

Additions[b]	$^{14}CO_2$	^{14}C-formate + $^{14}CO_2$
None	14.19 ± 0.18	36.02 ± 1.05
$FeCl_2$	26.80 ± 2.90***	46.99 ± 0.38***
Ascorbate	8.50 ± 0.75*	33.20 ± 1.44
2-Oxoglutarate	11.13 ± 0.83*	33.37 ± 2.84
Ascorbate + 2-oxoglutarate	14.53 ± 2.71	28.22 ± 2.15*
Ascorbate + $FeCl_2$	32.70 ± 5.17***	38.62 ± 6.72
2-Oxoglutarate + $FeCl_2$	28.40 ± 1.22***	58.86 ± 3.20*
Ascorbate + 2-oxoglutarate + $FeCl_2$	34.46 ± 2.83***	62.56 ± 0.24***

[a]Subcellular 150,000 × g_{max} fraction (0.1 mg protein) was incubated with 0.05 mM [1-^{14}C]palmitic acid in 50 mM HEPES, pH 7.4 in the absence and presence of the cofactors described in a total volume of 2.0 mL for 10 min at 25°C. One set of samples was assayed for $^{14}CO_2$ and the other set for ^{14}C-formate plus $^{14}CO_2$ production in the same experiment. The values represent the mean ± SEM (n = 3). Significantly different from control (None): ***P < 0.001; *P < 0.05.
[b]The concentrations of cofactors were: ascorbate 1.0 mM, 2-oxoglutarate 0.75 mM, and $FeCl_2$ 0.5 mM.

TABLE 2
Effect of $FeCl_2$, NADPH, and NADH on the Production of ^{14}C-Labeled CO_2 and Formate Plus CO_2 from [1-^{14}C]Palmitic Acid[a] [nmol × min^{-1} × (mg protein)$^{-1}$]

Additions[b]	$^{14}CO_2$	^{14}C-formate + $^{14}CO_2$
None	13.70 ± 0.52	29.97 ± 0.99
$FeCl_2$	33.03 ± 0.64***	42.88 ± 0.50***
NADH	15.95 ± 0.68	36.69 ± 1.13*
NADPH	17.60 ± 0.54**	43.63 ± 2.30***
NADH + $FeCl_2$	18.37 ± 0.48**	28.85 ± 0.30
NADPH + $FeCl_2$	27.64 ± 0.83***	49.48 ± 1.14***

[a]Incubation conditions as described in Table 1. The values represent the mean ± SEM of three experiments. Significantly different from control (None): ***P < 0.001; **P < 0.01; *P < 0.05.
[b]The concentrations of cofactors were: $FeCl_2$ 0.5 mM, NADH 0.5 mM, and NADPH 0.5 mM.

TABLE 3
Effect of H_2O_2, $FeCl_2$, and FAD on the Production of $^{14}CO_2$ and ^{14}C-Formate Plus $^{14}CO_2$ from [1-1^{4}C]Palmitic Acid[a] [nmol × min^{-1} × (mg protein)$^{-1}$]

Additions	$^{14}CO_2$	^{14}C-formate + $^{14}CO_2$
None	12.31 ± 0.26	31.15 ± 0.74
H_2O_2 (20 μM)	9.45 ± 0.13**	21.24 ± 1.10**
H_2O_2 (200 μM)	7.02 ± 0.08***	8.43 ± 0.79***
$FeCl_2$ (0.5 mM)	26.27 ± 0.27***	41.68 ± 0.85***
FAD (20 μM)	14.31 ± 1.73	33.98 ± 0.92
FAD (0.5 mM)	13.57 ± 1.01	31.94 ± 0.57
FAD (0.5 mM) + $FeCl_2$ (0.5 mM)	25.41 ± 0.43***	38.41± 0.31 ***

[a]Incubation conditions as described in Table 1. The values represent the mean ± SEM of three or two replicates. Significantly different from control (None): ***P < 0.001; **P < 0.01.

A more unstable 2-hydroperoxy α-oxidation intermediate was suggested in plant leaves (13) and mammalian brain microsomes (38). According to this proposal, the 2-hydroperoxyl-C_n intermediate is preferentially decarboxylated to the C_{n-1} fatty aldehyde, but to some degree reduced to 2-hydroxy C_n fatty acids. Recent substrate selectivity studies of the α-oxidation enzyme system in pea leaves show that pure (*R*)-2-hydroxy acids were enantiomerically formed (39). Consistent with the last proposed pathway, studies of long-chain aldehyde formation in green seaweeds showed the formation of 2-hydroperoxy-, 2-hydroxy-, and 2-oxopalmitic acid from palmitic acid, separated with reversed-phase HPLC, and that (*R*)-2-hydroperoxy acid was formed in high enantiomeric purity (40). We were not able to detect any 2-hydroperoxy fatty acid in the ethyl acetate extracts of the incubation mixtures with our HPLC separation system. Nor could the GC–MS analysis of the incubations with or without fatty acid derivatization give any evidence for a 2-hydroperoxypalmitate formation in cucumber enzyme fractions. In mammalian tissues, the existence of a 2-hydroxy α-oxidation intermediate was shown in early studies by Tsai *et al.* (41), and the formation of 2-hydroxy acyl-CoA was observed in broken cell systems, peroxisomes, and homogenates from rat liver (21,22). However, others were unable to confirm these findings in rat liver (42) or in intact liver cells (22). The most probable explanation for the difficulty in extracting the enzyme intermediate(s) is that channeling and/or tight binding of the intermediate(s) on the multimeric enzyme keeps the amount of intermediates at a low level.

The enzymatic formation of pentadecanal and $^{14}CO_2$ from [1-^{14}C]palmitic acid by our purified α-oxidation enzyme is in accordance with earlier studies. C_{n-1} long-chain aldehydes are well-established α-oxidation products of C_n long-chain fatty acids in higher plants (9,14), as well in algae (15). Very recently, the fatty end product was also identified as the C_{n-1} aldehyde in rat liver peroxisomes (31) and in human liver homogenate (30).

In the present study, formate was identified as a product of palmitic acid α-oxidation besides the previously described products, C_{n-1} long-chain aldehyde and CO_2, in cucumber $150{,}000 \times g_{max}$ subcellular fraction. The rather unusual decarboxylation product formate was not reported as an end product of the α-oxidation pathway in plants. However, our results are in agreement with recent mammalian studies, where α-oxidation of 3-methyl-substituted fatty acids was shown to result in the formation of formate as the end product besides CO_2 in human fibroblasts (27), in isolated rat hepatocytes (22,28), and *in vivo* in humans (43). Our experiments revealed a surprisingly low enzymatic activity for the conversion of formate into CO_2 in cucumber subcellular fractions. The overall high CO_2 production is not consistent with the low reaction rate of the formate step, indicating the existence of one pathway with direct liberation of CO_2 and another which could involve the enzymatic formation of formate. Furthermore, formate was not produced at all by purified α-oxidation enzyme fraction incubated with palmitic acid, which further supports our suggestion of two different α-oxidation pathways, at least in cucumber. These experiments clearly indicated that another enzyme other than the purified α-oxidation enzyme was responsible for the formate formation and the further conversion of formate into CO_2. The level of formate was reduced very slowly over time (Fig. 6), indicating no further metabolic conversion in the subcellular fractions studied. The putative formation of propionate as an α-oxidation product from saturated straight-chain fatty acids is not easily explained. Propionate is one of the mammalian oxidation products of branched-chain fatty acids which can undergo β-oxidation (27,44,45). The enzymatic production of the ^{14}C-labeled compound in our experiments was not due to peroxisomal or mitochondrial β-oxidation, as this would not be functional in our incubation systems. This was, however, an interesting observation which needs to be studied further before final conclusions can be drawn.

The fatty acid α-oxidation activity in plants, especially in cucumber, was found to be high without any added exogenous cofactors, compared to mammalian organellar fractions, e.g., rat liver peroxisomes, fortified with cofactors (21). The molecular oxygen requirement in our experiments suggests the involvement of an enzyme of the oxygenase family. In mammalian studies, the dioxygenase cofactors 2-oxoglutarate, Fe^{2+}, and ascorbate were reported to be required for optimal α-oxidation enzyme activity (21–23). In our experiments, Fe^{2+} stimulated the α-oxidation rate about twofold for both the $^{14}CO_2$ and the ^{14}C-formate plus $^{14}CO_2$ formation. Further addition of 2-oxoglutarate and ascorbate to the incubations with Fe^{2+} provided optimal rates of α-oxidation in cucumber $150{,}000 \times g_{max}$ fraction, indicating a dioxygenase reaction. This is in full agreement with the findings recently reported in mammalia (22). However, exogenous supply of these dioxygenase cofactors was not essential for the α-oxidation activity in cucumber. Most likely, minor quantities of cofactors are present in the cucumber subcellular fraction. Earlier studies of α-oxidation in cucumber suggested a metal-requiring enzyme, although 0.1 mM Fe^{2+} and Fe^{3+} inhibited $^{14}CO_2$ production, while Mg^{2+} stimulated the activity (14).

Monooxygenase cofactors were investigated in several tissues with different effects on the α-oxidation rate (5,13,14, 17,21,46,47). The stimulation of the α-oxidation activity with NADPH (0.5 mM) was consistent with earlier observations in plants (5,13) and in mammalian studies (46,47), where at least one of the α-oxidation steps is suggested to be mediated by a cytochrome P-450 monooxygenase (48). NADPH stimulated the rate of formate production better than it did CO_2 production. On the other hand, Fe^{2+} favored CO_2 formation, at the sacrifice of formate production. In summary, some discrepancies in the effects of the cofactors tested in this work on the $^{14}CO_2$ vs. the total ^{14}C-formate plus $^{14}CO_2$ production, respectively, were observed. These results indicate different routes for formate and CO_2 formation in palmitic acid α-oxidation in cucumber.

The inhibitory effect of H_2O_2 addition to our enzyme incubations can be explained by different inactivation

processes. The ability of H_2O_2 to convert Fe(II)-enzymes into the Fe^{3+} form and a reactive and denaturing hydroxyl radical was reported for dioxygenases (49). The involvement of a flavoprotein was proposed in the α-oxidation pathway involving a hydroperoxide intermediate in peanut cotyledons (13). In agreement with earlier studies in cucumber (5), no effect on CO_2 formation, nor on the formate production, was observed with FAD.

In summary, our results from the incubations of subcellular fractions and purified enzyme fraction suggest a multifunctional nature of the cucumber α-oxidation enzyme, which converts palmitic acid into CO_2 in a reaction involving two possible intermediates, a 2-hydroxy- and a 2-oxopalmitic acid, and which rate is stimulated by Fe^{2+} and Fe^{2+} in combination with ascorbate and 2-oxoglutarate. We demonstrate here, for the first time, that not only CO_2, but also HCOOH (formate), is an α-oxidation product in plant tissue. The possibility of more than one α-oxidation pathway in plants must be considered and investigated further.

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Proportion of Geometrical Hydroperoxide Isomers Generated by Radical Oxidation of Methyl Linoleate in Homogeneous Solution and in Aqueous Emulsion

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ABSTRACT: The proportion of geometrical hydroperoxide isomers generated by aerobic oxidation of methyl linoleate (18:2 Me) in either aqueous emulsion consisting of Tris-HCl buffer (pH 7.4) or in a homogeneous dichloromethane solution was determined to understand the mechanism of lipid oxidation in different reaction systems. Four geometrical isomers were generated after oxidation of 18:2 Me in dichloromethane: methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate, methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, and methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate in the ratios of 1:4:1:4, respectively. The ratios between each isomer did not change until the peroxide value (PV) increased to 58 meq/kg. Oxidation of 18:2 Me in aqueous emulsion yielded the same geometrical isomers of hydroperoxide. However, the ratios were different: 3:2:3:2 until the PV increased to 110 meq/kg. Predominant (60%) formation of *trans,trans* hydroperoxide isomers was obtained in the oxidation of a mixture of 18:2 Me and methyl laurate (12:0 Me). These results are interpreted to reflect the importance of the concentration of hydrogen atom-donating equivalents to the kinetic preference for different products. The high effective concentration of hydrogen donors in the oxidation of 18:2 Me in emulsions favored the formation of the less stable *cis,trans* isomers. The lower concentration of hydrogen donor in the dichloromethane solution effectively slowed hydrogen donation and led to the strong preference for the more stable *trans,trans* isomers. This interpretation was further tested by preparing emulsions of 18:2 Me and 12:0 Me to dilute concentration of hydrogen-donating species using the nonhydrogen-donating 12:0 Me. Consistent with the proposed hypothesis, the proportion of *trans,trans* isomers increased as a result of 12:0 Me addition.

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Lipid oxidation affects many properties of food, including texture, nutritive value, color, aroma, and functionality (1–4). Oxidation of cellular lipids also causes functional abnormalities and pathological changes in biological systems (5). Numerous promoting and inhibitory factors affect, to some extent, the oxidation of lipids in food systems. It has been generally accepted that the oxidizability of unsaturated fatty acids in homogeneous solution depends on the number of bis-allelic carbons available for hydrogen abstraction (6–9). The chemical environment in which oxidation occurs, however, can have an effect on the overall progress of oxidation.

This is perhaps most vividly seen by the observation that stability against oxidation of unsaturated fatty acids in aqueous micelles increases with increasing the number of bis-allelic carbons (10,11). Studies on aerobic oxidation kinetics explained the mechanism of the higher stability of polyunsaturated fatty acid in aqueous emulsion to be due to the polarity of the peroxyl radicals derived from polyunsaturated fatty acids (12,13). It is known that the ratio between *cis,trans* and *trans,trans* hydroperoxide products is affected by the concentration of hydrogen donor substrate and other conditions including temperature and stage of oxidation. For the oxidation of the mixture of linoleic acid and 1,4-cyclohexadiene (CHD), which is an excellent hydrogen-donating medium, almost 25 times higher *cis,trans* hydroperoxide isomer was generated in the system with 97% CHD. However, the ratio of the *cis,trans* isomer to *trans,trans* isomer decreased to 0.27 when cumene, a relatively poor hydrogen donor, was added to linoleic acid (14). In the present study, the effect of the colloidal reaction system in which lipid oxidation occurs on the distribution of unsaturated fatty acid hydroperoxide geometrical isomers was investigated by determining the geometrical isomers of methyl linoleate (18:2 Me) hydroperoxides.

MATERIALS AND METHODS

Lipids. 18:2 Me (over 99% purity) was purchased from Nu-Chek-Prep (Elysian, MN). The purchased 18:2 Me was dissolved in *n*-hexane and further purified by elution from a Sep-Pak silica cartridge (Waters Associates, Milford, MA) with 4.5% diethyl ether in *n*-hexane (15). Methyl laurate (12:0 Me, over 99.5% purity; Tokyo Chemical Ind., Tokyo, Japan) was reduced by sodium borohydride (Aldrich, Milwaukee, WI), followed by hydrogenation and further purification with a Sep-Pak silica cartridge.

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); CHD, 1,4-cyclohexadiene; DPPP, diphenyl-1-pyrenylphosphine; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; HPO, hydroperoxide; PV, peroxide value; TMS, trimethylsilyl; UV, ultraviolet; 12:0 Me, methyl laurate; 18:2 Me, methyl linoleate.

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Aerobic oxidation of lipids. For aerobic oxidation of 18:2 Me in a homogeneous solution, oxidation of 250 mg of the purified 18:2 Me was carried out in the presence of the free radical initiator 1 mM 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN; Wako Pure Chemicals, Osaka, Japan) in 10 mL of dichloromethane at 30°C in the dark. For aerobic oxidation of 18:2 Me in an aqueous emulsion, 625 mg of 18:2 Me was emulsified in 25 mL of Tris-HCl buffer (pH 7.4) by 0.3% Tween 20 under continuous and gentle stirring with a magnetic stirrer at 30°C in the dark. As a radical initiator, each of 1 mM AMVN or 1 mM 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH; Wako Pure Chemicals) was added to the aqueous emulsion (16,17). Aerobic oxidation of a mixture of 18:2 Me and 12:0 Me (1:28 in molar ratio) was initiated in the presence of 1 mM AMVN in bulk system at 30°C in the dark.

Determination of isomeric hydroperoxides of 18:2 Me. Geometrical isomers of the hydroperoxides generated by aerobic oxidation of 18:2 Me were analyzed by high-performance liquid chromatography (HPLC) equipped with a post-column diphenyl-1-pyrenylphosphine (DPPP) fluorescence detection system (18,19). In this system, the confounding effects of nonperoxy conjugated dienes such as hydroxy compounds were avoided by comparing fluorescence detection to ultraviolet (UV) detection at 235 nm. Briefly, geometrical isomers of hydroperoxides were separated along a Supelcosil LC-Si silica column (2.1 mm i.d. × 250 mm, 5 µm; Supelco, Bellefonte, PA) using a mixture of 500 mL of *n*-hexane and 34 mL of diethyl ether as a mobile phase at a flow rate of 0.6 mL/min. The eluting and separated hydroperoxides were mixed with DPPP solution (3 mg in a mixture of 200 mL 1-butanol and 200 mL methanol) pumped at a flow rate of 0.3 mL/min to form DPPP oxide in the postcolumn reaction coil. The fluorescence intensity of the DPPP oxide was monitored at the emission wavelength at 380 nm with the excitation wavelength of 352 nm.

The positional distribution of the hydroperoxy groups on 18:2 Me was determined by gas chromatography–mass spectrometry (GC–MS). Briefly, the corresponding trimethylsilyl ether (TMS) derivatives of the hydroxy methyl octadecanoates that were obtained by reduction followed by hydrogenation and subsequent TMS derivatization were separated and analyzed by GC–MS as described (18,19).

UV spectra of hydroperoxide isomers were determined with a photodiode array detector model 1050 (Hewlett-Packard, Boise, ID) positioned in the elution stream between the HPLC column and the reaction coil of the HPLC system.

Determination of peroxide value (PV). The PV of the oxidized 18:2 Me was determined photometrically by the ferric thiocyanate method (20). The PV of oxidized lipids in the aqueous emulsion was determined after extraction by *n*-hexane.

RESULTS

Changes in PV. In the aerobic oxidation of 18:2 Me in homogeneous dichloromethane solution, the PV increased almost rectilinearly to 70 meq/kg after 4 h of oxidation period when the hydroperoxide equivalent to 1.9% of the original 18:2 Me was generated (Fig. 1). In the aerobic oxidation of the lipid in aqueous solution, the trace of the PV did not show a significant induction period in either the AAPH- or AMVN-initiated aerobic oxidation (Fig. 1). When the reaction was terminated after 7 h, hydroperoxides equivalent to 5.5% of the original 18:2 Me had been formed.

Composition of geometrical hydroperoxide isomers of 18:2 Me. The various isomers formed from oxidized 18:2 Me were separated by HPLC and the hydroperoxide-containing isomers detected by postcolumn detection resolved into four distinct peaks (Fig. 2). No significant peaks other than hydroperoxy products of 18:2 Me were detected by either UV or fluorescent detection when the detection time was extended to more than 20 min. The positional distribution of hydroperoxy groups on these oxidation products of 18:2 Me was determined by GC/MS of the corresponding hydrogenated and reduced TMS-ether derivatives. The TMS-ether derivatives obtained from the peak component #1 and/or #2 on the HPLC chromatograms (Fig. 2, A and B) yielded two prominent fragment ions of *m/z* 173 and *m/z* 315. The TMS-ether derivatives obtained from peak component #3 and/or #4 yielded two prominent fragment ions of *m/z* 229 and *m/z* 259. These characteristic fragment ions were yielded by cleavage of C-C bond and to the carbon on which the TMS-ether group attached (21,22).

The geometrical configuration of the hydroperoxide isomers was determined by the UV spectra (data not shown). The bathochromic shift of the UV spectrum (from λ_{max} = 231 nm of peaks #1 and #3 to λ_{max} = 235 nm of peaks #2 and #4 in the present HPLC mobile phase) reflected the *trans,trans* and *cis,trans* isomers of conjugated fatty acid hydroperoxides (23,24).

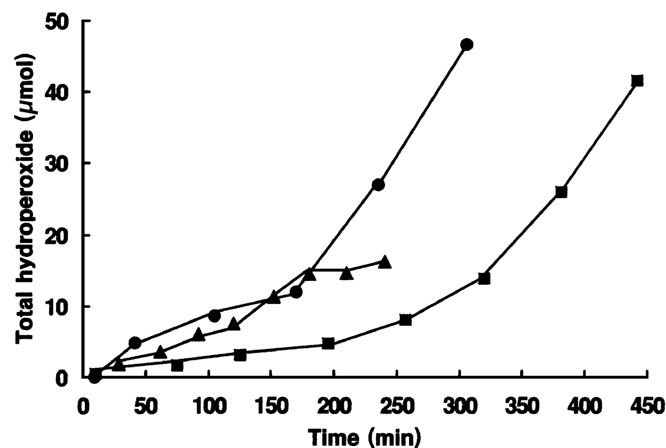

FIG. 1. Hydroperoxides formed with time during the oxidation of methyl linoleate in different physical states. Methyl linoleate in a Tween 20-stabilized emulsion (250 mg/10 mL) was initiated by the water-soluble free radical initiator, 2,2′-azobis(2-amidinopropane) dihydrochloride, AAPH (■) or by the oil-soluble free radical initiator, 2,2′-azobis(2,4-dimethylvaleronitrile, AMVN (●). Methyl linoleate in dichloromethane solution (625 mg/25 mL) was initiated by the oil-soluble free-radical initiator, AMVN (▲).

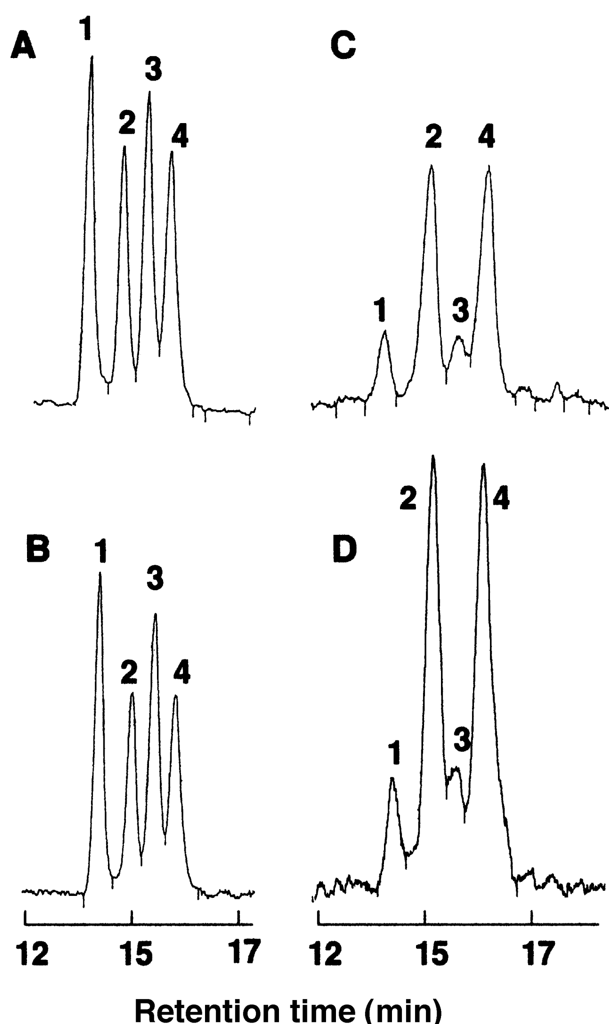

FIG. 2. Typical chromatograms of the positional and geometrical isomers of hydroperoxides generated by the oxidation of methyl linoleate. (A) Methyl linoleate in a Tween 20-stabilized aqueous emulsion initiated by the hydrophilic initiator AAPH; (B) methyl linoleate in a Tween 20-stabilized aqueous emulsion initiated by the hydrophobic initiator AMVN; (C) methyl linoleate in a dichloromethane solution initiated by AMVN; (D) methyl linoleate and methyl laurate (1:28 , w/w) initiated by AMVN. Peaks 1, 2, 3, and 4 were identified as methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate, 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate, and methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate, respectively. See Figure 1 for abbreviations.

These spectral data supported the assignments that the hydroperoxide isomers were methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate (13-*cis,trans*), methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate (13-*trans,trans*), methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate (9-*trans,cis*) and methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate (9-*trans,trans*), similar to those generated in the homogeneous solution. The ratio of these isomers was 3:2:3:2, respectively, in the aqueous emulsion as calculated from peak areas (Fig. 2, A and B) and did not change during 4 h of oxidation period as shown in Figure 3. When the oxidation in the aqueous emulsion was extended until the PV reached its maximal value (280 meq/kg, 9.2% of 18:2 Me was oxidized) after which PV decreased, the ratios of the four hydroperoxide isomers remained unchanged. In the aqueous emulsion, the yield of the *cis,trans*-hydroperoxide isomers was similar to that of *trans,trans*-hydroperoxide isomers.

Hydroperoxides generated from the oxidation of 18:2 Me in homogeneous solution included the same four positional and geometrical isomers, 13-*cis,trans*-, 13-*trans,trans*-, 9-*trans,cis*- and 9-*trans,trans*-isomers, as shown in Figure 2C. The ratio of these hydroperoxide isomers was determined from the peak areas to be 1:4:1:4, respectively. In homogeneous solution, the yield of the *trans,trans*-hydroperoxide isomers was greater than that of *cis,trans*- and *trans,cis*-hydroperoxide isomers. As shown in Figure 3, the composition of the positional isomers of the hydroperoxides did not change during 7 h of oxidation period. Similar distribution of the hydroperoxide isomers was obtained in the aerobic oxidation of 18:2 Me mixed with 12:0 Me as shown in Figure 2D. In this reaction system, the formation of *trans,trans* hydroperoxide isomers was predominant compared to *cis,trans*- and *trans,cis*-hydroperoxide isomers.

DISCUSSION

In the oxidation conditions used in these studies using azoradical initiators, the formation rate of hydroperoxides was higher than their decomposition rate. As a result, the monohydroperoxide products of 18:2 Me were the predominant oxidation products found in the present study. The hydroperoxide isomers obtained under these experimental conditions in aqueous emulsions were the same as those found in previous results (15,18–20,25–27) obtained from the autoxidation of 18:2 Me in bulk phase. When linoleic acid or its various esters oxidize, one of the bis-allelic hydrogens attached to the 11-carbon position is abstracted to yield a 1,4-pentadiene radical structure, followed by reaction with oxygen at the end-carbon positions to form a mixture of the terminal hydroperoxide isomers, including 9-hydroperoxy and 13-hydroperoxy 18:2 Me (14). The distribution of the four geometrical isomers, 9-*trans,cis*- and *trans,trans*-hydroperoxides and 13-*cis,trans*- and *trans,trans*-hydroperoxides, depends on the overall ability of coexisting molecules to donate hydrogen atoms to the peroxy radical. Free-radical oxidation of linoleic acid in bulk yields 9-hydroperoxy and 13-hydroperoxy products in a similar molar ratio (15,25,26). However, proportions of the geometrical isomers of hydroperoxides obtained in homogeneous solution and in the mixture with 12:0 Me were significantly different from the previous results obtained in the oxidation of 18:2 Me in bulk phase. It is well accepted that the *trans,trans*-isomers of peroxy radicals are formed by further oxidation of the initially formed *cis,trans* peroxy radicals (28). Therefore, the distribution of geometrical isomers, *cis,trans* and *trans,trans* hydroperoxides, depends on the ability of coexisting substrate to donate hydrogen to the peroxy radicals (14).

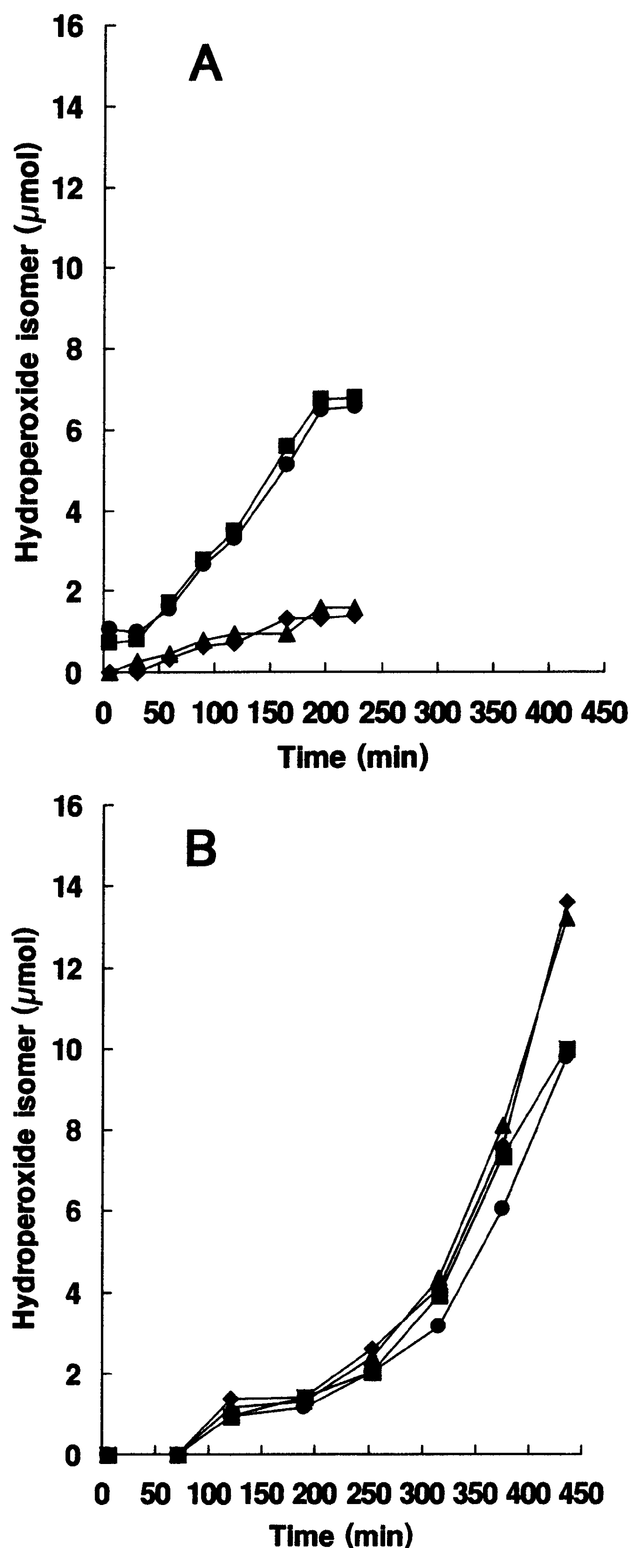

FIG. 3. Changes with time in the quantities of hydroperoxide isomers generated by aerobic oxidation of 250 mg of methyl linoleate in dichloromethane (A) and in aqueous emulsion (B). ■, Methyl 13-hydroperoxy-*trans*-9,*trans*-11-octadecadienoate; ●, methyl 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoate; ▲, methyl 13-hydroperoxy-*cis*-9,*trans*-11-octadecadienoate; ◆, methyl 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoate.

The formation of hydroperoxide comes about upon hydrogen atom donation to peroxy radicals. It is also known that only *cis,trans*-hydroperoxide isomers are produced in the presence of sufficiently high concentration of hydrogen atom donors. It is well accepted that the antioxidative activity of α-tocopherol is due to quenching the hydroperoxy radicals as an efficient hydrogen donor (29,30). Peers *et al.* (31) showed that α-tocopherol was effective in altering the distribution of *cis,trans*- and *trans,trans*-hydroperoxide isomers. The formation of *trans,trans*-hydroperoxide isomers was almost completely suppressed at a concentration of 0.1 M α-tocopherol in the autoxidation of methyl linolenate. When a large amount of dipalmitoyl phosphatidylcholine was incorporated into liposomes of soybean phosphatidylcholine and autoxidized, the yield of *trans,trans*-hydroperoxides was much greater than that of *cis,trans*-isomers (32). The dilution of soybean phosphatidylcholine rich in linoleic esters by dipalmitoyl phosphatidylcholine suppressed the hydrogen abstraction by peroxyradical yielded mostly the *cis,trans*-isomers as elaborated by Porter *et al.* (28), because dipalmitoyl phosphatidylcholine is a poor hydrogen atom donor relative to the polyunsaturated fatty acids linoleate and linolenate in soybean phosphatidylcholine.

The results from the present study showed that the *trans,trans*-isomers of 18:2 Me hydroperoxide were formed to a greater extent in the dichloromethane solution compared to oxidation in the aqueous emulsion. In the dichloromethane solution of 18:2 Me, the effective concentration of 18:2 Me as a hydrogen atom donor was expected to be lower compared to that of the aqueous emulsion. Thus, the distribution of the two kinds of geometrical isomers of hydroperoxide depends on the effective concentration of the hydrogen atom donors in the reaction system. It is therefore logical to expect that the formation of the *cis,trans*-products of oxidized 18:2 Me would be preferred in aqueous emulsions in which the 18:2 Me molecules exist as 100% of the oil phase. Contrary to this, the ratio of *trans,trans*-hydroperoxide isomers is larger than that of *cis,trans*-hydroperoxides in the diluted solution of linoleic acid, because the concentration of hydrogen atom donor is effectively lower. The predominant distribution of *trans,trans*-isomers in the oxidation mixture of 18:2 Me mixed with 12:0 Me strongly supports this assumption. Thus, we conclude that the actual concentrations of hydrogen atom donors in the lipid particles of the emulsion are in sufficient concentration to donate hydrogen atoms to the *cis,trans*-hydroperoxide isomers of oxidized 18:2 Me. Contrary to this, the concentration of hydrogen atom donor in the dichloromethane solution was too low to provide sufficient hydrogen atom to the *cis,trans*-peroxyradical of oxidizing methyl linoleate. This lack of hydrogen donor resulted in a net higher concentration of the *trans,trans*-hydroperoxide isomers. The present observation in which the predominant formation of hydroperoxide with *trans,trans* configuration can be explicitly controlled provides a powerful methodology to obtain all *trans* fatty acid hydroperoxides.

ACKNOWLEDGMENT

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19-Azasqualene-2,3-epoxide and Its *N*-Oxide: Metabolic Fate and Inhibitory Effect on Sterol Biosynthesis in *Saccharomyces cerevisiae*

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ABSTRACT: 19-Azasqualene-2,3-epoxide was more inhibitory than the corresponding *N*-oxide against 2,3-oxidosqualene cyclase (OSC) solubilized from *Saccharomyces cerevisiae* (IC_{50} 7 ± 2 and 25 ± 5 μM, respectively). Both compounds showed a reversible, noncompetitive-type inhibition on solubilized OSC. Different inhibitory properties between the compounds were especially evident when measuring [^{14}C]acetate incorporation into nonsaponifiable lipids extracted from treated cells. In cells treated with 19-azasqualene-2,3-epoxide at 30 μM, the radioactivity associated with the oxidosqualene fraction, which was negligible in the controls, rose to over 40% of the nonsaponifiable lipids, whereas it remained at a slightly appreciable level in cells treated with the *N*-oxide derivative under the same conditions. 19-Azasqualene-2,3-epoxide was also more effective than the *N*-oxide as a cell growth inhibitor (minimal concentration of compound needed to inhibit yeast growth: 45 and >100 μM, respectively). The two inhibitors underwent different metabolic fates in the yeast: while 19-azasqualene-2,3-epoxide did not undergo any transformation, its *N*-oxide was actively reduced to the corresponding amine in whole and in "ultrasonically stimulated" cells. The *N*-oxide reductases responsible for this transformation appear to be largely confined within the microsomal fractions and require NADPH for their activity. A possible relationship between the inhibitory properties of the two compounds and their metabolic fates is discussed.

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2,3-Oxidosqualene-lanosterol cyclase (OSC; EC 5.4.99.7) is a membrane-bound enzyme that plays a key role in the biosynthesis of sterols in animals and fungi because it catalyzes the conversion of an acyclic compound, (3*S*)-2,3-Oxidosqualene (OS), into lanosterol, the first cyclic precursor of C_{27}-sterols. The reaction, catalyzed by OSC and other OS cyclizing enzymes, is a multistep process, which starts with an initial protonation of the oxirane ring of the substrate and involves a series of transient carbocationic high-energy intermediates, to give a C-20 carbonium ion: this intermediate undergoes a series of 1,2 hydride and methyl shifts to form the cyclized product, after the loss of a proton (Scheme 1) (1–3).

Understanding this complex enzyme-mediated reaction at the molecular level requires knowledge of the active site structure and the precise hydrophobic interactions that control the conformation of the substrate during the cyclization process. In the last 10 yr, different and often partially overlapping strategies were employed to elucidate these points: (i) purification and characterization of OSC from different sources (4–6), (ii) determination of amino acid sequence from cloned and sequenced genes (7–10), and (iii) study of the effect of several types of rationally designed inhibitors (11–16).

In the field of OSC inhibitors, one of the most profitable designing strategies was to mimic the high-energy intermediates (formed during the cyclization process) by replacing their positively charged carbonium ion with a nitrogen atom, protonated at physiological pH (17). Several laboratories synthesized cyclic isoprenoid derivatives bearing a nitrogen atom corresponding to the C-10 or C-8 carbocationic intermediates formed during the cyclization process (1). Among these, mono- and bicyclic derivatives proved to be potent inhibitors of OSC, whereas the tricyclic derivatives showed only modest inhibition of OSC *in vitro* (17). Our strategy was to design acyclic aza-squalenoid derivatives possessing a nitrogen atom corresponding to the postulated C-2, C-8, and C-20 carbonium ions arising from the cyclization of OS: these derivatives act as potent inhibitors of OSC from mammals, fungi, and higher plants (17–20).

In this study we describe the biological effect of 19-azasqualene-2,3-epoxide (**1,** Scheme 2) and its *N*-oxide (**2,** Scheme 2) on OSC from *Saccharomyces cerevisiae*. The activity of these inhibitors was evaluated *in vitro* on solubilized OSC, and *in vivo* on proliferating cells, to estimate the effect on growth and ergosterol biosynthesis. The metabolic fate of these compounds was also carefully studied in different subcellular preparations, in order to find a possible correlation between biological activity and metabolic involvement.

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Abbreviations: IC_{50}, concentration of inhibitor that reduced the enzymatic conversion of 2,3-oxidosqualene to lanosterol by 50%; NMR, nuclear magnetic resonance; OS, 2,3-oxidosqualene; OSC, 2,3-oxidosqualene-lanosterol cyclase; TLC, thin-layer chromatography.

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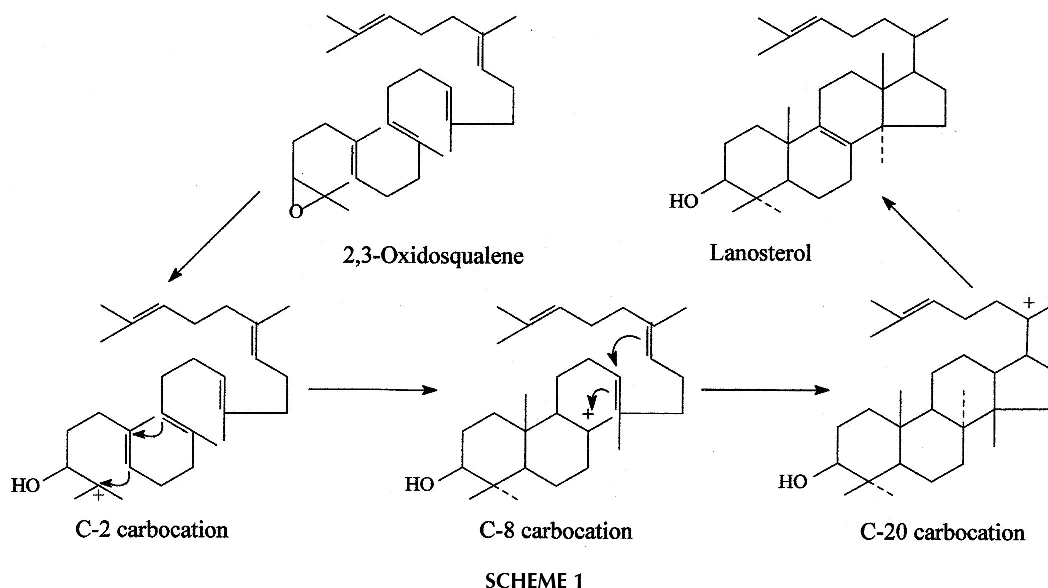


SCHEME 1

MATERIALS AND METHODS

Yeast strain, growth conditions, and microsome preparation. *Saccharomyces cerevisiae* ATCC 12341 was cultured as reported by Balliano *et al.* (18). For the preparation of microsomes, an earlier method was slightly modified (18). The yeast homogenate, obtained by sonication of washed cells in 2.5 vol of 0.1 M KH_2PO_4/NaOH buffer, pH 7.1 (Buffer A), was clarified by low-speed centrifugation (10,000 × *g* for 30 min) and then centrifuged at 150,000 × *g* for 60 min. The resulting supernatant (soluble or nonsedimentable fraction) was stored at −80°C. The pellet was resuspended in Buffer A and recentrifuged at 150,000 × *g* to give the washed microsomal fraction. The microsomal fraction was rapidly frozen in liquid N_2 and stored at −80°C. Protein was determined by the method of Bradford (21) using Bio-Rad Protein Assay with γ-globulin as standard. Microsomes, stored at −80°C, retained OSC activity for several months. In a typical preparation, 50 g cells (wet weight) gave 0.3 g microsomal protein.

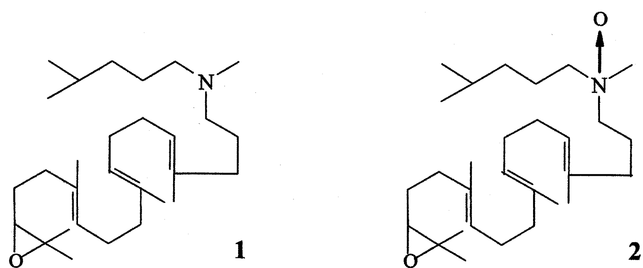
Preparation of ultrasonically stimulated cells. Ultrasonically stimulated cells were prepared as reported by Bujons *et al.* (22). Briefly: cells harvested at the late logarithmic phase were suspended in 3 vol of Buffer A and irradiated ultrasonically at 0°C for 1 h at intensities of 44 W/cm by using a Vibra Cell Ultrasonic Processor (Sonics & Materials, Danbury, CT) with a titanium immersion tip. The resulting dispersion was diluted at 4×10^6 cells/mL with Buffer A.

Removal of peripheral proteins and solubilization of OSC. Washed microsomes were sedimented at 150,000 × *g* and resuspended (12 mg/mL protein concentration) in 0.5 M KH_2PO_4/NaOH buffer, pH 6.2. The suspension was gently stirred for 30 min at 4°C and then centrifuged at 150,000 × *g* for 60 min. The supernatant (containing the peripheral proteins) was discarded, and the pellet was resuspended in 10 mg Polidocanol/mL at a protein concentration of 12 mg/mL. The solution was gently stirred for 60 min at 4°C and then centrifuged at 150,000 × *g* for 60 min, giving a supernatant containing the enzymatic activity (0.6 to 0.7 mg/mL protein concentration). The protein concentration of the solubilized sample was determined with Lowry's method, modified by Peterson (23). The solubilized OSC was stored at −80°C.

Isolation of mitochondria from yeast cells. Mitochondrial fraction was prepared as reported by Rickwood *et al.* (24). *Saccharomyces cerevisiae* was homogenized as described for microsome preparation. The cell lysate was centrifuged at 2,500 × *g* for 10 min at 5°C, to pellet unbroken cells, cell wall debris, and nuclei. The supernatant was centrifuged at 15,000 × *g* for 10 min at 5°C to pellet the mitochondria. Pellet was gently resuspended in 0.8 M sucrose, 1 mM EDTA, 10 mM Tris-HCl, pH 7.4, and 0.1% bovine serum albumin and loaded onto a continuous 1–2 M sucrose gradient containing 1 mM EDTA, 10 mM Tris-HCl, pH 7.4 and 0.1% bovine serum albumin.

The gradient was centrifuged in a swing-out rotor at 80,000 × *g* for 90 min at 5°C: the "mitochondrial fraction" was found as a brown band at a density of about 1.18 g/mL and collected using a Pasteur pipette.

Effects of inhibitors on yeast growth. The growth of *S. cerevisiae* with different concentrations of inhibitors as such was followed turbidimetrically, measuring the absorbance (*A*) of the culture at 640 nm (18). The evaluation was limited to



SCHEME 2

the logarithmic phase of the growth. A calibration curve of A_{640} vs. concentration of colonies forming cells was made in order to determine the yeast cell concentration during the logarithmic phase.

Acetate incorporation into nonsaponifiable lipids. Sterol biosynthesis in whole yeast cells was measured by incorporation of [2-^{14}C]acetate into nonsaponifiable lipids as described elsewhere (18). Briefly: washed cells (10–20 × 10^6 cells), resuspended in 5 mL of 25 mM KH_2PO_4/NaOH buffer (pH 6.5) containing 1% glucose, 0.1 mg/mL Tween 80 and inhibitors at different concentrations, were fed with 2 µCi [2-^{14}C]acetate (50 mCi/mM) and shaken for 2 h at 30°C. Nonsaponifiable lipids were extracted and separated on silica gel plates (Merck, Darmstadt, Germany) developed in *n*-hexane/ethyl acetate (85:15, vol/vol) with authentic references of ergosterol, lanosterol, dioxidosqualene, OS and squalene. The ^{14}C radioactivities of the chromatographed bands were evaluated by a System 2000 Imaging Scanner (Packard, Palo Alto, CA).

Assay of OSC activity and kinetic determinations. Enzyme activity of OSC was determined as elsewhere described (18). Briefly: the reaction mixture, containing (*R,S*)[3-^{3}H]-2,3-oxidosqualene (100,000 dpm, 25 µM), Tween 80 (0.2 mg/mL) and solubilized enzyme (0.3 mg protein/mL) in Buffer A, was incubated for 30 min at 35°C in a shaking water bath. The nonsaponifiable extract was separated by thin-layer chromatography (TLC), and bands co-migrating with 2,3-oxidosqualene and lanosterol were scraped off and monitored by liquid scintillation counting. The amount of product formed was calculated with respect to total recovered radioactivity.

IC_{50} values (the concentration of inhibitor that reduced the enzymatic conversion of 2,3-oxidosqualene to lanosterol by 50%) were determined at 25 µM substrate and different concentrations of inhibitors.

The kinetics of inhibition was analyzed by the Lineweaver-Burk graphical method (25).

Time-dependent inactivation and irreversible inhibition of OSC. Time-dependent inactivation was evaluated as described by Lindsey and Harwood (26). Solubilized OSC (0.3 mg protein/mL) was incubated for the set times at 35°C in Buffer A containing (*R,S*)[3-^{3}H]-2,3-OS (100,000 dpm, 200 µM), Tween 80 (0.2 mg/mL), and 0, 5, 20, and 100 µM inhibitor. Following incubation, OSC activity was calculated as described above and expressed as percentage of control (no inhibitor).

Irreversible inhibition was tested by determining the residual activity of preincubated enzyme after complete removal of inhibitors. Solubilized enzyme was preincubated for 20 min at 35°C with 200 µM of **1** or **2**. After preincubation, the enzyme solution (1 mL) was passed through a small column (1 mL) of DEAE Biogel A (Bio-Rad) equilibrated with 0.005 M KH_2PO_4/NaOH buffer pH 7.0, containing 2 mg Polidocanol/mL. After washing with the same buffer, the enzyme was eluted with 0.2 M KH_2PO_4/NaOH buffer buffer pH 7.0, containing 2 mg Polidocanol/mL, and the OSC activity was tested as described above. The activity of enzyme preincubated with inhibitors was compared with the activity of a control, preincubated without inhibitor and passed through the column.

*Metabolic fates of compounds **1** and **2***. The metabolism of azasqualene derivatives **1** and **2** was studied by incubating different preparations (2 mL each in Buffer A and 0.1 mg/mL Tween 80) with radioactive inhibitors (100 µM, 2 µCi) for 30 min at 35°C with or without a NADPH-generating system (0.15 mM $NADP^+$, 0.25 mM glucose 6-phosphate, and 0.5 unit glucose 6-phosphate dehydrogenase/mL). Nonsaponifiable lipid extracts were then chromatographed on silica gel plates (Merck) developed in two different TLC systems: (i) methanol and (ii) dichloromethane/methanol/NH_3 (97:1:2, by vol), with authentic samples of compounds **1** and **2**. Chromatographic bands were visualized with I_2. Transformation of compounds **1** and **2** was evaluated by scanning chromatographic plates with a System 2000 Imaging Scanner (Packard). To identify the main metabolite of **2**, 200 mL of cells were incubated with 100 µM unlabeled inhibitor **2** under the above conditions. The metabolite, recovered from TLC plates, was analyzed by ^{1}H nuclear magnetic resonance (NMR) and mass spectroscopy.

Chemistry. Synthesis of compounds **1**, **2,** and *R,S*-[3-^{3}H]-2,3-OS was described elsewhere (27,28). Polidocanol (polyoxyethylene 9 lauryl ether) and Tween 80 were from Sigma (Milan, Italy), [2-^{14}C]acetate was from Amersham International (United Kingdom), DEAE Biogel A and Bio-Rad Protein Assay were from Bio-Rad (Milan, Italy), and $NADP^+$, glucose 6-phosphate and glucose 6-phosphate, dehydrogenase were from Merck.

Tritium-labeled 19-azasqualene-2,3-epoxide **1** and its *N*-oxide **2** were synthesized as follows: pure C_{22} squalene aldehyde bromohydrin, synthesized as described recently (14), was added to a vial containing NaB^3H_4 (total activity 100 mCi, 200 × 10^9 dpm, specific activity 8.0 Ci/mmol). Purification on TLC afforded [1-^{3}H]C_{22} alcohol bromohydrin which was added to a suspension of pyridinium chlorochromate in dichloromethane. After workup and purification by column chromatography, pure [1-^{3}H]C_{22} aldehyde bromohydrin was obtained. Reductive amination of [1-^{3}H]aldehyde with *N*-methyl-4-methylpentylamine (27) and $NaBH_3CN$ directly afforded [18-^{3}H]-19-azasqualene-2,3-epoxide **1**. The large molar excess of *N*-methyl-4-methylpentylamine, with respect to the tritium-labeled aldehyde bromohydrin, afforded the contemporary reductive amination and closure of the bromohydrin to epoxide. Reaction with 30% hydrogen peroxide in methanol afforded [18-^{3}H]-19-azasqualene-2,3-epoxide *N*-oxide **2**. Inhibitors **1** and **2** (1 Ci/mmol) were obtained with high radiochemical yield and high purity. For determination of specific and total radioactivity, three samples of each pure product, diluted in benzene, were counted for radioactivity by liquid scintillation. Isotope counting was carried out as described elsewhere (28) and the mean value was used.

The ^{1}H NMR spectra were recorded on either a JEOL EX 400 (Tokyo, Japan) or a Bruker AC 200 instrument (Milton, Ontario, Canada) in $CDCl_3$ solution at room temperature, with tetramethylsilane as internal standard. Mass spectra were

recorded on a Finnigan MAT TSQ 700 spectrometer (Milan, Italy); infrared spectra were recorded on a Perkin-Elmer 781 spectrophotomer (Milan, Italy).

RESULTS

*Effects of compounds **1** and **2** on yeast growth.* MIC (= minimal concentration of compound that inhibits yeast growth) for 19-azasqualene-2,3-epoxide **1** was 45 ± 5 μM; the *N*-oxide **2** was ineffective on yeast growth even at the highest concentrations tested (100 μM), as judged by turbidimetric method.

Effects on yeast sterol biosynthesis. The influence of compounds **1** and **2** on sterol biosynthesis was evaluated by incorporation of [^{14}C]acetate into nonsaponifiable lipids, extracted from yeast cells cultured in the absence (control) or in the presence of different concentrations of inhibitors (Fig. 1). Compound **1** caused a dose-dependent accumulation of radioactivity in OS, accompained by a sharp drop in the radioactivity incorporated into the 4,4-desmethyl sterol fraction (ergosterol fraction). In cells treated with 30 μM compound **1**, the radioactivity associated with the OS fraction, which was negligible in the controls, increased to account for 40% of the nonsaponifiable lipids, and the ergosterol fraction, which accounted for 60% of the radioactivity in the control cultures, fell to 10% in treated cells. No increase in the radioactivity associated with the squalene fraction was noted after treatment of cells with **1** at concentrations up to 30 μM, suggesting that OS cyclase was the primary target of the inhibitory action. At higher concentrations (100 μM), compound **1** also inhibited squalene epoxidase, as suggested by the accumulation of radioactivity in the squalene fraction (Fig. 1).

The *N*-oxide derivative **2** was less efficient than **1** in inhibiting sterol biosynthesis. Its action seemed to be less specific, as shown by the complex distribution of radioactivity among the components of the nonsaponifiable extract (Fig. 1).

Both compounds seem to affect the uptake of the labeled acetate by the cells: radioactivity incorporated into nonsaponifiable fraction increased from 2% of the administered acetate in control culture to 5% in cells treated with either inhibitor.

Inhibition of solubilized OSC. The direct effect of compounds **1** and **2** on yeast oxidosqualene cyclase was evaluated by incubating solubilized enzyme with radioactive OS, in the presence of different amounts of inhibitors (no metabolization of either compound was observed in this enzymatic system). The results confirmed the differences in the inhibitory activities of the two compounds, compound **1** (IC_{50} 7 ± 2 μM) being more active than compound **2** (IC_{50} 25 ± 5 μM).

The kinetics of the inhibition of solubilized OSC by compounds **1** and **2** were apparently noncompetitive, as shown by the Lineweaver-Burk plot (K_i 14 and 20 μM, respectively).

The ability of compound **1** to inactivate yeast OSC in a time-dependent manner was evaluated as described by Lindsey and Harwood (26), using the solubilized enzyme. As shown in Figure 2, inhibition of OSC activity by **1** seems not to be strictly dependent on the duration of incubation.

To evaluate the ability of compounds **1** and **2** to inactivate yeast OSC irreversibly, solubilized enzyme was preincubated with **1** or **2** as described in the Materials and Methods section. After removal of the inhibitor by chromatographic passage through DEAE column, the recovery of enzyme activity was above 85% of control for both inhibitors.

*Metabolism of compounds **1** and **2** in cell cultures.* Two possible metabolic fates of compounds **1** and **2**, namely, interconversion (29) and cyclization (30), were monitored by feeding either whole or ultrasonically stimulated cells with radiolabeled inhibitors. While compound **1** was always recovered unchanged, compound **2** was found to be extensively transformed into a metabolite cochromatographing with **1**. The metabolite cochromatographing with **1**, isolated from cells incubated with cold compound **2**, had the same biological activity (IC_{50} and MIC) as an authentic sample of **1**. Of this metabolite 1.5 mg was analyzed by ^{1}H NMR and electron impact mass spectrometry and gave the following data: ^{1}H NMR ($CDCl_3$) δ 0.89 [*d*, 6 H, $(CH_3)_2CH$], 1.18 [*m*, 2 H, $(CH_3)_2CHCH_2$], 1.27 and 1.31 (two peaks, 6 H epoxidic CH_3), 1.43–1.64 [*m*, 16 H allylic CH_3, $(CH_3)_2CHCH_2CH_2CH_2NCH_2CH_2$ and epoxide-CH_2], 1.96–2.12 (*m*, 12 H, allylic CH_2), 2.22 (*s*, 3 H, CH_3N), 2.30 (*t*, 4 H, CH_2NCH_2), 2.71 (*t*, 1 H, epoxidic CH), 5.12–5.18 (*m*, 3 H vinylic CH); electron impact mass spectrum *m/z* 431 (8), 360 (35), 278 (25), 210 (42), 128 (100). Spectroscopic data were identical to those of an authentic sample of **1** (27). Tracer experiments and spectroscopical results suggest that an *N*-oxide reductase activity is responsible for the transformation of **2** into **1**.

Attempts at identifying minor transformation products of compound **2**, appearing as more polar bands on TLC plates, were unsuccessful. However, it was ruled out that they were cyclization products arising from catalytic involvement of OSC, as they were also formed in the presence of a powerful inhibitor of yeast oxidosqualene cyclase, the 2-aza-2,3-dihydrosqualene 100 μM (31).

Subcellular localization of N*-oxide reductase activity.* The results of the search for the subcellular localization of *N*-oxide reductase activity are summarized in Table 1. The ultrasonically stimulated cells retained almost the same activity as the intact cells. Among the fractions recovered by cellular fractionation, microsomes supplemented with NADPH generating system proved to be able to reduce actively 19-azasqualene-2,3-epoxide *N*-oxide **2** into the corresponding amine **1**. A weaker *N*-oxide reductase activity was detectable in mitochondrial fraction.

DISCUSSION

19-Azasqualene-2,3-epoxide was designed by our group as 19-aza analog of the C-20 carbocationic intermediate formed during the enzymatic cyclization of OS. Such inhibitor was previously tested on purified pig liver OSC and on microsomes from rat and pig liver, *S. cerevisiae* and *Candida albicans* (27), as well as in hepatoma cells (20), proving to be a

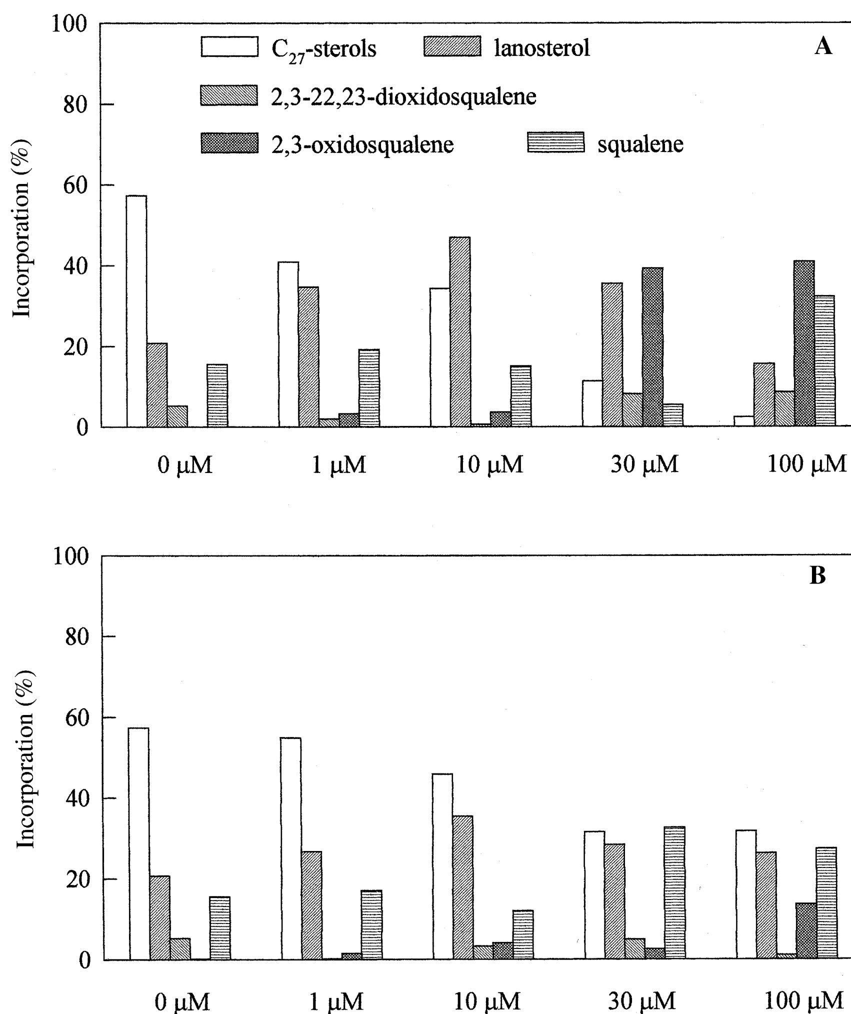


FIG. 1. Effect of (A) 19-azasqualene-2,3-epoxide (**1**) and its (B) *N*-oxide (**2**) on the incorporation of [^{14}C]acetate into the nonsaponifiable lipid fraction of *Saccharomyces cerevisiae*. Cells were incubated as described in the Materials and Methods section. Nonsaponifiable lipids were extracted and analyzed by thin-layer chromatography (TLC) on silica gel plates developed in *n*-hexane/ethyl acetate (85:15, vol/vol) with authentic references of ergosterol, lanosterol, dioxidosqualene, oxidosqualene, and squalene. Average incorporation of radioactivity into total nonsaponifiable extract ranged from 2% of initial acetate in control culture to 5% in cells treated with 100 μM inhibitors (see text). Results are means of two separate experiments, each with duplicate incubation. Maximal deviations from the mean were less than 10%.

good inhibitor of OSC. In the present study we focused our attention exclusively on *S. cerevisiae*, examining the inhibitory properties of the azasqualene derivative **1** either on OSC solubilized from microsomes (an enzyme preparation more homogeneous than microsomal pellet previously tested) or in whole cells.

Assays on solubilized enzyme confirmed the effectiveness of 19-azasqualene-2,3-epoxide as inhibitor of yeast OSC and revealed that its kinetic behavior was of reversible noncompetitive type. Tracer experiments with cell cultures fed with labeled acetate accounted for a highly specific action of the inhibitor. In fact, the most remarkable effect of treating cells with increasing concentration of inhibitor was the accumulation of radioactivity into OS, the substrate of OSC. The effect, already appreciable at 1–10 μM inhibitor, burst at 30 μM, a concentration approaching that causing the inhibition of cell growth.

The activity of 19-azasqualene-2,3-epoxide **1** was compared with that of the corresponding *N*-oxide **2**, a molecule designed on the basis of the hypothesis that squalenoid molecules bearing amine *N*-oxide group could mimic the positively charged intermediates involved in the enzymatic cyclization of OS (31). *N*-Oxide derivative **2** proved to be less-effective than the corresponding amine **1** as inhibitor of solubilized OSC. The difference, however, did not appear dramatic especially if evaluated through kinetic analysis in carefully controlled steady-state conditions (K_i of **1** and **2**, 14 and 20 μM, respectively).

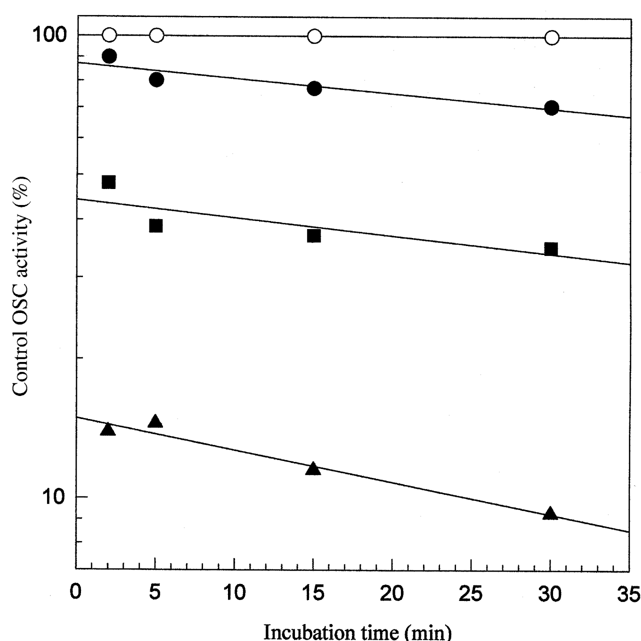


FIG. 2. Time-dependent inhibition of 2,3-oxidosqualene-lanosterol cyclase (OSC) by compound **1**. Solubilized OSC (0.3 mg/mL) was incubated for the given time at 35°C in the presence of the inhibitor 0 μM (○), 5 μM (●), 20 μM (■), and 100 μM (▲). Residual activity (% of the control incubated without the inhibitor for the same duration) was determined as described in the Material and Methods section. Points are means of two separate experiments, each with duplicate incubation. Maximal deviation from the mean was less than 10%.

The most impressive difference between **1** and **2** arose from experiments with whole cells. *N*-Oxide derivative **2** was found to be a poorer and less specific inhibitor of sterol biosynthesis as well as almost ineffective as inhibitor of cell growth at up to 100 μM concentration.

A comparison between the properties of two inhibitors of OSC of *S. cerevisiae* led us to investigate the reasons of the differences observed.

Our interest in this subject began more than 10 yr ago, from the observation that, among the azasqualene derivatives, the *N*-oxides were always less effective inhibitors of yeast OSC than the corresponding amines (18). This difference was especially evident in cell cultures treated with inhibitors: *N*-oxide derivatives inhibited cell growth as well as produced an accumulation of OS in the lipid fraction at much higher concentrations of inhibitor than the amine derivatives (18,29,32).

In a preceding paper (29) we observed that, when cells were treated with 2-aza-2,3-dihydrosqualene or 22,23-epoxy-2-aza-2,3-dihydrosqualene, inhibitors were recovered unchanged, whereas under the same conditions, *N*-oxide derivatives were actively transformed into compounds cochromatographing with the corresponding amines, i.e., into products of a putative *N*-oxide reductase activity. Experimental proof supporting the transformation did not, however, go beyond the TLC level. In the present investigation, the transformation of 19-azasqualene-2,3-epoxide *N*-oxide into the

TABLE 1
Subcellular Localization of *N*-Oxide Reductase Activity[a]

Assayed preparations	Starting compound	Products obtainted
Whole cells (4 × 10^6 cells/mL)	19-Azasqualene epoxide *N*-oxide	100% 19-azasqualene epoxide
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Ultrasonically stimulated (44 W) cells (4 × 10^6 cells/mL)	19-Azasqualene epoxide *N*-oxide	100% 19-azasqualene epoxide
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Mitochondria (12 mg/mL protein)	19-Azasqualene epoxide *N*-oxide	100% 19-azasqualene epoxide *N*-oxide
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Microsomes (4 mg/mL protein)	19-Azasqualene epoxide *N*-oxide	90% 19-azasq. epox. *N*-oxide, 10% 19-azasq. epox.
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Soluble fraction (10 mg/mL protein)	19-Azasqualene epoxide *N*-oxide	95% 19-azasq. epox. *N*-oxide, 5% 19-azasq. epox.
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Mitochondria (12 mg/mL protein) + NADPH-generating system	19-aAzasqualene epoxide *N*-oxide	85% 19-azasq. epox. *N*-oxide, 15% 19-azasq. epox.
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Microsomes (4 mg/mL protein) + NADPH-generating system	19-Azasqualene epoxide *N*-oxide	50% 19-azasq. epox. *N*-oxide, 50% 19-azasq. epox.
	19-Azasqualene epoxide	100% 19-azasqualene epoxide
Soluble fraction (10 mg/mL protein) + NADPH-generating system	19-Azasqualene epoxide *N*-oxide	90% 19-azasq. epox. *N*-oxide, 10% 19-azasq. epox.
	19-Azasqualene epoxide	100% 19-azasqualene epoxide

[a]The different preparations (2 mL each in Buffer A and 0.1 mg/mL Tween 80) were incubated, in the absence or in the presence of a NADPH generating system, for 30 min at 35°C, with [18-^{3}H]-19-azasqualene-2,3-epoxide (100 μM, 2 μCi) or [18-^{3}H]-19-azasqualene-2,3-epoxide *N*-oxide (100 μM, 2 μCi). The cells were then collected by centrifugation, and nonsaponifiable lipids were analyzed by thin-layer chromatography (CH_3OH) with authentic references of the two compounds. Radioactivity on the plates was evaluated with a radiochromatogram scanner. Boiled extracts were used as controls.

amine was unambigously demonstrated by mass spectrometry and NMR analysis of the recovered metabolite, as well as by its biological activity. Although on one hand these results confirmed the occurrence in yeast of a *N*-oxide reductase activity similar to that described in mammals (33), on the other hand they complicated the question of the different effects of two types of inhibitor: why should cells treated with the less active compound respond differently from cells treated with the more active compound, if the former is rapidly transformed into the latter within the cell? Why should the behavior of an active compound produced within the cell be different from that of the same molecule taken up from the outside? A careful examination of the subcellular localization of *N*-oxide reductase and OSC activity could provide the answers. Reducing enzymes appeared to be confined mainly within the microsomal compartment, whereas OSC is shared between the microsomes and the nonsedimentable fractions of the cell, the latter retaining the bulk of the activity (34). One could postulate that the inhibitor 19-azasqualene-2,3-epoxide, after entering the cell, is immediately ready to hit the target enzyme wherever it is located, while the *N*-oxide derivative is readily captured by the endoplasmic reticulum, where it is actively reduced to the corresponding more active amine. If this is the case, the only target enzyme left to the inhibitor formed *in situ* is microsomal OSC activity, which is only a small part of the total activity of the cell.

We very recently observed that the localization of OSC in yeast cells seems even more complex than previously thought. Part of the activity, which had simply been defined as a "nonsedimentable form" of the enzyme in a previous investigation (34), was found to be associated with lipid particles (Balliano, G., unpublished results), a subcellular compartment involved in lipid metabolism and trafficking (35), containing other enzymes of sterol biosynthesis, such as Δ^{24}-methyltransferase and squalene epoxidase (36–38). Leber *et al.* (35), who studied lipid particles in yeast in considerable depth, observed that their structural characteristics resemble those of mammalian serum lipoproteins, with protein exposed at the surface of the particle. If this is true of OSC, any inhibitor able to enter the cell can easily reach its target enzyme, provided the molecule is not captured by some different compartment of the cell, rapidly and specifically.

To summarize, the lower effectiveness of 19-azasqualene-2,3-epoxide *N*-oxide as inhibitor of yeast OSC in cell cultures may be ascribed to the combined effect of the following factors: (i) intrinsic properties of the molecule, (ii) rapid removal of the inhibitor from soluble compartment of the cell by the action of microsomal *N*-oxide reductases, and (iii) trapping of the reduction product within microsomal membranes.

In conclusion, the differences observed between the new inhibitors, 19-azasqualene-2,3-epoxide **1** and the *N*-oxide **2**, of yeast OSC make it clear that designing new inhibitors and evaluating their effects in whole cells require knowledge not only of the structural properties of the molecules but also of the subcellular distribution of both the target enzymes and any enzymes possibly involved in their metabolic transformation.

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C_2-Ceramide Attenuates Phenylephrine-Induced Vasoconstriction and Elevation in $[Ca^{2+}]_i$ in Rat Aortic Smooth Muscle

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ABSTRACT: In the present study, we examined the effects of cell-permeable C_2-ceramide on contraction of aortic smooth muscle and intracellular free Ca^{2+} ($[Ca^{2+}]_i$). C_2-ceramide (10^{-7} to 10^{-4} M) alone did not elicit any significant changes in either basal tension or resting levels of $[Ca^{2+}]_i$ in rat aortic smooth muscle. However, C_2-ceramide (10^{-7} to 10^{-4} M) attenuated phenylephrine-induced contractions in isolated rat aortic rings in a concentration-related manner, and inhibited elevations in $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells induced by phenylephrine. C_2-ceramide-induced relaxation was found to be only slightly endothelium-dependent. However, nitric oxide inhibitors (L-NNA, L-NMMA), an inhibitor of prostanoid synthesis (indomethacin), an inhibitor of opiate actions, and several inhibitors of the pharmacologic actions of various vasoactive amines all failed to interfere with the vasorelaxant responses of C_2-ceramide. Three different inhibitors of protein kinase C, when used in a wide concentration range, also failed to interfere with the ceramide-induced relaxations. Our results suggest that the sphingomyelin-signaling pathway may play an important regulatory role in arterial wall tone.

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Sphingolipids are invariably present in the outer leaflet of the plasma membrane, and well over 300 types of sphingolipids were identified in eukaryotic cells (1). Sphingolipid metabolites participate in key events of signal transduction and cell regulation. The sphingomyelin pathway is a new signal transduction system. Numerous extracellular agonists, including 1,25-dihydroxyvitamin D_3, tumor necrosis factor alpha, endotoxin, interferon, interleukin I, Fax ligands, dexamethasone, retinoic acid, progesterone, ionizing irradiation, chemotherapeutic agents, heat, and nerve growth factor (2,3) cause the activation of neutral sphingomyelinase, which hydrolyzes plasma membrane sphingomyelin to produce ceramide (4–6). Ceramide, released as a consequence of sphingomyelinase, is now thought to play roles in fundamental processes such as cell proliferation, membrane-receptor functions, oncogenesis, and immune inflammatory responses (1–6).

Recently, ceramide was implicated in atherogenesis (7,8). The diverse effects of ceramide suggest that it may serve as a pleotrophic modulator of many types of cell functions. However, little is known about the role for this lipid molecule in vascular smooth muscle tone. Since intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) plays a key role in regulating vascular tone, and α_1-adrenergic responses are potently elicited by the agonist phenylephrine (9), on vascular muscle, the present studies were designed to investigate the effects of ceramide on phenylephrine-induced contraction of isolated rat aorta and Ca^{2+} mobilization in cultured rat aortic smooth muscle cells.

MATERIALS AND METHODS

Animals, vessels, cell preparations, and solutions. The experiments were performed on thoracic aortae obtained from adult Wistar male rats (weighing 200–250 g). The animals were killed by stunning and subsequent decapitation. Aortae were isolated according to previously established methods (10,11). The vessels for the ring segments were carefully excised and cleaned, and the tissues cut into *ca.* 3 to 4 mm lengths. For intact tissue preparations, extreme care was taken to avoid damage of endothelial cells. For denuded arteries, the intima of the vessels were gently rubbed with a wire to rub off endothelial cells (11), and the tissues were placed in normal Krebs-Ringer bicarbonate solution (NKRB) at room temperature. The composition of the NKRB was (in mM): NaCl 118, KCl 4.7, $KHPO_4$ 1.2, $MgSO_4$ 1.2, $CaCl_2$ 2.5, glucose 10, and $NaHCO_3$ 25.

The procedure employed to isolate and culture single aortic vascular smooth muscle cells, and the use of digital imaging microscopy with the fluorescent indicator, fura-2, were reported (12,13). Briefly, aortic vascular smooth muscle cells were cultured in Dulbecco's modified Eagle's medium mixed 1:1 with Ham's (vol/vol) nutrient mixture F-12, penicillin 100 U/mL, streptomycin 100 μg/mL, 0.1% lipid mixture and supplemented with 20% fetal bovine serum at 37°C in a humidi-

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Abbreviations: CAPP, ceramide-activated protein phosphatase; NKRB, normal Krebs-Ringer bicarbonate solution; NO, nitric oxide; PKC, protein kinase C; PPase 2A, protein phosphatase 2A.

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fied atmosphere composed of 95% air, 5% CO_2. Morphological examination of confluent cultures revealed vascular smooth muscle cells exhibiting a crisscross pattern, hills-and-valleys, and nodular structures when examined by phase-contrast microscopy. Immunohistochemical staining with a monoclonal antibody, recognizing exclusively α-smooth muscle actin, indicated that over 97% of the cultures were pure vascular smooth muscle cells (13). Cells were maintained in the culture medium through passage 3 and harvested with 1% trypsin-EDTA and then seeded onto 12-mm circular coverslips at an almost confluent density.

Assessment of vascular reactivity, denudation of endothelium, and drugs used. The ring segments of rat thoracic aortae were arranged, isometrically, under resting tensions of 1.5–3.0 g, respectively. All tissues were equilibrated, initially, for 2 h in chambers containing 20 mL of NKRB at 37°C, gassed continuously with a 95% O_2–5% CO_2 mixture, and connected to force displacement transducers using Grass Model 7 polygraphs (Grass Model FT 03, Grass Instrument Company, Quincy, MA) before data collection. The loading tensions were adjusted periodically and maintained throughout the equilibration period. The incubation media were routinely changed every 10–15 min as a precaution against interfering metabolites (14), and only then were changes in isometric tensions of the vascular smooth muscle preparations recorded. The stable level of tension developed in response to the addition of 60 mM KCl was always measured prior to collection of the data; this was utilized as a reference contractile maximal response. To examine the functional viability of an intact endothelium, a concentration-response curve to phenylephrine (10^{-8}–10^{-5} M) was determined for each ring in order to determine the concentration (EC_{50}) producing a half-maximal contractile response to phenylephrine. After the effects of the concentration-response curves were washed out, vascular rings were precontracted with EC_{50} doses of phenylephrine (10^{-6} M), and the presence and absence of endothelium were confirmed by testing for relaxation to acetylcholine (10^{-7}–10^{-4} M), which generally resulted in 90% relaxation in aorta with intact endothelium.

Experimental procedures for aortic rings. Before phenylephrine EC_{50} doses were added, the ring aortic segments were exposed to NKRB containing various concentrations of C_2-ceramide (10^{-7}–10^{-4} M), for each dose, for at least 15 min to determine whether or not the sphingolipid derivative had any effects on base-line tension and/or development of spontaneous mechanical activity. A C_2-ceramide inactive analog (i.e., C_2-dihydroceramide) was used as above, as a negative control. C_2-ceramide and C_2-dihydroceramide were dissolved in dimethyl sulfoxide, and aliquots were added to an aqueous medium with sonication to a stock concentration of 1 mM. The stock solution was diluted in the NKRB solution and aliquots added to the chamber and allowed to rapidly mix at concentrations over the range of 10^{-7}–10^{-4} M. In other experiments, phenylephrine EC_{50} doses were added, and when the contraction reached a plateau phase the C_2-ceramide was added cumulatively to the bath, as well as C_2-dihydroceramide. The results of these experiments are expressed in percentage relaxation of the stable contraction induced by phenylephrine (10^{-6} M) (reference contraction). For studies on the role of endothelium in C_2-ceramide-induced relaxation, the rings were exposed to various, specific pharmacologic antagonists for 20 min prior to stimulation with ceramide. To determine whether the induced relaxations were affected by endogenous release of prostanoids and nitric oxide (NO), from the blood vessels, various inhibitors of NO formation were added, e.g., N^G-nitro-L-arginine (L-NNA), and L-N^G-monomethyl-arginine (L-NMMA), as well as indomethacin (5×10^{-5} M). These specific pharmacologic antagonists were added to the baths for at least 20–30 min periods prior to ceramide. Other pharmacologic amine antagonists, such as atropine (10^{-6} to 10^{-5} M), diphenhydramine (10^{-5} M), cimetidine (10^{-5} M), propranolol (10^{-5} M), and methysergide (10^{-6} to 10^{-5} M) were also used (added 20 min prior to ceramide) to determine whether the ceramide responses were due to release of different types of vasoactive amines. To determine whether protein kinase C (PKC) activation plays any role in ceramide-induced relaxation, three different inhibitors of PKC (i.e., staurosporine, H7, and bisindolylmaleimide I) were tested over a wide concentration range; these drugs were also added 15–20 min prior to phenylephrine and before ceramide addition.

$[Ca^{2+}]_i$ studies in single cultured vascular smooth muscle cells. $[Ca^{2+}]_i$ in single vascular smooth muscle cells was measured according to previously established methods (12,13). Cultured cells were preloaded with 2 μM fura-2 acetoxymethyl ester in the culture medium without fetal bovine serum for 45–60 min in a humidified 37°C air incubator. To improve the loading efficiency, 0.12% pluronic F-127 was used in the loading medium. Following dye-loading, the coverslips, containing fura-2-loaded cells, were washed three times with HEPES buffer solution and placed in a tissue chamber on a temperature-controlled stage of a Nikon fluorescence microscope and subsequently superfused with HEPES buffer solution (pH 7.4, 37°C). The HEPES buffer solution contained (in mM): NaCl 118, KCl 4.8, $CaCl_2$ 2.5 , KH_2PO_4 1.2, $MgSO_4$ 1.2, HEPES 5, and glucose 10. The pH was brought to 7.4 with NaOH. Measurement of $[Ca^{2+}]_i$ was performed using a PTI model 4000 Deltascan, LPS-220 Image Analyzer, Video Scope (Photon Technology International, Inc., South Brunswick, NJ), and the $[Ca^{2+}]_i$ was measured 20 min after C_2-ceramide or C_2-dihydroceramide adminstration without superfusion. The cultured cells, preloaded with fura-2 acetoxymethyl ester, were excited, alternately, at 340 and 380 nm. The emission intensity was recorded at 510 nm using a silicon intensified target camera.

Fluorescence ratios (R) were obtained by dividing the 340-nm images by the 380-nm images. Then $[Ca^{2+}]_i$ of single vascular smooth muscle cells was calculated by using the equation:

$$[Ca^{2+}]_i = K_d \times B \times \left[(R - R_{\min})/(R_{\max} - R)\right] \quad [1]$$

A K_d (dissociation constant) of 224 nM was used for the fura-2/Ca^{2+} complex. B is the ratio of fluorescence intensity of

fura-2 to Ca-bound fura-2, with excitation at 380 nm. Calibration parameters were determined using small volumes of buffered calibrating solutions (pH 7.2, 37ºC) containing 3 μM fura-2 pentapotassium salt and various $[Ca^{2+}]$ (12,13,15). The intensity of the recorded images at 340 and 380 nm was corrected by subtracting them from background fluorescence recorded at the corresponding wavelengths. The resulting images were then used to calculate $[Ca^{2+}]_i$ in vascular smooth muscle cells. Calibration showed that our 340:380 ratio fell on the linear portion of the calibration curve. Particular care was taken to minimize photobleaching of the dye. Experiments were carried out in total darkness, and exposure to excitation light was less than 2 s in all experiments.

Chemicals and reagents. C_2-ceramide and naloxone HCl were obtained from Sigma Chemical Co. (St. Louis, MO). Indomethacin was purchased from Merck (Rahway, NJ); C_2-dihydroceramide, acetylcholine, L-NNA, L-NMMA) propranolol HCl, and diphenhydramine HCl were obtained from Calbiochem Co. (La Jolla, CA). Phenylephrine HCl and atropine sulfate were obtained from Mann Research Lab. Inc. (New York, NY). Fura-2 acetoxymethyl ester was purchased from Molecular Probes Inc. (Eugene, OR). Cimetidine HCl was obtained from Smith-Kline Beecham (Philadelphia, PA). Staurosporine, H7, and bisindolylmaleimide I were all obtained from Sigma Chemical Co. All other chemicals and reagents were obtained from Fisher Scientific (Fairlawn, NJ); these were commercial products of the highest grade available.

Statistical analyses. Where appropriate, results are expressed as means ± SEM. Differences between means were analyzed using nonpaired *t*-tests or analysis of variance followed by a Newman-Keule test. Statistical significance was assumed when $P < 0.05$.

RESULTS

Effects of C_2-ceramide on rat thoracic aorta vasomotor tone. C_2-ceramide (10^{-7}–10^{-4} M), added to organ chambers containing resting rat thoracic aortic rings, did not elicit any significant changes in basal tension (data not shown, $n = 8$). However, treatment of phenylephrine-contracted rat aortic rings with and without endothelium, with C_2-ceramide (10^{-7}–10^{-4} M), resulted in concentration-dependent relaxation, respectively, during stable contraction evoked by 10^{-6} M phenylephrine (Fig. 1C, D; Fig. 2). The relaxation responses in endothelium intact rings were of a somewhat greater magnitude ($P < 0.001$) compared to endothelium-denuded rings, indicating that the responses were only partially dependent on intact endothelial cell function. At lower concentrations (i.e., 10^{-7}–10^{-6} M), C_2-ceramide also elicited a 25% reduction (relaxation), which could be deemed physiologic, of phenylephrine-induced contractions (Fig. 2). In con-

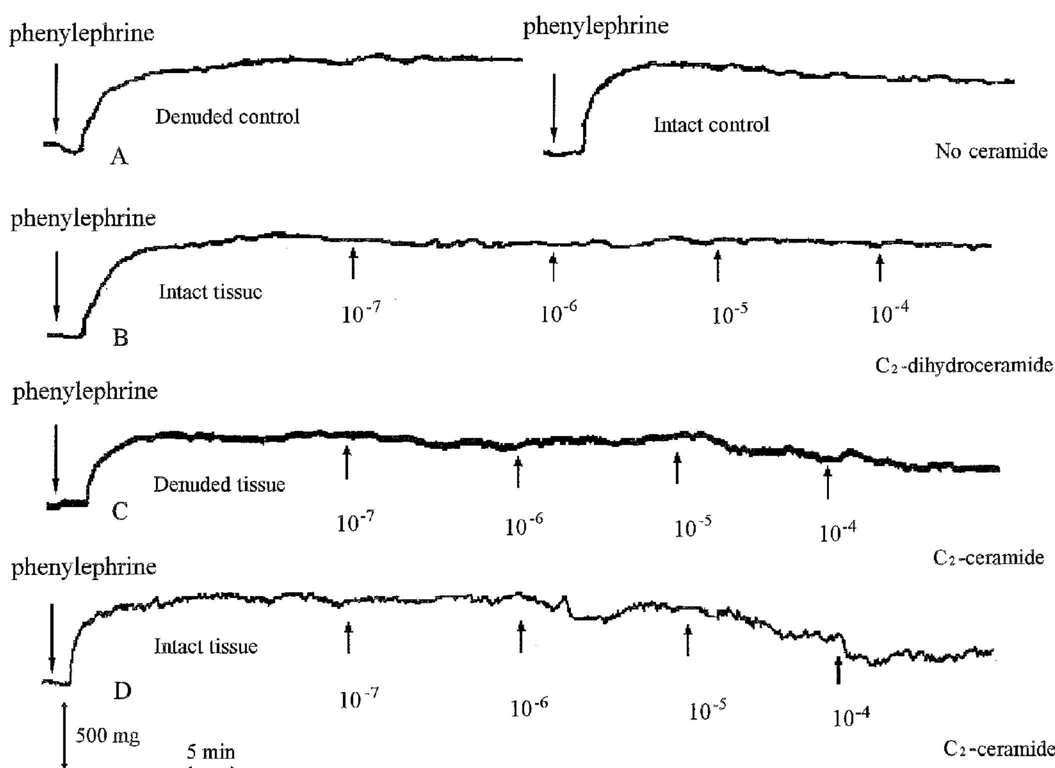

FIG. 1. Typical recording of C_2-ceramide-induced relaxations of phenylephrine-contracted aortic rings in intact and denuded tissue and inactive C_2-dihydroceramide controls. Vertical bar = tension; horizontal bar = time. C_2-dihydroceramide, unlike active C_2-ceramide, failed to elicit any relaxation. (A) Timed controls with phenylephrine added but no ceramide; (B) addition of C_2-dihydroceramide after phenylephrine; (C) C_2-ceramide added to denuded tissue; (D) C_2-ceramide added to intact tissue.

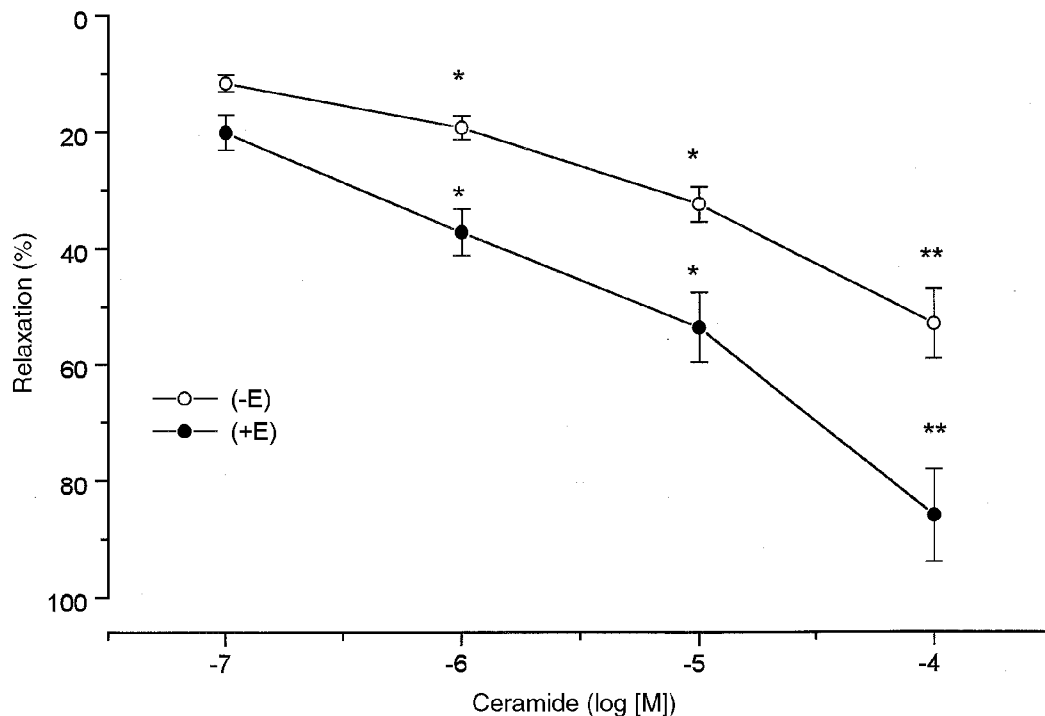

FIG. 2. Effects of ceramide on phenylephrine (10^{-6} M)-induced contractions in rat aortic segments in the absence or presence of endothelium. Values are expressed as means ± SE. All concentrations of ceramide at 10^{-6} to 10^{-4} M are significantly different between rings with and without endothelium (*$P < 0.05$, **$P < 0.01$), $n = 8$ each.

trast, C_2-dihydroceramide, an inactive analog of ceramide, had no relaxant effects (Fig. 1B).

Effects of NO inhibitors, indomethacin, and pharmacologic antagonists on C_2-ceramide-induced vasorelaxation. As normal endothelial cells can release several vasoactive substances, such as NO and prostacyclin, and endogenous amines can also be released from the arterial wall, we determined whether C_2-ceramide-induced relaxations were due to release of any these substances. In the presence of two different inhibitors of NO [i.e., L-NNA (5×10^{-5} M), L-NMMA (5×10^{-5} M)] or the cyclooxygenase inhibitor, indomethacin (5×10^{-5} M), the vasorelaxations in response to C_2-ceramide were not attenuated significantly ($n = 6$ each, $P > 0.05$, data not shown). These results suggest that the partial endothelium-dependent relaxations to C_2-ceramide probably do not depend on either an L-NNA-sensitive or L-NMMA-sensitive NO release from endothelium, or cyclooxgenase-sensitive prostacyclin generation. Thus, there may exist another possible vasodilator(s) which are synthesized and released from endothelium in response to C_2-ceramide. Because endothelial cells may also release a variety of vasorelaxation factors other than NO (e.g., endothelium-dependent hyperpolarizing factors, epoxyeicosatrienoic acids, etc.) (16), potential vasorelaxant effects of active ceramide metabolites must be postulated until either ruled in or out. That the response to C_2-ceramide was only slightly attenuated, but not abolished, after de-endothelialization implies that C_2-ceramide exerts most of its relaxant effects by direct actions on smooth muscle cells that are not dependent on endothelium-derived relaxing factor(s). Although not shown, a wide variety of amine pharmacologic antagonists (i.e., atropine, diphenhydramine, cimetidine, propranolol, methysergide) and an opiate antagonist (naloxone) all failed to attentuate or interfere with the ceramide-induced relaxations ($n = 6$–8 each, $P > 0.05$) (data not shown).

Effects of protein kinase C (PKC) inhibitors, staurosporine, H7, and bisindolylmaleimide I, on C_2-ceramide-induced vasorelaxation. The purpose of these experiments was to determine whether the C_2-ceramide-induced relaxation is dependent on activation of PKC. Various PKC inhibitors were added 15 min before and 15 min after treatment of phenylephrine-contracted intact and denuded aortic rings, using staurosporine (5×10^{-8}–5×10^{-6} M), H7 (5×10^{-8}–5×10^{-6} M) or bisindolylmaleimide I (5×10^{-7}–5×10^{-6} M), respectively. We found that all of these agents, which collectively act on all active sites of PKC, did not significantly attenuate or interfere with the C_2-ceramide-induced relaxations ($n = 6$ each, $P > 0.05$, data not shown). These results suggest that activation of a PKC pathway cannot explain ceramide's relaxant action on phenylephrine-induced vasoconstriction.

Effects of C_2-ceramide on phenylephrine-induced changes in $[Ca^{2+}]_i$ in cultured rat aortic smooth muscle cells. Since Ca^{2+} plays a critical role in the regulation of smooth muscle tone, we examined the effect of ceramide on phenylephrine-induced changes in $[Ca^{2+}]_i$ .Table 1 shows that addition of 10^{-6} M phenylephrine to cultured rat aortic smooth muscle cells caused a significant rise in $[Ca^{2+}]_i$ from a mean resting level of 84.8 ± 6.0 nM (35–40 cells) to 116.7 ± 4.8 nM

TABLE 1
Ceramide Attenuates Rises in $[Ca^{2+}]_i$ Concentration Induced by Phenylephrine in Single Rat Aortic Smooth Muscle Cells

Treated with	$[Ca^{2+}]_i$ (nM)
Control	84.8 ± 6.0
Ceramide alone (10^{-5} M)	89.9 ± 6.3[a]
Phenylephrine alone (10^{-6} M)	116.7 ± 4.8*,[b]
Phenylephrine + ceramide	77.2 ± 5.3**,[a]

[a]The $[Ca^{2+}]_i$ was measured 20 min after ceramide was added to the chamber. [b]Phenylephrine-induced $[Ca^{2+}]_i$ value, alone, was measured 5 min after its addition to the organ bath. Values are means ± SEM of at least 15–20 cells. Significantly different from control (*P < 0.01). Significantly different from phenylephrine alone (**P < 0.01).

(15–20 cells) ($P < 0.01$). Exposure of cultured rat aortic smooth muscle cells to 10^{-5} M C_2-ceramide, alone did not significantly change the resting levels of $[Ca^{2+}]_i$. However, after addition of C_2-ceramide, phenylephrine-induced elevations of $[Ca^{2+}]_i$ were inhibited (from 116.7 ± 4.8 nM to 77.2 ± 5.3 nM, n = 20, $P < 0.01$). Although not shown, lower concentrations of C_2-ceramide (e.g., 10^{-7}, 10^{-6} M) exerted weaker inhibitory actions on phenylephrine-induced rises in $[Ca^{2+}]_i$. Figure 3A shows that addition of 10^{-6} M phenylephrine to cultured rat aortic smooth muscle cells caused a biphasic rise in $[Ca^{2+}]_i$. The first phasic component reached a peak fairly quickly and then fell rapidly to a lower steady-state level which is the second component; this is clearly above the basal steady-state level. Chelation of extracellular Ca^{2+} in the complete absence of $[Ca^{2+}]_o$, with EGTA (2 mM) added, almost abolished the phenylephrine-induced secondary phase rise of $[Ca^{2+}]_i$ (Fig. 3B), indicating attenuation of influx of extracellular Ca^{2+}. Treatment with C_2-ceramide (10^{-5} M), before or after phenylephrine was added, resulted in an inhibition of the secondary phase rise of $[Ca^{2+}]_i$ in a manner clearly distinguishable from that of control cells cultured for the same period of time (Fig. 3C, D). The time course of this reduction in $[Ca^{2+}]_i$ was similar to the time required for ceramide-induced relaxation. Although not shown, the inactive analog, C_2-dihydroceramide, did not significantly inhibit phenylephrine-induced elevation in $[Ca^{2+}]_i$.

DISCUSSION

To our knowledge, the present results represent the first demonstration that C_2-ceramide can attenuate phenylephrine-induced contractions in isolated rat aortic rings, as well as inhibit elevations of $[Ca^{2+}]_i$ produced by a contractile agent in cultured, single primary rat aortic smooth muscle cells. This action may be of real physiologic importance, as the biologically active concentrations of ceramide shown herein (e.g., 10^{-7} to 10^{-6} M) are in the range thought to be generated *in vivo*. The concentration of C_2-ceramide used here for $[Ca^{2+}]_i$ measurement in cultured rat aortic smooth muscle cells (10^{-7} to10^{-5} M) is consistent with those used in earlier studies on HL-60 cell differentiation (1–10 μM) (17), phospholipase D inhibition in fibroblasts (10–50 μM) (18), and rat pinealocytes (10–100 μM) (21). However, the mechanism(s) for the inhibitory action of C_2-ceramide on phenylephrine's action are not as yet fully understood.

The rise in cytosolic Ca^{2+} induced by α_1-adrenergic stimulation of vascular smooth muscle is due initially to the release of Ca^{2+} from internal stores, resulting in transient elevation of $[Ca^{2+}]_i$ (primary Ca^{2+} spike). The internal release of Ca^{2+} is responsible for the initial phasic component of contraction, which is succeeded by a slower tonic component dependent on influx of extracellular Ca^{2+} and involving increased Ca^{2+} permeability of the plasma membrane (9). As shown in Figure 3B, chelation of extracellular Ca^{2+} with

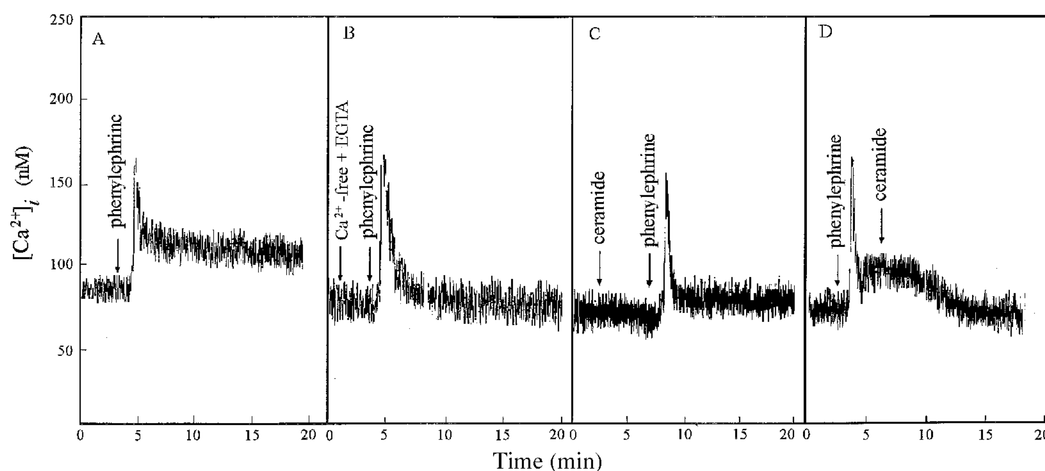

FIG. 3. Effects of C_2-ceramide (10^{-5} M) on phenylephrine-induced rises in $[Ca^{2+}]_i$ in single aortic smooth muscle cells. Effects of phenylephrine alone on $[Ca^{2+}]_i$ in single aortic smooth muscle cells (A). Phenylephrine-induced rises in $[Ca^{2+}]_i$ in the absence of extracellular Ca^{2+} with 2 mM EGTA added (B). Effects of C_2-ceramide addition before (C) and after (D) phenylephrine (10^{-6} M) on rises in $[Ca^{2+}]_i$ in single aortic smooth muscle cells. Traces shown are representative examples of typical responses of single cells from at least 10 similar separate experiments in various assay conditions. Phenylephrine—(10^{-6} M); ceramide—(10^{-5} M).

EGTA (in the absence of $[Ca^{2+}]_o$) markedly reduced the phenylephrine-stimulated secondary phase rise of $[Ca^{2+}]_i$, indicating diminution of influx of extracellular Ca^{2+}. Treatment with C_2-ceramide (10^{-5} M), before or after phenylephrine was added, did not affect the initial increase in $[Ca^{2+}]_i$ or the peak value (Fig. 3A, C, D), but did cause a rapid decline in the secondary phase rise of $[Ca^{2+}]_i$ in a manner distinguishable from that of control cells cultured for the same period of time (Fig. 3C, D). The phenylephrine-induced $[Ca^{2+}]_i$ increase under such conditions is due to its release from internal stores by myo-inositol-1,4,5-trisphosphase (9). This agrees with the result that Ag^+-induced formation of myoinositol-1,4,5-trisphosphase is not affected by C_2-ceramide (19). The $[Ca^{2+}]_i$ profiles obtained herein in the presence of C_2-ceramide were nearly the same as those observed in the Ca^{2+}-depleted cells in the presence of EGTA (Fig. 3B). Thus, these results lead us to consider that C_2-ceramide blocked phenylephrine-stimulated Ca^{2+} influx from the extracellular source.

Wong *et al*. (20) reported that C_2-ceramide can inhibit human peripheral blood polymorphonuclear leukocyte Ca^{2+} influx induced by *n*-formyl-methionyl-leucyl-phenylalanine and suggested that ceramide-activated protein phosphatase (CAPP), activated by ceramide, may dephosphorylate a calcium influx factor and thereby inactivate it. Very recently, ceramide was shown to inhibit L-type Ca^{2+} channels in rat pinealocytes (21) and L-type Ca^{2+} channel current in rat ventricular myocytes (22). Our findings, in the present study, are thus in line with these suggestions. Whatever its precise molecular mechanism of action, C_2-ceramide inhibition of Ca^{2+} influx promotes a decrease of $[Ca^{2+}]_i$ and may result in dephosphorylation of myosin, resulting in relaxation.

In recent *in vitro* investigations, CAPP, a serine/threonine protein phosphatase, was shown to be activated directly and specifically by ceramide (2,3). CAPP is related to and regarded as a subtype of the protein phosphatase 2A (PP2A) family (2,3). Dobrowsky *et al*. (23) reported that C_2-ceramide can activate heterotrimeric PP2A. Moreover, PP2A was shown to catalyze the dephosphorylation of calponin and caldesmon (24,25). Calponin and caldesmon, in smooth muscle cells, inhibit smooth muscle contraction by decreasing the actin-activated Mg-ATPase activity of myosin; and the phosphorylation of calponin and caldesmon by PKC or Ca^{2+}/calmodulin-dependent kinase II results in loss of their ability to inhibit the actomyosin Mg-ATPase (26,27). Therefore, an increased activity of smooth muscle PP2A induced by ceramide might result in relaxation *via* a dephosphorylation of calponin and caldesmon.

In conclusion, our experiments demonstrate that ceramide, a putative sphingolipid second messenger, can inhibit phenylephrine-induced contractions and phenylephrine-induced elevations in $[Ca^{2+}]_i$ in rat aortic smooth muscle. Although the mechanisms underlying the precise relaxant actions of ceramide remain to be established, a reduction in intracellular Ca^{2+} is clearly a key factor in causing relaxation. Our results suggest that a sphingomyelin-signaling pathway may play an important regulatory role in arterial wall tone.

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Specific Modifications of Phosphatidylinositol and Nonesterified Fatty Acid Fractions in Cultured Porcine Cardiomyocytes Supplemented with n-3 Polyunsaturated Fatty Acids

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ABSTRACT: Mechanisms for the antiarrhythmic effect of n-3 polyunsaturated fatty acids (PUFA) are currently being investigated using isolated cardiac myocytes. It is still not known whether the incorporation of n-3 PUFA into membrane phospholipids is a prerequisite for its protective action or if n-3 PUFA exert antiarrhythmic effects in their nonesterified form as demonstrated by recent studies. Adult porcine cardiomyocytes were grown in media supplemented with arachidonic acid (AA), eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA). After 24 h, analysis of total lipids showed that the myocytes were enriched with the respective fatty acids compared to control cells. Large proportions of all three fatty acids supplemented (69% AA, 72% DHA, and 66% EPA) remained unesterified. Fatty acid analyses of total phospholipids (PL) revealed that the incorporation of EPA and DHA, though small, was significantly different ($P < 0.05$) from that of the control cells. The PL fraction was further separated into phosphatidylinositol (PI), phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine to study the pattern of incorporation of the fatty acids in these fractions. It became apparent that EPA and DHA were selectively incorporated into the PI fraction. This study demonstrates that in adult porcine cardiomyocytes, the n-3 PUFA supplementation selectively modulates two important lipid fractions, nonesterified fatty acid and PI, which were implicated in the mechanisms of prevention of cardiac arrhythmias.

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The role of dietary n-3 polyunsaturated fatty acids (PUFA) in the prevention of cardiac arrhythmia in animal models and in cardiomyocytes is now well established. The mechanisms by which these dietary n-3 PUFA may confer their protective effects were reviewed (1–3). The n-3 PUFA in fish oil can cause a reduction in the rate of sudden cardiac death by decreasing lethal ventricular arrhythmias in patients with coronary heart disease (4). Early pioneering studies by McLennan (5) and Charnock *et al.* (6) reported that n-3 PUFA exhibit their beneficial effects *via* incorporation into sarcolemmal phospholipids (PL), thereby changing membrane properties and altering eicosanoid metabolism. In a study by Hallaq *et al.* (7), the protective effect of n-3 PUFA was demonstrated by its prevention of ouabain-induced rise of free cytosolic calcium to toxic levels within myocytes. Studies conducted by Leaf *et al.* (8,9) demonstrated the antiarrhythmic effects of the free (nonesterified) form of the n-3 PUFA on isolated rat neonatal ventricular cardiomyocytes. A recent study by Billman *et al.* (10) performed on conscious, exercising mongrel dogs involved intravenous infusion of fish oil fatty acids as an emulsion prior to ischemic challenge. They report statistically significant prevention of ischemia-induced ventricular arrhythmias in dogs by acute infusions of n-3 PUFA.

Cardiac membrane PL play a central role in electrical signal generation and conduction. Many of the biological effects of n-3 and n-6 fatty acids were attributed to the result of the alteration of membrane PL by their inclusion in the diet. Altering membrane PL has a direct consequence for the maintenance of cardiac rhythm through several pathways (11). Less attention, however, was given to the effect of n-3 PUFA supplementation on the cellular nonesterified fatty acid (NEFA) compartment. Recently, it was established that NEFA interacts with a variety of intracellular factors (12,13), and its role in cell signaling without metabolic transformation is particularly relevant to our investigation of its role in the antiarrhythmic mechanism of fish oils. Most studies used isolated neonatal ventricular cardiomyocytes from rats to investigate the effect of n-3 PUFA supplementation; very few studies were using adult cardiomyocytes. The purpose of this experiment was therefore to study the effect of n-3 PUFA supplementation in isolated adult porcine cardiomyocyte and the extent of incorporation into myocyte membrane PL and NEFA. These stud-

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Abbreviations: AA, arachidonic acid; DMEM, Dulbecco's Modified Eagle Medium; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IP_3 inositolphosphates; NEFA, nonesterified fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PS, phosphatidylserine; PUFA, polyunsaturated fatty acids.

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ies demonstrate for the first time that in adult cardiomyocytes a major proportion of n-3 PUFA remains in the NEFA fraction, and a significant amount is selectively incorporated into the phosphatidylinositol (PI) fraction.

MATERIALS AND METHODS

Cell culture. Freshly harvested adult pig hearts were obtained from a local abbatoir (F.C. Nicholls, New South Wales, Australia). Cardiomyocytes were isolated from ventricles of adult pigs using the method of Spinale *et al*. (14) with some modifications. After the ventricles were excised from the heart, they were washed free of blood using a modified Krebbs solution (composition in mmol/L: NaCl 145, KCl 5, NaH_2PO_4 1.2, HEPES 20, glucose 10, taurine 30, succinate 5, L-glutamine 25, adenosine 1, and creatine 5). The tissue was then dissociated in two successive enzyme treatments. The first incubation was carried out at 37°C with gentle shaking in a water bath using collagenase (0.11 mg/mL; Sigma, St. Louis, MO). After 35 min, the supernatant was discarded and the tissue was minced very finely. This was followed by the second incubation in a solution containing collagenase (0.11 mg/mL), DNase (type II, 50 Kunitz/mL; Sigma), 300 mM $CaCl_2$, and 2% bovine serum albumin for 15 min in similar conditions as the first incubation. The tissue and solution were filtered, centrifuged, and the pellet resuspended in Dulbecco's Modified Eagle Medium (DMEM; CSL, New South Wales, Australia) supplemented with 10% (vol/vol) fetal calf serum and 2.0 mM Ca^{2+}.

Cell viability. The number of viable cells was counted at 100× magnification by trypan blue exclusion in a hemocytometer. Only cells that were rod-shaped and excluding trypan blue were defined as viable. Proportion of viable cells was between 88 and 90% in each cell culture. The cells were plated at a live cell density of 1.5×10^6 cells/mL and cultured in a tissue culture incubator at 37°C (5% CO_2, 95% humidity) for 48–72 h until the cells had all adhered.

Fatty acid incubations. After 5–6 d in culture, the plates containing cells were divided into four groups. The cells in each group were incubated for 24 h in a medium (DMEM containing 10% fetal calf serum) supplemented with either 400 µM arachidonic acid (AA), 400 µM eicosapentaenoic acid (EPA), or 400 µM docosahexaenoic acid (DHA). The fourth group of cells was incubated in unsupplemented DMEM and treated as the control group. The fatty acids used for the incubations were obtained from Sigma and were >99% pure. They were dissolved in a small volume of ethanol before addition into medium. The final concentration of ethanol in the medium was 0.04%. Before commencement of this study, numerous experiments were conducted to determine the concentration of fatty acids required for incubation as well as the length of incubation time needed to ensure maximal incorporation in the adult myocytes. Fatty acid concentrations of 50, 100, and 250 µM in the incubation medium were not enough to enrich the myocytes even after 48 h. Increasing the concentration of the fatty acids to 400 µM ensured a significant incorporation (compared to controls) in only 24 h. Before the start of fatty acid analyses, the incubation medium was carefully and completely removed by at least three successive washes to ensure that the fatty acids were not carried over during lipid extraction. Albumin was not included in the wash media because in preliminary experiments addition of albumin caused considerable loss of cells from the plates. Since it was very essential to maintain cell numbers for lipid extraction, addition of albumin was discontinued.

Fatty acid analyses. Fatty acid composition of total lipids of cells after 24-h incubation was determined by direct transesterification using the method of Lepage and Roy (15). Fatty acid composition of cellular PL and nonesterified fatty acid (NEFA) was determined after extraction of lipid from the cells (16) and separation by thin-layer chromatography (TLC). Briefly, lipid was extracted with chloroform/methanol (2:1, vol/vol) containing butylated hydroxytoluene (0.005% wt/vol) to prevent oxidation. The samples were dried under nitrogen, reconstituted in a small volume of chloroform, and separated on TLC plates (Silica gel 'G'; Alltech, New South Wales, Australia), using a solvent system comprising hexane/diethylether/acetic acid (85:15:1, by vol) (17). Heptadecanoic acid (200 µg) was added as a reference standard. After spraying the plates with 2,7-dichlorofluoresceine (0.1% wt/vol in methanol; Ajax Chemicals, New South Wales, Australia) for identification, the PL and NEFA bands were scraped from the plates and redissolved in hexane. The fractions were methylated using BF_3/methanol (14% wt/vol; Sigma) for at least 45 min to 1 h at 100°C (18) to ensure complete transesterification (19) and analyzed by gas chromatography.

Fatty acid composition of the PL fraction of myocytes was determined by separation on TLC plates (Silica gel 'H'; Alltech), using a solvent system comprising chloroform/methanol/2-propanol/triethylamine/0.25 KCl (15:4.5:12.5:9:3, by vol). The PL fractions were identified using authentic individual standards as reference and R_f values as per Touchstone *et al.* (20). The R_f values from our experiments were 0.647, 0.47, 0.382, 0.232, and 0.176 for PI, phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylcholine (PC), and sphingomyelin, respectively. The separated PL were methylated as above and analyzed by gas chromatography. A fused carbon-silica column (30 m × 0.25 mm; J&W Scientific, Folsom, CA) was used to analyze the derivatized fatty acids in a Hewlett-Packard 6890 gas chromatograph (Palo Alto, CA) (21). The injector and detector port temperatures were set at 250°C. The oven temperature was initially held at 170°C for 2 min, then increased by 10°C/min to 190°C, held for 1 min, and then increased by 3°C/min up to 220°C and maintained for 15 min. The total run time was 30 min. A split ratio of 10:1 and an injection volume of 5 µL were used. Sample fatty acid peaks were identified by comparing their retention times with those of authentic standards (Nu-Chek-Prep, Elysian, MN) and area percentage resolved.

Statistical analyses. All data are shown as means ± SEM. The results of fatty acid supplementation were analyzed using analysis of variance using Statview 4.5 and Fisher's Exact Test.

RESULTS

Total fatty acid content. Analysis of total fatty acid content of myocytes after 24 h in media supplemented with 400 μM fatty acid confirmed that incorporation had taken place (Table 1). To distinguish cells grown in control media from cells grown in media supplemented with the three different fatty acids, they have been referred to as control cells (c-cells), EPA-supplemented cells (EPA-cells), DHA-supplemented cells (DHA-cells), and AA-supplemented cells (AA-cells). The results indicate that there was a significant increase in the fatty acid content of supplemented cells compared to controls. AA increased from 11.83 ± 0.05 to 26.37 ± 1.05% in AA-cells, EPA increased from 0.36 ± 0.3 to 18.68 ± 0.55% in EPA-cells, and DHA increased from 1.37 ± 0.03 to 19.88 ± 0.23%.

Myocyte NEFA content. There was a very large increase in the amounts of all three supplemented fatty acids, AA, EPA and DHA, in the NEFA fraction of all the three fatty acid-supplemented groups compared to c-cells ($P < 0.001$). The highest incorporation was in DHA-cells (72.8 ± 0.1% DHA) followed by AA in AA-cells (69.5 ± 5.0%) and then EPA in EPA-cells (66.3 ± 0.2%). The increase in these fatty acids in all groups was accompanied by the decrease in palmitic and stearic acids (saturated fatty acids), oleic acid (monounsaturated fatty acid), and linoleic acid compared to c-cells ($P <$ 0.0001) (Table 2).

Myocyte PL composition. The incorporation of the supplemented fatty acids was modest in the cellular PL compared to the NEFA fraction. In the cellular PL, EPA in EPA-cells was significantly different from that in c-cells ($P < 0.05$). The same difference was observed for DHA in DHA-cells, but interestingly this trend was not seen in the AA-cells. Unlike the NEFA, there was no significant difference in palmitic, stearic, oleic, and linoleic acids among groups (Table 3).

Myocyte triacylglycerol composition. The incorporation of supplemented fatty acids in the triacylglycerol fraction was very small compared to the other fractions, and only AA in AA-cells was significantly different ($P < 0.05$) from control (Table 4).

Fatty acid composition of PL fractions. Total PL were fractionated into PI, PE, PC, and PS, the results of which are shown in Table 5 and Figure 1. Incorporation of EPA and DHA in EPA-cells and DHA-cells, respectively, was highest in the PI fraction (36.9 ± 1.1% EPA and 52.5 ± 1.7% DHA) followed by the PE fraction compared to the c-cells ($P <$ 0.05).The increase of EPA and DHA in the PI fraction was associated with a decrease in the linoleic acid and AA content. In the PC and PS fractions, there was little or no change in the incorporation of EPA and DHA. AA levels in AA-cells were significantly different from c-cells only in the PC fraction ($P < 0.05$) but not in the PI, PE, and PS fractions (Table 5).

DISCUSSION

In order to elucidate the mechanism(s) by which dietary n-3 PUFA exhibit antiarrhythmic properties, we examined the modification of cardiomyocytes isolated from adult pigs by incubating them in the presence of long-chain n-6 and n-3 PUFA. Most studies investigating the role of n-3 PUFA in the prevention of cardiac arrhythmia used neonatal ventricular myocytes isolated from rats. Studies of fatty acid incorporation in porcine cardiac myocytes are not common. A review of literature indicates that our study is the first of its kind using adult porcine myocytes. Therefore, comparison with other similar studies is difficult. Pigs have a cardiac physiology and fatty acid metabolism similar to humans (22). Neonatal cardiac myocytes have a variety of structural and functional differences from adult myocytes (23). Unlike myocytes from neonatal hearts, myocytes from adult heart do not undergo cell division but remain differentiated in culture (24). Earlier studies (25–30) measured fatty acid composition of

TABLE 1
Fatty Acid Composition of Total Lipids of Porcine Cardiomyocytes After 24-h Incubation with Various Fatty Acids[a]

	Fatty acid composition (wt%)				
Fatty acid	Control	AA	EPA	DHA	*P* value
16:0 DMA	9.6 ± 0.1[a]	7.6 ± 0.03[b]	7.9 ± 0.02[b]	7.5 ± 0.4[b]	0.0057
16:0	14.5 ± 0.4[a]	11.5 ± 0.3[b]	10.5 ± 0.2[b]	11.2 ± 0.4[b]	0.003
18:0 DMA	6.8 ± 0.1[a]	5.3 ± 0.01[b]	5.5 ± 0.1[b]	5.2 ± 0.3[b]	0.0063
18:0	13.2 ± 0.9	11.7 ± 0.3	11.3 ± 0.03	11.8 ± 0.2	ns
18:1n-9	12.9 ± 0.04[a]	11.4 ± 0.3[b]	10.4 ± 0.04[b]	11.2 ± 0.04[b]	0.0012
18:1n-7	4.3 ± 0.1[a]	3.6 ± 0.1[b]	3.6 ± 0.02[b]	3.6 ± 0.04[b]	0.0005
18:2n-6	23.4 ± 0.3[a]	18.6 ± 0.2[b]	19.1 ± 0.2[b]	18.3 ± 0.3[b]	0.0003
20:4n-6	11.8 ± 0.1[a]	26.4 ± 1.1[b]	9.6 ± 0.1[c]	9.2 ± 0.1[d]	<0.0001
20:5n-3	0.4 ± 0.4[a]	0.3 ± 0.3[a]	18.7 ± 0.6[b]	0.3 ± 0.3[a]	<0.0001
22:5n-3	1.6 ± 0.02[a]	1.4 ± 0.1[b]	1.3 ± 0.01[b]	1.3 ± 0.02[b]	0.0052
22:6n-3	1.4 ± 0.03[a]	1.2 ± 0.1[a]	1.2 ± 0.01[a]	19.9 ± 0.2[b]	<0.0001

[a]Data are expressed as means ± SEM. Values without a common superscript across individual rows indicate statistically significant difference. $n = 6$ in each group. Only major fatty acids are listed. DMA, dimethylacetals; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; ns, not significant.

TABLE 2
NEFA Profiles of Porcine Cardiomyocytes After 24-h Incubation with Various Fatty Acids[a]

	NEFA composition (wt%)				
Fatty acid	Control	AA	EPA	DHA	*P* value
16:0 DMA	3.8 ± 1.2	0.7 ± 0.4	0.7 ± 0.4	0.2 ± 0.2	ns
16:0	20.9 ± 1.1^{a}	6.4 ± 0.9^{b}	6.1 ± 0.4^{b}	5.4 ± 0.9^{b}	<0.0001
18:0 DMA	1.6 ± 1.4	0.2 ± 0.03	0.1 ± 0.1	1.6 ± 1.6	ns
18:0	15.4 ± 0.8^{a}	6.1 ± 1.3^{b}	5.1 ± 0.5^{b}	4.8 ± 0.8^{b}	<0.0001
18:1n-9	27.5 ± 0.2^{a}	7.5 ± 0.9^{b}	7.7 ± 0.8^{b}	8.5 ± 1.8^{b}	<0.0001
18:2n-6	8.9 ± 1.1^{a}	4.0 ± 1.1^{b}	4.2 ± 0.3^{b}	3.5 ± 0.8^{b}	0.0080
18:3n-3	5.2 ± 0.8^{a}	1.5 ± 0.4^{b}	1.3 ± 0.3^{b}	1.4 ± 0.3^{b}	0.0009
20:4n-6	1.1 ± 0.2^{a}	69.5 ± 5.1^{b}	1.1 ± 0.1	0.6 ± 0.2	<0.0001
20:3n-3	1.8 ± 0.7	0.4 ± 0.1	2.5 ± 2.4	0.1 ± 0.01	ns
20:5n-3	0.9 ± 0.5^{a}	0.5 ± 0.2^{a}	66.3 ± 3.6^{b}	0.5 ± 0.2^{a}	<0.0001
22:5n-3	0.6 ± 0.4	0.3 ± 0.01	0.4 ± 0.01	0.2 ± 0.1	ns
22:6n-3	2.1 ± 0.3^{a}	0.5 ± 0.1^{b}	0.7 ± 0.1^{b}	72.8 ± 4.0^{d}	<0.0001

[a]Data are expressed as means ± SEM. Values without a common superscript across individual rows indicate statistically significant difference. *n* = 6 in each group. Only major fatty acids are listed. NEFA, nonesterified fatty acid. See Table 1 for other abbreviations.

total lipid and PL fractions after growing cardiomyocytes in media enriched with individual fatty acids and in combinations of n-3 and n-6 PUFA. While some looked at the effect of n-3 and n-6 fatty acid supplementation of cardiomyocytes to investigate the essential fatty acid metabolism (27,28), others studied their effect on electrophysiology and contraction (30,31) and on hypoxia and hypoxia-induced phospholipase A_2 activity (25,26). These studies all used neonatal rat ventricular myocytes grown in media supplemented with fatty acid concentrations ranging from10 to 100 μM and incubated for periods of 24 h up to 4 d. Of these studies, two examined the total fatty acid content of modified myocytes (27,28), four examined only the PL fatty acid composition of modified myocytes (25,26,29,30) and only one analyzed the fatty acid content of total lipids and PL fatty acid composition of myocytes after supplementation (32).

None of these previous studies measured the NEFA content and composition in the myocytes. The results presented here documented for the first time the incorporation of n-3 PUFA in the NEFA fraction in an adult myocyte model. We found that the n-3 PUFA were selectively incorporated into the NEFA and PI fraction after growing myocytes for 24 h in media supplemented with these fatty acids. Using growing pigs, we recently demonstrated that cell lipid fractions (NEFA, PL, and triglycerides) are uniformly enriched with n-3 PUFA following feeding of n-3 PUFA-enriched diets (Nair, S., Leitch, J., and Garg, M., unpublished observations). Whether specific modifications of NEFA and PI fractions occur in myocytes isolated from adult animals fed a diet supplemented with n-3 PUFA remains to be determined. If the same is true for adult human myocytes following dietary n-3 PUFA supplementation, then the accumulation of n-3 PUFA

TABLE 3
Composition of Membrane Phospholipids in Porcine Myocytes After 24-h Incubations with Fatty Acids[a]

	Phospholipid fatty acid composition (wt%)				
Fatty acid	Control	AA	EPA	DHA	*P* value
16:0 DMA	4.1 ± 1.2^{a}	4.9 ± 0.1^{b}	5.8 ± 0.2^{b}	2.5 ± 1.2^{b}	0.0195
16:0	14.3 ± 0.2^{a}	13.6 ± 0.5^{a}	13.1 ± 0.2^{a}	13.7 ± 0.5^{a}	ns
18:0 DMA	2.0 ± 0.8	3.2 ± 0.9	3.9 ± 0.2	1.7 ± 0.9	ns
18:0	12.8 ± 0.6	13.9 ± 0.4	13.5 ± 0.3	14.4 ± 0.6	ns
18:1n-9	14.3 ± 0.1	13.7 ± 0.6	12.9 ± 0.3	14.1 ± 0.5	ns
18:1n-7	5.6 ± 0.2	5.4 ± 0.1	5.3 ± 0.1	5.7 ± 0.01	ns
18:2n-6	27.2 ± 0.8	26.0 ± 0.4	25.6 ± 0.4	26.8 ± 0.3	ns
18:3n-3	0.7 ± 0.1	0.6 ± 0.04	0.6 ± 0.1	0.7 ± 0.1	ns
20:4n-6	11.6 ± 0.1	11.6 ± 0.1	11.4 ± 0.2	12.0 ± 0.2	ns
20:3n-3	0.3 ± 0.0	0.2 ± 0.1	0.3 ± 0.02	0.3 ± 0.02	ns
20:5n-3	1.1 ± 0.1^{a}	1.1 ± 0.02^{a}	1.7 ± 0.2^{a}	1.1 ± 0.03^{b}	0.0192
22:5n-3	2.1 ± 0.05	2.1 ± 0.1	2.1 ± 0.04	2.2 ± 0.01	ns
22:6n-3	1.9 ± 0.04^{a}	1.9 ± 0.1^{a}	1.9 ± 0.04^{a}	3.2 ± 0.7^{b}	0.0489

[a]Data are expressed as means ± SEM. Values without a common superscript across individual rows indicate statistically significant difference. *n* = 6 in each group. Only major fatty acids are listed. See Table 1 for abbreviations.

TABLE 4
Composition of Membrane Triglycerides in Porcine Myocytes After 24-h Incubations with Fatty Acids[a]

	Triglyceride fatty acid composition (wt%)				
Fatty acid	Control	AA	EPA	DHA	*P* value
14:0	1.30 ± 0.53	1.52 ± 0.58	0.72 ± 0.12	0.53 ± 0.53	ns
16:0	26.39 ± 0.41	27.30 ± 1.44	26.72 ± 0.62	23.35 ± 1.45	ns
18:1n-9	39.03 ± 1.34	36.85 ± 2.93	35.48 ± 1.11	38.62 ± 0.65	ns
18:1n-7	3.79 ± 0.10[a]	4.34 ± 0.05[b]	4.38 ± 0.19[b]	4.68 ± 0.13[b]	0.0072
18:2n-6	4.67 ± 2.67	6.62 ± 0.28	7.09 ± 0.63	7.11 ± 0.32	ns
18:3n-3	0.67 ± 0.28	0.51 ± 0.03	0.56 ± 0.07	0.55 ± 0.05	ns
20:4n-6	0.31 ± 0.03[a]	0.62 ± 0.02[b]	0.59 ± 0.05	0.63 ± 0.05	0.006
20:5n-3	0.03 ± 0.03	0.00 ± 0.00	0.14 ± 0.14	0.60 ± 0.40	ns
22:5n-3	0.49 ± 0.30	0.65 ± 0.02	0.77 ± 0.12	0.82 ± 0.07	ns
22:6n-3	1.52 ± 0.71	1.15 ± 0.60	2.15 ± 1.01	1.58 ± 0.80	ns

[a]Data are expressed as means ± SEM. *n* = 6 in each group. Values with different superscripts across individual rows indicate statistically significant difference. Only major fatty acids are listed. See Table 1 for abbreviations.

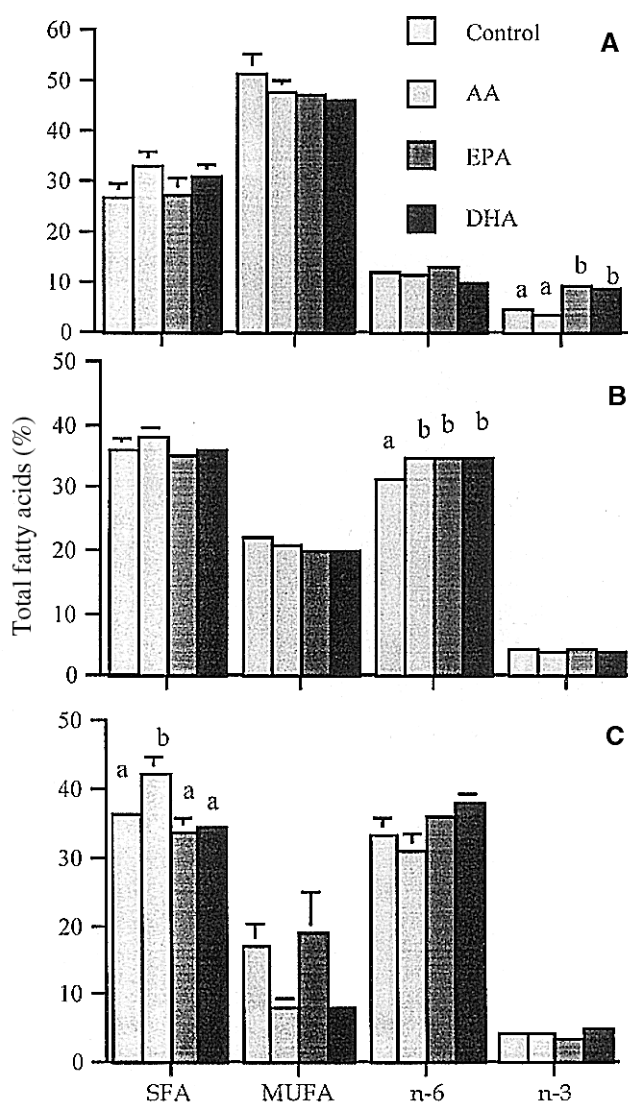

FIG. 1. The results of total phospholipids fractionated into (A) phosphatidylethanolamine, (B) phosphatidylcholine, and (C) phosphatidylserine. SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid.

in the NEFA fraction would be available to interact with ion channels for the prevention of cardiac arrhythmias.

Early evidence for the role of NFA in ischemia was mostly obtained from studies using myocardial tissue (33–35). The "free fatty acid" hypothesis, as it was called, was considered controversial because of the conflicting values obtained by the different groups. Recent studies (9,10,36–38) using neonatal isolated cardiomyocytes renewed the interest in NEFA by demonstrating that n-3 PUFA in their nonesterified forms are able to exert antiarrhythmic action by their effect on Na^+ and Ca^{2+} channels. They also found that AA by itself was antiarrhythmic, but because of its rapid metabolism to arrhythmogenic eicosanoids, it exhibits proarrhythmic activity. In a recent study by Billman *et al.* (10), after infusion of a fatty acid emulsion containing n-3 PUFA to exercising dogs, the NEFA content of plasma increased considerably, supporting the hypothesis that the action of nonesterified PUFA is sufficient to prevent arrhythmia.

The incorporation of n-3 PUFA into the different PL (particularly PI) fractions was studied with a view to understanding their role in the inositol lipid cycle. The inositol lipid cycle is an important aspect of membrane lipid metabolism which generates the second messengers inositol phosphate (IP_3) and diacylglycerol that are involved in intracellular cell signaling and consequently causing Ca^{2+} oscillations within the cells. Modulation of the PI substrate for phospholipase C is a prerequisite for alterations in the release of IP_3.

Studies on the effect of n-3 PUFA supplementation on IP_3 generation in cardiomyocytes are limited. Initial evidence for the role of dietary PUFA in regulation of Ca^{2+} release from endoplasmic reticulum was provided by Kinsella *et al.* (2). Their study revealed that there was an increase in Ca^{2+} uptake by endoplasmic reticulum that could be associated with the prevention of arrhythmias in rats raised on fish oil-enriched diet. A study by Medini *et al.* (39) showed that the administration of diets enriched with oleic, linoleic, or with n-3 PUFA, EPA, and DHA in rabbits resulted in modified production of IP_3 in platelets when stimulated. They found that stimulation of IP_3 generation was lower in the n-3 PUFA-fed animals compared

TABLE 5
Fatty Acid Composition of the Phosphatidylinositol Fraction in Porcine Cardiomyocytes[a]

	Fatty acid (wt%)				
Fatty acid	Control	AA	EPA	DHA	*P* value
16:0 DMA	1.3 ± 0.2	0.3 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.0001
16:0	27.2 ± 2.1[a]	36.4 ± 4.8[b]	24.2 ± 0.4	18.2 ± 0.7[b]	0.0078
16:1	3.6 ± 0.5	4.0 ± 0.8	1.7 ± 0.3	2.0 ± 0.3	0.0405
17:0	1.7 ± 0.1	1.0 ± 0.1	0.9 ± 0.4	0.4 ± 0.0	0.0071
18:0 DMA	2.5 ± 0.41	2.0 ± 2.4	9.5 ± 0.8	6.6 ± 0.7	0.0058
18:0	11.2 ± 0.7[a]	6.5 ± 0.2[b]	4.5 ± 0.2[b]	2.9 ± 0.1[b]	<0.0001
18:1n-9	13.5 ± 0.6[a]	6.2 ± 0.7[b]	3.2 ± 0.3[b]	2.5 ± 0.2[b]	<0.0001
18:1n-7	4.5 ± 0.5	3.7 ± 0.1	2.0 ± 0.1	1.8 ± 0.2	0.0002
18:2n-6	25.3 ± 4.2[a]	23.0 ± 1.0 b	2.7 ± 0.4[b]	10.2 ± 0.6[b]	0.0028
18:3n-3	0.2 ± 0.2	0.8 ± 0.1	0.2 ± 0.0	0.4 ± 0.1	0.0477
20:2n-6	0.6 ± 0.3	0.7 ± 0.4	0.4 ± 0.0	0.4 ± 0.0	ns
20:4n-6	1.8 ± 0.2[a]	1.8 ± 0.5	1.1 ± 0.1	0.6 ± 0.0[b]	0.0244
20:5n-3	1.4 ± 0.7[a]	1.2 ± 0.9	36.9 ± 1.1[b]	0.1 ± 0.0	<0.0001
22:5n-3	0.0 ± 0.0	0.1 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.0002
22:6n-3	1.2 ± 1.2[a]	0.1 ± 0.1	0.4 ± 0.0	52.5 ± 1.7[b]	<0.0001

[a]Data are expressed as means ± SEM. Values with different superscripts across individual rows indicate statistically significant difference. *n* = 3 in each group. Only major fatty acids are listed. See Table 1 for abbreviations.

to other groups. Similar inhibitory effect of fish oil administration on IP_3 was also reported in guinea pig epidermis (40) and in stimulated platelets after *in vitro* incubation of EPA (41). In a study by Woodcock *et al*. (42) involving left atrial myocytes isolated from rats fed n-3 PUFA- and n-6 PUFA-enriched oils for 8 wk, the total release of IP_n was reduced both with and without stimulation by norepinephrine. All these studies indicate a role for n-3 PUFA in cell signaling.

Our results indicate that in adult porcine cardiomyocytes enriched with n-3 and n-6 PUFA, the pattern of incorporation of fatty acids is very different from that observed in neonatal rat cardiomyocytes. We recently completed an *in vivo* study where cardiac myocytes were isolated from pig hearts after a 6-wk dietary supplementation with fish oil (Nair, S., Leitch, J., and Garg, M., unpublished observations). The data are surprisingly similar to that of the *in vitro* data where the incorporation of the fatty acid AA is greater in the PI fraction than in the PC and the incorporation of fatty acids EPA and DHA is greater in the PE and PI compared to the PC. The difference in pattern of incorporation in the different PL fractions may be attributed to the age and the animal species used. A study that might illustrate the difference in incorporation is that by Lamers *et al*. (43). They studied the PL modification in neonatal cardiac myocytes grown in media supplemented with different fatty acids like 18:2n-6 and EPA. In the PI fraction of cell supplemented with EPA (214 μM), they found that EPA incorporation was highest (20.6 mol%) compared to the other fractions PC, PE, and PS. They also found that cardiomyocyte PC more readily incorporated 18:2n-6 than 20:5n-3 which might explain the pattern of incorporation seen in our study. Another study that illustrates the preferential uptake of DHA by cardiac myocyte is that by Bordoni *et al*. (44), where growing neonatal rat cardiac myocytes in a DHA-rich medium (60 μM) increased the content of DHA from 0.67 ± 0.36 to 3.13 ± 0.69 nmol/mg protein in the PI fraction. This was accompanied by a decrease in the content of AA from 13.4 ± 3.59 to 7.73 ± 0.70 nmol/mg protein, but there was no mention of the other PL fractions. Meij *et al*. (45) also found in their study that the PI was not enriched in 20:4n-6 (no values were provided), and they speculate that this could be due to the preferential degradation of this fatty acid in the PI fraction by kinases.

In our study, the increased incorporation of the fatty acids in the NEFA fraction and the selective incorporation of the n-3 PUFA into the PI fraction indicate that the n-3 and n-6 PUFA could be adopting different routes after entry into cell membrane. The results could also indicate a possible route through which these fatty acids could modulate the inositol lipid cycle. This study links two of the mechanisms proposed to explain the antiarrhythmic effect of n-3 PUFA from fish oil: (i) the direct effect of NEFA on the myocardium and (ii) effect on inositol lipid cycle and cell signaling *via* the modification of membrane PL. Studies on the effect of n-3 PUFA on IP_3 release and calcium oscillations are currently being conducted in our lab. In true sense, in the study of the antiarrhythmic mechanisms of n-3 PUFA, supplementations are warranted for adult human beings only, therefore research efforts should be focused on examination of the n-3 PUFA effects in cells/tissues from adult animals or adult human subjects.

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Dose-Dependent Inhibition of Cell Proliferation Induced by Lipid Peroxidation Products in Rat Hepatoma Cells After Enrichment with Arachidonic Acid

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ABSTRACT: Polyunsaturated fatty acids (PUFA) are important constituents of membrane phospholipids, whose levels are decreased in some tumor cells. This deficiency may cause alterations in signal transduction and an interruption of normal cellular events. The enrichment of tumor cells with PUFA may stimulate or inhibit tumor growth, probably depending on the type of PUFA and the cellular concentration of aldehydes derived from restored lipid peroxidation. We examined the effect of several doses of prooxidant on the growth of hepatoma cells with different aldehyde dehydrogenase activities, enriched with arachidonic acid. Two doses of prooxidant were sufficient to reduce growth of hepatoma cells with low aldehyde dehydrogenase activity, whereas three doses were necessary for those with high enzyme activity. In both cases, lipid peroxidation products blocked the cells in the S phase.

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Polyunsaturated fatty acids (PUFA) are important constituents of membrane phospholipids. After conversion to prostaglandins (PG), leukotrienes, or oxygen-derived free radicals, they mediate a variety of pathological processes (1). The PUFA content is drastically decreased in hepatoma cells in comparison with normal hepatocytes (2), in glioma cells in comparison with oligodendroglia cells (3,4) and in melanoma cells (5). This deficiency may cause alterations in signal transduction and an interruption of normal cellular events (3).

During differentiation, the fatty acid pattern shifts toward that of normal cells (6). This may be spontaneously occurring, or induced by several substances in glioma and neuroblastoma cells. Thus, there is great interest in the fields of chemoprevention and chemotherapy exploring in the effect of PUFA on tumors.

It appears certain that n-3 PUFA inhibit the induction and growth of tumors, such as carcinoma of the breast, colon, and pancreas (7–10). This inhibitory effect may be related to the accumulation of lipid peroxidation products in the tumor tissues (8) or to a decrease of oncogene expression (11), production of PGE_2, tumor necrosis factor, and interleukin-1 (11,12).

The effect of n-6 PUFA appears more contradictory. Studies have shown that arachidonic acid (ARA) is an effective stimulator of tumor growth *in vitro* and *in vivo* (13–15), by promoting cell proliferation or by suppressing apoptosis (16); it is also required for the invasive activity of tumor cells (17). The stimulatory effect of ARA is mediated by its metabolites, i.e., $PGF_{2\alpha}$ and 5-hydroperoxy-eicosatetraenoic acids (17). However, other studies have shown that exogenously supplied n-6 PUFA kill malignant cells selectively (18,19). For example, 7,12-dimethylbenz[a]anthracene-initiated mouse skin failed to result in a tumor if treated with ARA (20). HepG2 cells also undergo cytotoxicity and apoptosis when treated with ARA (21). The cytotoxic and apoptogenic effects are probably due to the involvement of lipid peroxidation (22,23).

Changes in PUFA content in membrane phospholipids have been shown to contribute to the resistance of tumors to chemotherapy (24). Many anticancer therapies, including irradiation, chemotherapy, hyperthermia and photodynamic therapy, kill tumor cells by processes involving free-radical generation leading to lipid peroxidation. Malignant cell resistance to such therapy may be due, in part, to the altered PUFA composition. It has been demonstrated that increasing the PUFA content in tumor cells increases the effect of some types of therapy (25–28).

In view of the importance of PUFA in controlling cell proliferation, we decided to concentrate on the fact that ARA levels are lower in hepatoma cells than in normal hepatocytes. Parallel to the decrease of PUFA, hepatoma cells show a decrease of lipid peroxidation, and in consequence, also of its products, especially the 4-hydroxyalkenals, which are probably involved in growth regulation. 4-Hydroxynonenal (HNE) added to leukemia HL-60 cells reduced cell growth and induced cell differentiation in granulocytes (29); when added to hepatoma cells it reduced their capability to form colonies (2). In previous research, we showed that enriching hepatoma cells with ARA to the normal value induced susceptibility to lipid peroxidation and increased adenylate cyclase activity (30,31). Subsequent exposure to prooxidants caused an in-

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Abbreviations: ALDH, aldehyde dehydrogenase; ARA, arachidonic acid; 4-HNE, 4-hydroxynonenal; LDH, lactate dehydrogenase; MDA, malondialdehyde; PG; prostaglandins; PUFA, polyunsaturated fatty acids.

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crease in lipid peroxidation products and, in parallel, a reduction in cell proliferation and death.

In view of the contradictory results obtained in regard to the effects of n-6 PUFA, we decided to continue our research into the cytotoxic effects of ARA, mediated by lipid peroxidation. The different behavior of n-6 PUFA might be related to the type of stimulatory agent used to induce PUFA metabolism, or to the different concentrations of lipid peroxidation products inside the cells (30). In this study, we considered the second hypothesis, and we carefully examined the effects of different concentrations of lipid peroxidation products on cell proliferation. At the same time, we aimed to identify the cell-cycle phase at which tumor cells are blocked.

MATERIALS AND METHODS

Culture conditions. Hepatoma cell lines (7777 and JM2) were routinely grown as monolayers under an atmosphere of 5% CO_2 and 95% air in 25 cm^2 tissue culture flasks. The 7777 hepatoma cell line was seeded (time 0) at 20,000 cells/cm^2, and JM2 hepatoma cell line at 15,000 cells/cm^2. Both cell lines were maintained for 24 h in medium A (DMEM/F12 plus 2 mM glutamine and 1% antibiotic/antimycotic solution) supplemented with 10% newborn calf serum. After 24 h, medium A was removed and replaced with medium B [medium A plus 0.4% albumin, 1% ITS (insulin, transferrin, sodium selenite), 1% nonessential amino acid solution, 1% vitamin solution]. The cells were divided into two groups: controls and ARA-treated. ARA, dissolved in newborn calf serum (0.2% final concentration in the flasks) with mechanical stirring, was added to the latter group of cells, so as to have a concentration of 250 or 400 nmoles/10^6 cells in each flask. ARA from porcine liver, approximately 98% pure, was purchased from Sigma Chemical Company. α-Tocopherol was not present in the ARA preparation as determined by high-performance liquid chromatography. To avoid the autooxidation of ARA during storage, fresh solution was prepared for each experiment. Twenty-four hours later (time 48 h), ARA-supplemented medium B was removed and replaced with ARA-free medium B. Over the next 48 h, the two groups (control and ARA) were divided into five subgroups each, as in Figure 1, and received 0, 1, 2, 3, or 4 doses of prooxidant (500 μM ascorbate and 100 μM iron sulfate) at 12-h intervals. Cells from each of the 10 subgroups were harvested with 0.5 mL of trypsin (0.25%) at the times noted in Figure 1. When malondialdehyde (MDA) determination was carried out, the cells were collected with trichloroacetic acid at the times shown in Figure 1.

Lipid peroxidation. Lipid peroxidation was measured as the production of MDA as described by Canuto *et al.* (30). Briefly, cold 50% TCA (0.1 mL/mL of culture medium) was added to the flasks maintained in ice. After 15 min, cells were harvested and centrifuged at 2500 × g for 15 min. Supernatants were used for MDA determination by spectrophotometric thiobarbituric acid assay.

Viability test. Cell viability was determined as lactate dehydrogenase (LDH) release. LDH was evaluated in the culture medium as described by Kornberg (32). Results are expressed as nmoles of NADH consumed per mL of medium.

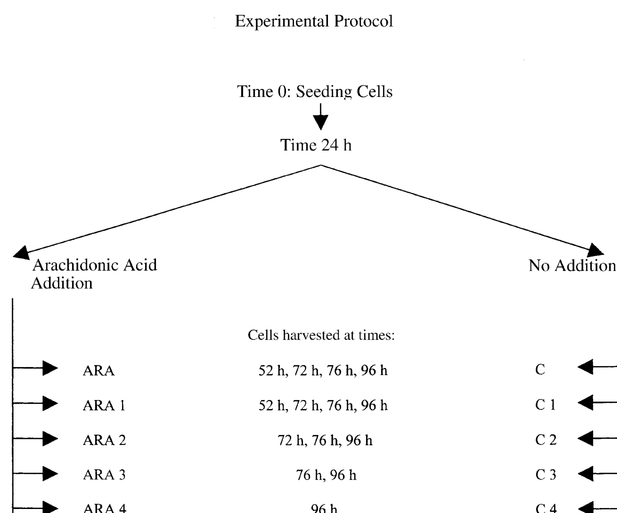


FIG. 1. Times of arachidonic acid (ARA) and ascorbate/iron sulfate addition and times of cell harvest for determination of the various parameters. ARA 1 and C 1, cells exposed to 1 dose of ascorbate/iron sulfate (asc/Fe^{2+}) at time 48 h; ARA 2 and C 2, cells exposed to 2 doses of asc/Fe^{2+} at times 48–60 h; ARA 3 and C 3, cells exposed to 3 doses of asc/Fe^{2+} at times 48–60–72 h; ARA 4 and C 4, cells exposed to 4 doses of asc/Fe^{2+} at times 48–60–84 h.

DNA content. Flow cytometric analysis of DNA was done with a FACScan (Becton Dickinson Immunocytometry Systems USA, San Jose, CA) on cells stained with propidium iodide (18 μg/mL final concentration), as indicated by Dolbeare *et al.* (33).

Fatty acid content. The percentage content of fatty acids in phospholipids extracted from cells was measured as described in Canuto *et al.* (30).

Enzyme activity determination. Aldehyde dehydrogenase (ALDH) activity was determined as described in Canuto *et al.* (2). Benzaldehyde (2.5 mM), HNE (0.1 mM), or propionaldehyde (1 mM) was used as substrate.

Statistical analysis. All data are expressed as means ± standard deviation. The significance of differences between group means was assessed by variance analysis, followed by the Newman-Keuls test.

RESULTS

We used two hepatoma cell lines, 7777 and JM2, both of which have a low content of ARA in the membrane phospholipids and low susceptibility to lipid peroxidation. However, the two cell lines differ in terms of ALDH content, which was the reason we chose them: this enzyme is involved in the metabolism of lipid peroxidation products and gives the hepatoma cells a different susceptibility to these products (2,30). Table 1 shows that JM2 hepatoma cells have an higher content of ALDH than 7777 hepatoma cells. The enzyme activity was measured in the cytosol using benzaldehyde, HNE, or propionaldehyde as substrate.

TABLE 1
Aldehyde Dehydrogenase Activity in Cytosol Isolated From Hepatocytes and Hepatoma Cell Lines[a]

Cells	BA	4-HNE	PA
Hepatocytes	2.27 ± 0.51[a]	1.64 ± 0.32[a]	2.33 ± 0.17[a]
7777 hepatoma	1.23 ± 0.19[b]	3.32 ± 0.51[b]	4.35 ± 0.46[b]
JM2 hepatoma	597.63 ± 93.21[c]	12.25 ± 4.71[c]	41.73 ± 6.89[c]

[a]Data are expressed as nmoles of NAD(P)H produced/min/mg of protein and represent mean ± SD of three experiments. For each substrate, means with different letters are statistically different ($P < 0.001$) from one another as determined by variance analysis followed by the Newman-Keuls test. BA, 2.5 mM benzaldehyde; 4-HNE, 0.1 mM 4-hydroxynonenal; PA, 1 mM propionaldehyde.

TABLE 2
Percentage of Arachidonic Acid (ARA) in Phospholipids Extracted from Hepatocytes and Hepatoma Cell Lines[a]

	ARA (nmoles/10^6 cells)		
	Unenriched cells	250	400
Hepatocytes	23.49 ± 1.91[a]		
7777 hepatoma	5.43 ± 2.01[b]	18.97 ± 2.88[c]	24.62 ± 1.98[a]
JM2 hepatoma	4.82 ± 0.93[b]	16.50 ± 5.66[c]	24.50 ± 2.67[a]

[a]Data are expressed as percentage of total fatty acids and are means ± SD of 5 experiments. Means with different letters are statistically different ($P < 0.001$) from one another as determined by variance analysis followed by the Newman Keuls test

Externally added ARA was incorporated into the phospholipids of hepatoma cells. Both the doses used increased the percentage content in both cell lines to the same extent. The higher dose completely restored the ARA level of normal hepatocytes (Table 2). However, the lower concentration was used in the following experiments, to avoid cytotoxicity.

7777 hepatoma cells. Figure 2 shows lipid peroxidation in 7777 hepatoma cells treated with ARA and different doses of ascorbate/iron sulfate. The production of MDA was proportional to the number of ascorbate/iron sulfate doses, and decreased over time after interruption of prooxidant administration. In this and the following figures, only control cells and cells treated with ARA and ascorbate/iron sulfate (ARA 1, ARA 2, ARA 3, and ARA 4) are reported, since cells treated with ARA alone and cells treated with ascorbate/iron sulfate alone (control cells 1–4) behaved in a similar way to control cells. Cell numbers (Fig. 3) indicated a reduction in growth correlated to the quantity of MDA produced, but no increase in growth even when only one dose of prooxidant was used. Growth of cells exposed to only one dose of ascorbate/iron sulfate was slower than that of controls, although they did continue to grow. When cells were exposed to two doses, growth was reduced and had stopped almost entirely by the end of the experimental time, although cells remained alive. On the other hand, three and four doses of ascorbate/iron sulfate caused cell death, evidenced by cells detached from the monolayer and the presence of LDH in the culture medium. Very few cells were still alive in the monolayer. About 20,000/cm^2 remained after three doses, 15,000/cm^2 after four doses. Table 3 shows the trend of LDH, which was present in the medium in large quantities only after three and four doses of prooxidant. The content of LDH in the culture medium was expressed as percentage of LDH present in the control cells. The cells enriched with ARA did not undergo modifications of LDH activity.

To investigate the cell-cycle phases and to determine at which phase lipid peroxidation products may affect cell proliferation, we used flow cytometry to analyze DNA content in treated cells. Figure 4 shows that the percentage of S phase cells increased with the number of doses of ascorbate/iron sulfate until dose three. After that, a below-normal percentage of the cells still alive was in the S phase, while more were in the G_0/G_1 phase than the untreated cells. In cells treated with one or two doses of prooxidant (ARA 1 and ARA 2) there was an increase in the percentage of cells in the S phase, which was larger with two doses than with one dose. The DNA distribution subsequently returned to a pattern similar to that of control cells. Moreover, cells treated with one dose continued to grow, although more slowly than controls, whereas growth of cells treated with two doses of prooxidant ceased almost entirely (Fig. 2).

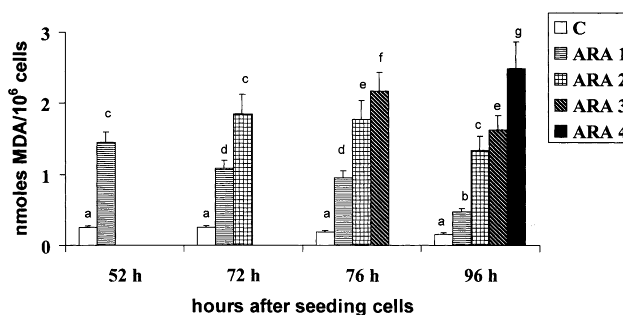


FIG. 2. Lipid peroxidation in 7777 hepatoma cells after enrichment with ARA. Lipid peroxidation was determined as malondialdehyde (MDA) production per 10^6 cells. ARA 1, 2, 3, 4 cells were harvested for MDA determination at the times indicated in the figure. Means with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. For abbreviations see Figure 1.

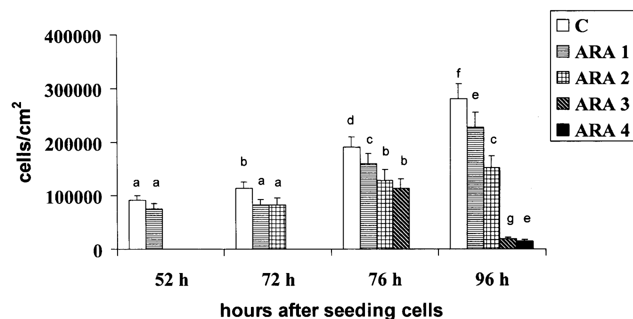


FIG. 3. Number of 7777 hepatoma cells after enrichment with ARA. ARA 1, 2, 3, 4 cells were harvested for counting at the times indicated in the figure. Means with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. For abbreviations see Figure 1.

Cells treated with the third dose of prooxidant maintained increase in the S-phase cells seen with two doses for 4 h after dose three; by the end of the experiment (24 h after dose 3), they were almost all in the G_0/G_1 phase, as were cells after four doses. With four doses, however, the number of cells in the G_0/G_1 was higher than after three doses. Thus, for 7777 cells, three doses of prooxidant are enough to cause inhibition of cell proliferation and death.

JM2 hepatoma cells. JM2 hepatoma cells enriched with ARA and exposed to ascorbate/iron sulfate showed an increase in lipid peroxidation over the controls, similar to that found with 7777 cells, but to a lesser extent (Fig. 5). Production of MDA decreased over time in cells exposed only to one or two doses of prooxidant. The effect of lipid peroxidation products on growth began to be evident after the third dose of prooxidant, and was more marked after the fourth dose (Fig. 6). It is of note that JM2 cells showed a reduction of growth after the third and fourth doses of prooxidant in comparison with the control cells, and that no cells died. Determination of both cell number and MDA production showed that these cells were more resistant than 7777 cells to lipid peroxidation products, and like 7777, they showed no increase in growth. The difference between the two cell lines is probably due to the different content of aldehyde deydrogenase, as reported elsewhere (34).

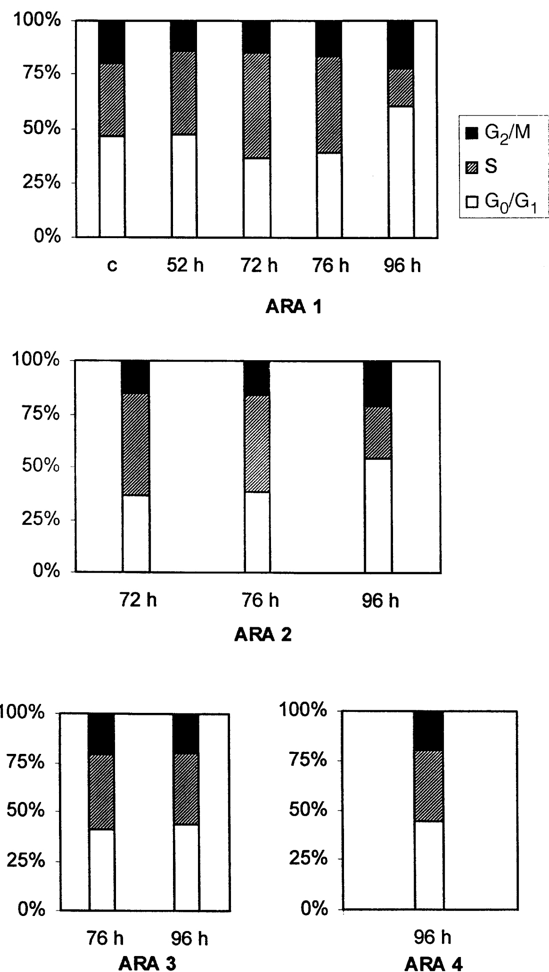


FIG. 4. Cell-cycle analysis in 7777 hepatoma cells. ARA 1, 2, 3, 4 cells were harvested for DNA analysis by cytofluorimetry at the following times: 52 h; 72 h; 76 h; 96 h, after seeding cells, as explained in the figure. Control data (c) are means of values obtained at each of the four harvesting times, since there was no significant variation. For abbreviations see Figure 1.

Analysis of DNA distribution showed that JM2 cells were blocked in the S phase by lipid peroxidation products, as were the 7777 cells, but only after three doses of prooxidant. DNA

TABLE 3
Lactate Dehydrogenase (LDH) Release from 7777 Hepatoma Cells After Enrichment with ARA[a]

	Hours after seeding cells			
Cells	52	72	76	96
C	0.85 ± 0.06^a	1.70 ± 0.15^a	1.20 ± 0.24^a	2.80 ± 0.35^b
ARA 1	1.30 ± 0.11^a	9.08 ± 1.34^b	5.58 ± 0.89^b	4.36 ± 0.71^b
ARA 2		8.09 ± 0.92^b	6.53 ± 0.78^b	6.81 ± 1.21^b
ARA 3			8.51 ± 1.10^b	92.23 ± 5.19^c
ARA 4				94.33 ± 6.67^c

[a]LDH was determined in the culture medium and expressed as percentage of total activity present in the control cells, where LDH activity was 204.73 ± 36.95 nmoles of NADH consumed per minute per 10^6 cells. C, cells not enriched with ARA and not exposed to ascorbate/iron sulfate; ARA 1, 2, 3, 4, cells enriched with ARA and exposed to 1, 2, 3 or 4 doses of 500 μM ascorbate and 100 μM iron sulfate, as in Figure 1. Culture media were removed for LDH determination at the times indicated in the table. Means with different letters are statistically different ($P < 0.001$) from one another as determined by variance analysis followed by the Newman-Keuls test. For abbreviation see Table 2.

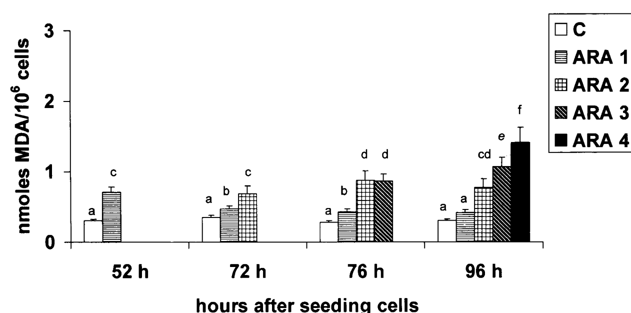


FIG. 5. Lipid peroxidation in JM2 hepatoma cells after enrichment with ARA. Lipid peroxidation was determined as MDA production per 10^6 cells. ARA 1, 2, 3, 4 cells were harvested for MDA determination at the times indicated in the figure. Means with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. For abbreviations see Figures 1 and 2.

distribution in JM2 cells treated with one or two doses of prooxidant was similar to that of control cells (Fig. 7). With four doses, the number of JM2 cells in the S phase tended to decrease, while cell numbers in the G_0/G_1 phase increased. This occurred to a lesser extent than with the 7777 cells. With JM2 cells the number of cells in the S phase was always higher than that of control cells. After four doses the JM2 cells were still able to proliferate; inhibition of cell growth was about 30% of the control growth.

DISCUSSION

ARA is an important substrate for lipid peroxidation, a process that may be considered not only a mechanism of cell damage, but also a mechanism of cell cycle regulation through its aldehyde products (35–37).

The decrease in ARA content in hepatoma cells may be seen as an advantage for cell proliferation. Thus, increasing its percentage content may affect hepatoma cell growth in a

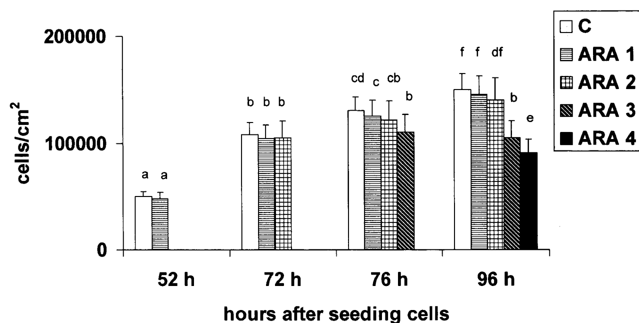
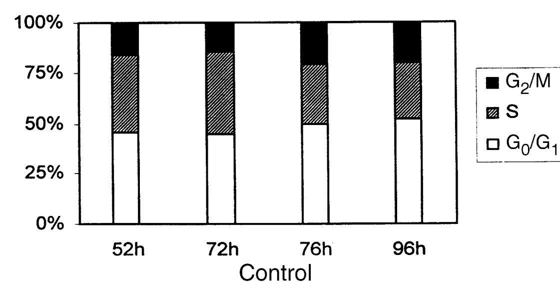
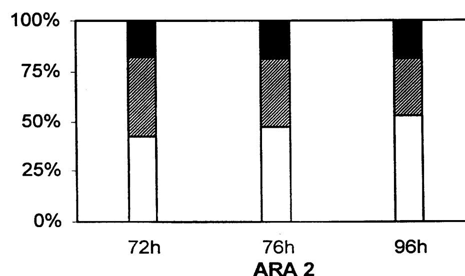


FIG. 6. Number of JM2 hepatoma cells after enrichment with ARA. ARA 1, 2, 3, 4 cells were harvested for counting at the times indicated in the figure. Means with different letters are statistically different ($P < 0.05$) from one another as determined by variance analysis followed by the Newman-Keuls test. For abbreviations see Figure 1.





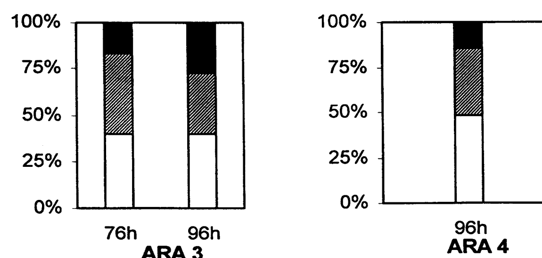


FIG. 7. Cell-cycle analysis in JM2 hepatoma cells. ARA 2, 3, 4 cells were harvested for DNA analysis by cytofluorimetry at the following times: 52 h; 72 h; 76 h; 96 h, after seeding cells, as explained in the figure. Data of cells enriched with ARA and exposed to one dose of prooxidant (ARA 1) were not reported, since the values were similar to those of control cells. For abbreviations see Figure 1.

dose-dependent manner. We used a concentration (250 nmoles/10^6) that does not affect cell growth. With this concentration, the percentage content of ARA inside hepatoma cell phospholipids did not quite reach that of hepatocytes, but this concentration was used to avoid the cytotoxic effect of ARA. At 24 h after addition of ARA, the number of cells was similar to that of control cells both for 7777 and for JM2 cells. Higher concentrations inhibit cell growth, as shown in our previous work (38,39) and by other researchers who have demonstrated that ARA inhibits cell growth (20,21,23). On the other hand, other groups have found that ARA induces cell growth and stimulates metastasis through PG or 5-hydroperoxy-eicosatetraenoic acid production (17). The different effects are probably due to the different concentrations of ARA used.

In the study reported here, 250 nmoles/10^6 cells ARA alone does not inhibit cell growth, but it can, after the addition of prooxidant, induce lipid peroxidation. If enrichment with ARA is followed by exposure to the prooxidant system, there is an increase in lipid peroxidation, and as a conse-

quence, an increase in aldehyde products, which inhibit cell proliferation or induce cell death, depending on the quantities existing within the cells.

This effect may be seen clearly by comparing 7777 and JM2 cells. In 7777 cells, two doses of prooxidant are enough to reduce cell proliferation and maintain the cells in a stable and alive state. The addition of the third and fourth doses of prooxidant causes cell death, drastically reducing the number of cells still alive.

The behavior of JM2 cells is different, these cells have a high specific activity of ALDH, which is an aldehyde-metabolizing enzyme. The effect on JM2 cell growth begins to be evident only after several doses of prooxidant (three or four), at which time ALDH activity is inhibited (36).

Since aldehyde products of lipid peroxidation reduce cell proliferation, we investigated the effect on the cell cycle, evaluating DNA content by flow cytometry. Very little is known about the effects of lipid peroxidation products on the cell cycle. Hammer *et al.* (40) have shown that lipid peroxidation inhibits the incorporation of ^{3}H-thymidine into DNA in hepatoma cells; Barrera *et al.* (41) have reported that administration of HNE to HL-60 cells inhibits proliferation, blocking them in the G_0/G_1 phase. In the work reported here, with both cell lines used, aldehydes produced by lipid peroxidation appear to block the cells in the S phase: the percentage of cells in the S phase increases with the number of doses of prooxidant used. In the case of 7777 cells, cells in the S phase are then detached from the monolayer when subsequent doses of prooxidant are added, and the cells remaining in the monolayer are mostly in the G_0/G_1 phase. This is evident 24 h after the third dose of prooxidant, and still more so after the fourth dose. In the case of JM2 cells, which are more resistant to the antiproliferative effect of aldehydes, the increase of cells in the S phase is less marked; the number of cells in the S phase is, however, above the control value at all times. The marked increase in cells in the G_0/G_1 phase found with the 7777 cells is not present with the JM2 cells: the cells' ability to proliferate is reduced, but they are all alive.

How the aldehydes block the cells in the S phase is unknown at present; we can hypothesize that they interfere with cyclins or cyclin-dependent kinases. Pizzimenti *et al.* (37) found that HNE, an active product of lipid peroxidation, inhibits cyclin D1, D2, and A in HL-60 cells; HNE, externally added, blocks the cells in the G_0/G_1 phase, interfering with these cyclins. It thus appears likely that production of aldehydes inside the cells, which blocks them in the S phase, inhibits cyclins involved in this specific phase.

The decrease in PUFA content in hepatoma cells is important for growth and viability. The reduction in PUFA decreases susceptibily to lipid peroxidation and consequently decreases cytotoxic and cytostatic aldehydes. This is one of the mechanisms able to induce tumor resistance. Enrichment with ARA followed by administration of prooxidant restores lipid peroxidation, which causes a decrease of growth, blocking the cells in the S phase, or killing them, depending on the aldehyde content. Aldehyde content is also correlated with the capability of cells to metabolize aldehydes, as is shown by the different behavior of the two types of hepatoma cells with different contents of ALDH (JM2 cells are less susceptible to lipid peroxidation than 7777 cells). However, lipid peroxidation aldehydes affect cell growth of both cell lines, blocking them in the S phase.

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Ethanol-Extracted Soy Protein Isolate Results in Elevation of Serum Cholesterol in Exogenously Hypercholesterolemic Rats

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ABSTRACT: Soy protein preparations were reported to have hypocholesterolemic actions in experimental animals and humans, while the active components and the mechanism by which this occurs are not clarified yet. The objective of this study is to address these issues by using exogenously hypercholesterolemic rats which are susceptible to dietary cholesterol. Two groups of five rats (male, 12-wk-old) were fed on AIN 93G-based diet with soy protein isolate (SPI) or ethanol-extracted SPI (EE-SPI) for 2 wk. EE-SPI was prepared by ethanol extraction to remove isoflavones and other components. Concentrations of serum and liver total cholesterol were lower in rats fed SPI than in those fed EE-SPI. The abundances of mRNA for 7α-hydroxylase and low density lipoprotein receptor in the liver were lower in EE-SPI group than those in SPI group. These results suggest that the ethanol extract from SPI has a factor(s) to alleviate hypercholesterolemia by increasing the removal of cholesterol from serum through the receptor pathway and then from liver through enhancement of bile acid synthesis.

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Soy protein preparations were reported to have a factor(s) to lower serum cholesterol in experimental animals (1) and humans (2). Active principles, however, have not been clarified yet. Recently, soy ethanolic extract containing isoflavones, saponins, phytic acid, and phospholipids was reported to have both hypocholesterolemic and antiatherogenic actions in rhesus monkeys (3). In contrast, Tovar-Palacio *et al.* (4) reported that, in gerbils, consumption of an isoflavone-containing extract does not contribute to the hypocholesterolemic effect of soy protein preparations. In our previous work, neither soy protein isolate (SPI) or the ethanol-extracted SPI (EE-SPI) showed hypocholesterolemic action in comparison with casein in apo E-deficient mice (5). Since the cholesterol metabolism in apolipoprotein E (apo E)-deficient mice is quite different from wild-type animal or humans (6), it seems that this is not an appropriate model for studying dietary SPI or its ethanol extract's effect on cholesterol metabolism. Kirk *et al.* (7) also reported that dietary soy protein did not reduce plasma cholesterol in low density lipoprotein (LDL) receptor-deficient mice.

Exogenously hypercholesterolemic (ExHC) rats, initially isolated from the Sprague-Dawley (SD) strain by Imai and Matsumura (8), show a ready hypercholesterolemia upon cholesterol feeding in the absence of hypothyroidism and bile salt in diet (9–12). It is considered that polygenes would be involved in stable hypercholesterolemia in this strain (8), but detailed mechanisms have not been clarified yet. So in this study, by using ExHC rats, the effects of ethanol extraction of SPI on the concentration of cholesterol in the serum and liver and the liver mRNA abundances for LDL receptor and cholesterol 7α-hydroxylase (7α-hydroxylase) were evaluated in comparison with intact SPI.

MATERIALS AND METHODS

Reagents. Human actin cDNA was purchased from Wako Pure Chemicals Co. (Osaka, Japan). Nylon membrane was purchased from Bio-Rad Japan (Tokyo, Japan). A multiprime DNA labeling system was purchased from Amersham Japan (Tokyo, Japan). [α-^{32}P] Deoxycytidine triphosphate (1.48 MBq/mmol) was from ICN Co. (Costa Mesa, CA). 18S ribosomal RNA was obtained from American Type Culture Collection (Rockville, MD). The sources of cDNA for apo A-IV, apo B and apo E, LDL receptor, and 7α-hydroxylase have been described (11).

Animals and diets. Two male (*Rattu rattus*, F55-1.2) and six female (F55-1.2.3.4.5.6.) ExHC rats were kindly donated by Takeda Chemical Industries, Ltd. (Osaka, Japan) in 1987; then they were bred and maintained in the Laboratory of Animal Experiments in Kyushu University, Faculty of Medicine (Fukuoka, Japan). They had free access to a commercial non-purified chow diet (NMF; Oriental Yeast Co., Tokyo, Japan) and nonionized water and were maintained in a temperature-controlled room at 22–25°C with a 12-h light cycle (0800 to 2000) until initiation of the experimental diets. In this experiment, ExHC rats (male, 12-wk-old, weighed 330–342 g) were

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Abbreviations: Apo, apolipoprotein; EE-SPI, ethanol extracted soy protein isolate; ExHC, exogenously hypercholesterolemic; HDL, high density lipoproteins; 7α-hydroxylase, cholesterol 7α-hydroxylase; LDL, low density lipoproteins; SD, Sprague-Dawley; SPI, soy protein isolate; VLDL, very low density lipoproteins.

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allowed free access to two experimental diets for 2 wk. Because ExHC rats, in comparison with SD rats, develop hypercholesterolemia as early as 3 d after a high-cholesterol diet, and gradually increased in concentration until 2 wk, they were fed the diets for 2 wk (9,10).

The composition of the experimental diets was based on AIN 93G formulation (13). As a source of dietary protein, SPI (in wt%: protein 90.5; fat 0.3; ash 4.3; water 4.9; Fujipro-R, Fuji Oil Co., Osaka) and EE-SPI (in wt%: protein 90.8; fat 0.7; ash 4.0; water 4.5) were used. The preparation of EE-SPI was described previously (5). The protein content in both experimental diets was adjusted to be the same at the expense of corn starch. To both experimental diets, 0.5% cholesterol was supplemented to induce hypercholesterolemia. As a source of dietary fat, olive oil was used instead of soybean oil, because previous experiments showed that olive oil induced higher serum total cholesterol levels in this animal model, compared with soybean oil (Sato, M., Yoshida, S., Nagao, K., and Imaizumi, K., unpublished observations). From 100 g SPI, 143 mg isoflavones were removed, composed (in wt%) of 15.2% genistein, 52.1% genistin, 8.1% daidzein, and 24.5% daidzin. Isoflavones were determined by high-performance liquid chromatography as described (5).

Diets were removed at 0800 and rats were killed by decapitation at 1200–1300. Serum was prepared by centrifugation at 1,000 × *g* for 15 min. The liver was immediately excised and frozen at −80°C until use for the determination of liver lipids and mRNA abundance. This experiment was carried out under the guidelines for Animal Experiments in the Faculty of Agriculture and the Graduate Course, Kyushu University, Fukuoka, Japan, and the Law (No. 105) and Nortification (No. 6) of the Government (20).

Determination of serum and hepatic lipid levels. Serum and liver lipids were analyzed as described previously (5). Briefly, serum cholesterol, triacylglycerols, phospholipids, and high density lipoprotein (HDL) cholesterol were determined by using commercially available kits from Wako Pure Chemicals. Liver lipids were initially extracted by Folch *et al.* method (14) and chemically determined.

Determination of hepatic LDL receptor, 7α-hydroxylase, and apo A-IV, B and E mRNA abundance. Total cellular RNA was isolated using the guanidinium thiocyanate/cesium chloride ultracentrifugal method according to Chirgwin *et al*. (15). Northern blotting was carried out as described in Nagao *et al.* (11). The hybridization was performed in hybridization solution containing cDNA labeled with [α-^{32}P]dCTP by multiprime DNA labeling system, according to Amersham's instruction. After quantification of the hybridization signals using a bio-imaging analyzer (BAS-1000; Fuji Photo Film Co., Tokyo, Japan), autoradiography was performed on X-ray film. Results were expressed as a relative value after normalization to 18S ribosomal RNA.

Statistical analyses. Data were expressed as means with pooled SEM for five rats in each group and analyzed by Student's *t*-test. Differences were considered significant at $P < 0.05$.

RESULTS

Food intake and growth parameters. There were no differences in body weight gain (24.0 and 24.4 g for SPI and EE-SPI group, respectively, pooled SEM = 2.9 g), liver weight (12.1 and 13.0 g for SPI and EE-SPI group, respectively, pooled SEM = 0.4 g), and food intake (18.8 and 20.7 g/d for SPI and EE-SPI group, respectively, pooled SEM = 0.6 g) between two groups.

Serum and liver lipid concentrations. Rats fed on the EE-SPI-containing diet had an elevated serum total cholesterol concentration, compared with those fed an intact SPI-containing diet (Table 1). There were no significant differences in the concentration of serum triacylglycerols, phospholipids, and HDL cholesterol between EE-SPI and SPI group. There was the same trend in liver lipid concentrations as those in the serum. Liver total cholesterol concentration in EE-SPI group was significantly higher than that of the SPI group, and the elevation was attributed to esterified cholesterol. There were no significant differences in liver free cholesterol, triacylglycerols, and phospholipids concentrations between the two groups.

Hepatic LDL receptor, 7α-hydroxylase and apo A-IV, B and E mRNA abundance. Northern blotting patterns of mRNA for LDL receptor, 7α-hydroxylase, apo A-IV, apo B, apo E, and 18S ribosome are shown in Figure 1. The abundance of 18S ribosome mRNA was similar between SPI and EE-SPI group. The relative abundance of the mRNA against the 18S ribosome mRNA is shown in Table 2. The level of 7α-hydroxylase mRNA was significantly lower in rats fed an EE-SPI-containing diet than those fed a SPI-containing diet. Similarly, the EE-SPI group had a lowered LDL receptor mRNA level, compared with the SPI group. There were no differences in apo A-IV, apo B, and apo E mRNA levels between the two groups.

TABLE 1
Serum and Liver Lipid Concentrations in ExHC Rats Fed SPI or EE-SPI Containing Diet[a]

	SPI	EE-SPI	Pooled SEM
Serum lipids (mmol/L)			
Total cholesterol	4.25	7.78[b]	0.818
Triacylglycerols	0.696	0.769	0.064
Phospholipids	2.09	2.43	0.167
HDL cholesterol	0.574	0.458	0.0352
Liver lipids (μmol/g liver)			
Total cholesterol	61.9	76.0[b]	3.21
Esterified cholesterol	56.7	70.0[b]	3.13
Free cholesterol	5.20	6.00	0.361
Triacylglycerols	19.6	25.5	1.89
Phospholipids	33.7	33.8	0.665

[a]Values are means with pooled SEM for five rats in each group. EE-SPI, ethanol-extracted soy protein isolate; HDL, high density lipoproteins; SPI, soy protein isolate; ExHC, exogenously hypocholesterolemic.
[b]Significantly different from SPI group by Student's *t*-test at $P < 0.05$.

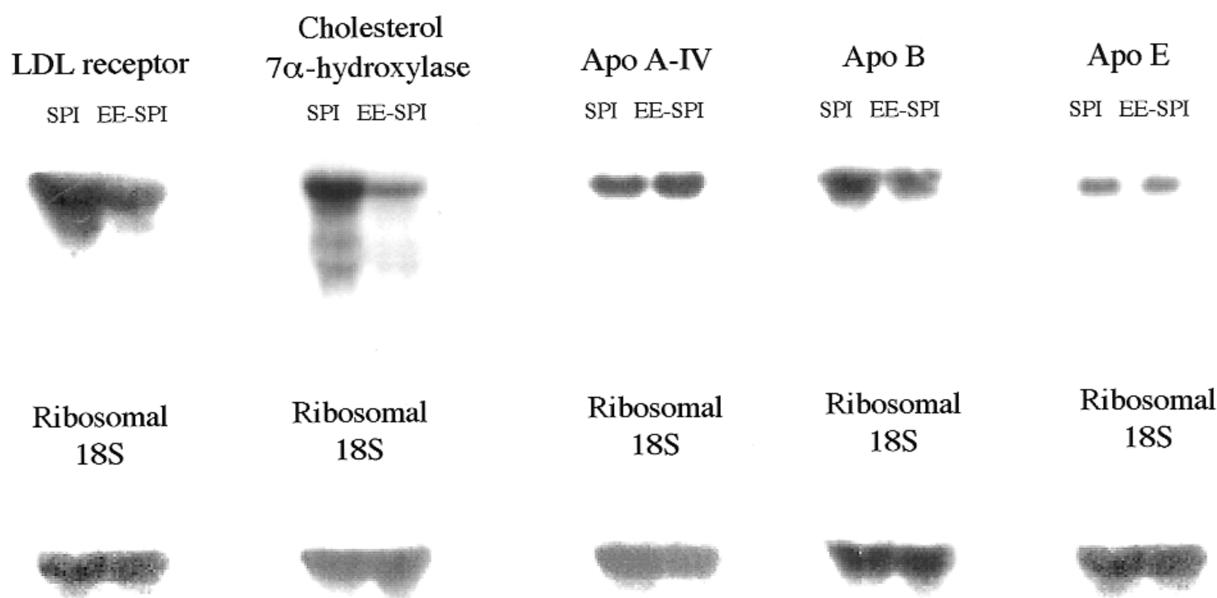

FIG. 1. Northern blot hybridization of hepatic 7α-hydroxylase, low density lipoprotein (LDL) receptor, apo A-IV, B and E mRNA. Apo, apolipoprotein; EE-SPI, ethanol-extracted soy protein isolate; 7α-hydroxylase, cholesterol 7α-hydroxylase; SPI, soy protein isolate.

DISCUSSION

In this study, ExHC rats fed the EE-SPI-containing diet had about twofold the elevated serum total cholesterol concentration in comparison with those fed intact SPI. The results are in agreement with those reported by Kirk *et al*. (7). They had a similar outcome in C57BL/6 mice fed soy protein with and without isoflavones. In the present study, there were no differences in serum HDL cholesterol concentrations between the two groups, thereby indicating increased cholesterol mass in the very low density lipoproteins (VLDL) and LDL fractions in EE-SPI group. It is therefore likely that the EE-SPI group had a higher concentration of the serum apo B than did the SPI group.

LeBoeuf *et al*. (16) suggested that in the liver of rodent and perhaps avian species, apo A-IV is needed for storage of triacylglycerol and/or packaging into VLDL, but that increased expression of apo A-IV does not lead to enhanced secretion of VLDL. In the present experiment, it is likely that removal of isoflavones from SPI exerted no significant influence on the expressions of apo A-IV, apo B, and apo E.

The EE-SPI group had a lower abundance of hepatic 7α-hydroxylase mRNA, suggesting that SPI loses most of its effectiveness in enhanceing bile acid synthesis by withdrawal of the ethanol extract. This is consistent with reports by several groups (17,18) that intact SPI preparations increase bile acid excretion, compared with casein. Although bile acid synthesis was not measured in the present experiment, it was reported that the abundance of 7α-hydroxylase mRNA correlates to the activity of 7α-hydroxylase, which is a rate-limiting enzyme of bile acid synthesis (19). Most likely the increased concentration of esterified cholesterol in the liver in EE-SPI-fed rats, in comparison with SPI-fed rats, was attributed to decreased synthesis of bile acids.

In addition, EE-SPI group also had a reduced hepatic LDL receptor mRNA level, compared with that of intact SPI group, which suggests that SPI ethanolic extract has the effect of increasing the transcription of the LDL receptor gene, probably by blocking the down-regulation of the LDL receptor by increasing cholesterol excretion as bile acid. Alternatively, it remains a possibility that the ethanol extracts directly influenced the LDL receptor gene expression.

In our preliminary experiment, the serum cholesterol concentration in ExHC rats fed a casein-containing diet supplemented with isoflavones which were derived from soybean germ hypocotyl was significantly lower than that from groups fed the diet without isoflavones, but still significantly higher than that from groups fed SPI-containing diets (Sato, M., Tsuda, Y., Takashima, S., and Imaizumi, K., unpublished observation). The major isoflavones of soybean germ hypocotyl are daidzein and its glycoside (88.9 wt%), while those of SPI are rich in genistein and the glycoside (67.3 wt%). Therefore, a type of isoflavone may differently influence the serum cho-

TABLE 2
Hepatic mRNA Abundance for LDL-Receptor, 7α-Hydroxylase, Apo A-IV, Apo B, and Apo E in ExHC Rats Fed SPI or EE-SPI Contained Diet[a]

	SPI	EE-SPI	Pooled SEM
mRNA (Arbitrary units)			
LDL receptor	100	73.0[b]	7.09
7α-Hydroxylase	100	36.5[b]	16.6
Apo A-IV	100	104	8.70
Apo E	100	87.6	3.38
Apo B	100	101	7.95

[a]Values are means with pooled SEM for five rats in each group. 7α-Hydroxylase, cholesterol 7α-hydroxylase; LDL, low density lipoproteins. See Table 1 for other abbreviations.
[b]Significantly different from SPI group by Student's *t*-test at $P < 0.05$.

lesterol concentration. Likely our ethanol extraction procedure inactivated other components of SPI which might be responsible for lowering serum cholesterol concentration.

In summary, the present results demonstrate that ethanol extract from SPI contains a factor(s) to lower serum total cholesterol concentration or non-HDL cholesterol and suggest that this may be mediated by increasing the transport of serum cholesterol into the liver through LDL receptor-mediated pathway and the subsequent excretion as bile acids. Hence, ExHC rats are an appropriate animal model to identify the active component(s) in SPI responsible for the hypocholesterolemic effect and the mechanism involved.

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The Effects of a Dietary Oxidized Oil on Lipid Metabolism in Rats

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ABSTRACT: This study was carried out to investigate the effects of a dietary oxidized oil on lipid metabolism in rats, particularly the desaturation of fatty acids. Two groups of rats were fed initially for a period of 35 d diets containing 10% of either fresh oil or thermally treated oil (150°C, 6 d). The dietary fats used were markedly different for lipid peroxidation products (peroxide value: 94.5 vs. 3.1 meq O_2/kg; thiobarbituric acid-reactive substances: 230 vs. 7 μmol/kg) but were equalized for their fatty acid composition by using different mixtures of lard and safflower oil and for tocopherol concentrations by individual supplementation with DL-α-tocopherol acetate. In the second period which lasted 16 d, the same diets were supplemented with 10% linseed oil to study the effect of the oxidized oil on the desaturation of α-linolenic acid. During the whole period, all the rats were fed identical quantities of diet by a restrictive feeding system in order to avoid a reduced food intake in the rats fed the oxidized oil. Body weight gains and food conversion rates were only slightly lower in the rats fed the oxidized oil compared to the rats fed the fresh oil. Hence, the effects of lipid peroxidation products could be studied without a distortion by a marked reduced food intake and growth. To assess the rate of fatty acid desaturation, the fatty acid composition of liver and heart total lipids and phospholipids was determined and ratios between product and precursor of individual desaturation reactions were calculated. Rats fed the oxidized oil had reduced ratios of 20:4n-6/18:2n-6, 20:5n-3/18:3n-3, 20:4n-6/20:3n-6, and 22:6n-3/22:5n-3 in liver phospholipids and reduced ratios of 20:4n-6/18:2n-6, 22:5n-3/18:3n-3, and 22:6n-3/18:3n-3 in heart phospholipids. Those results suggest a reduced rate of desaturation of linoleic acid and α-linolenic acid by microsomal Δ4-, Δ5-, and Δ6-desaturases. Furthermore, liver total lipids of rats fed the oxidized oil exhibited a reduced ratio between total monounsaturated fatty acids and total saturated fatty acids, suggesting a reduced Δ9-desaturation. Besides those effects, the study observed a slightly increased liver weight, markedly reduced tocopherol concentrations in liver and plasma, reduced lipid concentrations in plasma, and an increased ratio between phospholipids and cholesterol in the liver. Thus, the study demonstrates that feeding an oxidized oil causes several alterations of lipid and fatty acid metabolism which might be of great physiologic relevance.

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Polyunsaturated fatty acids (PUFA) are labile compounds which, under the promoting effect of oxygen, high temperature or metallic catalysts, undergo peroxidative damage. In the course of this reaction, various products such as peroxides, aldehydes, acids or polymerization products are formed. To study metabolic effects of these compounds, experiments with animals were carried out which in most cases used highly oxidized vegetable oils or fish oil (1–7) as well as isolated lipid peroxidation products (8). Those experiments exhibited some effects of oxidized oils on lipid metabolism, with alterations of tissue fatty acid compositions being observed (1–7). Increased levels of arachidonic acid in tissue lipids suggested an elevated desaturation of linoleic acid in animals fed oxidized oils (1,4,5,7). However, experiments using highly oxidized oils must be regarded critically from some points of view. First, feeding diets with highly oxidized oils usually reduces the animals' food intake (1,2,6,9,10). Additionally, the digestibility of fatty acids from highly oxidized oils is affected as a consequence of fatty acid polymerization (2,7,10). Therefore, animals fed such oils usually exhibit a markedly lower growth rate than control animals fed equivalent fresh oils (1–3,5–7,9,10). Oxidized oils also contain significantly less PUFA and tocopherols than the equivalent fresh oils (1–7). Therefore, the effects observed after feeding oxidized oils are not caused solely by lipid peroxidation products but by a complex mixture of a different nutritional status and different growth rates between animals fed fresh oils and animals fed oxidized oils.

The present study was performed to investigate the effects of a dietary oxidized oil under a controlled feeding system. Thereby, differences in feed intake were avoided by a paired feeding system; dietary fats used were equalized for their fatty acid composition by using mixtures of safflower oil and lard in various ratios, and tocopherol concentrations of the oils were equalized by individual supplementation of both fats with DL-α-tocopherol acetate.

The main objective of this study was to study the effect of an oxidized oil on the desaturation of essential fatty acids which play an important role for the synthesis of eicosanoids.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acids; POV, peroxide value; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TBARS, thiobarbituric acid-reactive substances.

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Studies in the literature considered concentrations of arachidonic acid in animal tissues as a parameter of linoleic acid desaturation (1,4,5,7). However, this desaturation assessment method has a disadvantage in that levels of arachidonic acid are relatively high even at the beginning of the experiment. Therefore, it is to be expected that there is only a small change in arachidonic acid levels in tissue lipids in response to an altered desaturation rate. Besides the conversion of linoleic acid into arachidonic acid, the present study also considered the conversion of α-linolenic acid into highly unsaturated n-3 PUFA, particularly eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) as an additional measure of essential fatty acid desaturation. This was done by including linseed oil as a source of α-linolenic (18:3n-3) acid in the diets. However, before linseed oil was used, an initial period was preceded in order to begin the metabolic effects of an oxidized oil. In that period, rats were fed either fresh or oxidized oils with similar fatty acid compositions which were poor in α-linoleic acid. This concept was based on the idea that an early marked incorporation of n-3 desaturation products into tissue lipids at a phase when the metabolic effects of the oxidized oil were not yet occurring could confound the investigation of the oxidized oil on desaturation of α-linolenic acid. In the second phase, after metabolic effects of the oxidized oils might have been induced, diets were supplemented with linseed oil which is rich in α-linolenic acid. At the end of this period, levels of highly unsaturated fatty acids deriving from desaturation of both linoleic acid and α-linolenic acid were determined in tissue lipids in order to assess desaturation of essential fatty acids.

Our recent studies using moderately oxidized oils revealed that lipid peroxidation products reduce lipid concentrations in plasma as well as the osmotic fragility of red blood cells (11–13). This study also aimed to investigate whether these surprising metabolic alterations can be reproduced in animals fed a stronger oxidized oil.

MATERIALS AND METHODS

Animals and diets. An experiment was carried out using 20 male Sprague-Dawley rats with an average initial body weight of 107 ± 5 g (±SD) which were divided into two groups of 10 rats each. In the first period, which lasted 35 d, the rats were administered diets with fresh or oxidized oil. The oxidized oil was prepared by the following procedure: 5 kg of a mixture of lard and safflower oil (2:1, w/w) was put into a glass beaker with a capacity of 10 L; the fat mixture was heated at a temperature of 150°C using a temperature-controlled hotplate. The temperature of the oil was checked several times per day using a glass thermometer. During the entire heating period, air was continuously bubbled through the fat mixture *via* a glass pipette. To control for the degree of oxidation, aliquots of the heated oil were taken at several time points and peroxide values (POV), and concentrations of thiobarbituric acid-reactive substances (TBARS) were determined. After 6 d of continuous heating, the peroxidation procedure was terminated. This treatment caused a marked loss of PUFA, a complete loss of tocopherols, and a marked elevation of the POV (Table 1). It was planned to equalize fatty acid composition and tocopherol concentrations of both fats. Therefore, the control diet included a fat mixture composed of lard and safflower oil in a ratio of 94.5:5.5 (w/w). This fat mixture had a similar fatty acid composition as the oxidized oil; the POV of this fat mixture was 2 meq O_2/kg. Both fat mixtures were adjusted to a vitamin E activity of 170 mg α-tocopherol equivalents per kg. In the second phase of the experiment which lasted 16 d, those diets were supplemented with 10% linseed oil. The composition of the experimental diets is shown in Table 2; analyzed concentrations of fatty acids and tocopherols are shown in Table 3. In both periods, a restrictive feeding system was used in which all the rats were administered identical quantities of diet. In order to achieve complete consumption of the diet, the quantity administered was 10 to 20% lower than expected for *ad libitum* consumption of the control diet according to a previous study using similar diets (14). The quantity of diet administered was gradually increased with increasing body weight of the rats from 11

TABLE 1
Chemical Changes of the Dietary Fat (lard/safflower oil, 2:1 w/w) After Heating at a Temperature of 150°C for 6 d with Continuous Bubbling of Air[a]

	Fresh oil	Heated oil
Peroxide value (meq O_2/kg oil)	3.1	94.5
TBARS (μmol/kg oil)	7	230
Fatty acid composition (g/100 g fatty acids)		
16:0	21.5	30.0
18:0	11.6	15.8
18:1	30.3	35.9
18:2	32.2	13.7
α-Tocopherol (mg/kg)	159	<1

[a]Abbreviation: TBARS, thiobarbituric acid-reactive substances.

TABLE 2
Composition (g/kg) of the Experimental Diets

	Period 1		Period 2	
Component	Control oil	Oxidized oil	Control oil	Oxidized oil
Casein	200	200	200	200
Corn starch	300	300	250	250
Saccharose	298	298	248	248
Lard	94.5	66.7	94.5	66.7
Safflower oil	5.5	33.3	5.5	33.3
Linseed oil	—	—	100	100
Fiber	40	40	40	40
Mineral mixture[a]	40	40	40	40
Vitamin mixture[b]	20	20	20	20
DL Methionine	2	2	2	2

[a]Minerals supplemented per kg diet: 13.16 g KH_2PO_4; 0.86 g MgO; 12.37 g $CaCO_3$; 3.82 g NaCl; 224 mg $FeSO_4 \cdot 7H_2O$; 167 mg $ZnSO_4 \cdot 7H_2O$; 31.0 mg $MnSO_4 \cdot H_2O$; 23.7 mg $CuSO_4 \cdot 5H_2O$; 0.64 mg KI; 0.60 mg $Na_2Se_2O_3$; 5.16 mg $CrCl_3 \cdot 6H_2O$.
[b]Vitamins supplemented per kg diet: 1.38 mg all-*trans*-retinyl palmitate; 25 μg cholecalciferol; 0.75 mg menadion sodium bisulfite; 5 mg thiamin-HCl, 6 mg riboflavin; 6 mg pyridoxine-HCl; 30 mg nicotinic acid; 15 mg Ca pantothenate; 2 mg folic acid, 0.2 mg biotin; 0.025 mg cyanocobalamin; 1000 mg choline chloride.

TABLE 3
Fatty Acid Composition and Tocopherol Concentration of the Diets

	Period 1		Period 2	
	Control oil	Oxidized oil	Control oil	Oxidized oil
Fatty acids (g/100 g of fatty acids)				
14:0	1.6	1.9	0.9	0.9
16:0	26.3	30.0	16.8	16.7
16:1	2.3	1.6	1.0	0.7
18:0	15.5	15.8	9.7	9.1
18:1	37.8	35.9	27.8	25.1
18:2n-6	14.8	13.7	14.7	14.5
18:3n-3	0.3	0.2	28.4	33.3
20:1	0.6	0.4	0.3	0.3
22:0	0.8	0.5	0.4	0.3
Tocopherols				
α-Tocopherol (mg/kg)	3.6	—[a]	3.5	—
γ-Tocopherol (mg/kg)	—	—	43.3	39.7
DL-α-Tocopherol acetate (supplemented, mg/kg)	20	25	20	25
Total tocopherol actvity[b]	17.0	16.8	21.2	20.7

[a]Not detectable (<0.1 mg/kg).
[b]α-Tocopherol equivalents (mg/kg).

to 22 g per day in period 1. Because of the higher fat concentration of the diet used, the quantity of diet administered in period 2 was between 16 and 18 g per day. Animals were kept individually in Macrolon cages in a room with a temperature of 23°C and a relative humidity between 50 and 60%. Water was provided *ad libitum* by nipple bottles.

Analyses. After the feeding period of 51 d was completed, all the rats were killed by decapitation after light anesthesia with diethyl ether. Blood was collected in heparinized polyethylene tubes; liver and heart were excised and stored at a temperature of −20°C until analysis. Lipids from liver and heart were extracted using a mixture of hexane and isopropanol (3:2, vol/vol) (15). Phospholipids of the extracts were isolated by high-performance liquid chromatography using a Merck-Hitachi (Darmstadt, Germany) system consisting of a gradient pump (L-6200), an ultraviolet detector (L-4250), a 25 × 0.4 cm (internal diameter) Si-60 (5 µm) cartridge (LiChroCART, Merck), and a fraction collector (L-5200). The gradient system was based on acetonitrile and methanol as mobile phases (16). Total lipids of liver and heart as well as isolated phospholipid fractions were methylated with boron fluoride/methanol reagent (17). Fatty acid methyl esters were separated by gas–liquid chromatography using a Varian system (model 3400; Darmstadt, Germany) equipped with a very polar capillary column (SP-2340, 60 m × 0.25 mm i.d., Supelco, Bellefonte, PA) and a flame-ionization detector. Helium was used as carrier gas. Fatty acid methyl esters were identified by comparing their retention times with those of individual purified standards and quantified using heptadecanoic acid methyl ester as an internal standard (18).

Concentrations of tocopherols in liver, plasma, oils, and diets were determined by high-performance liquid chromatography (19). Samples were mixed with 1 mL 1% pyrogallol solution (in ethanol, absolute) and 150 µL saturated sodium hydroxide solution. This mixture was heated for 30 min at 70°C. Then tocopherols were extracted three times with *n*-hexane. Individual tocopherols were separated using a mixture of *n*-hexane and 1,4-dioxane (94:6, vol/vol) as mobile phase and a LiChrosorb Si 60 column (5 µm particle size, 250 mm length, 4 mm internal diameter; Merck) and detected by fluorescence (excitation wavelength: 295 nm, emission wavelength: 320 nm).

For determination of liver and heart total cholesterol, triglycerides and phospholipids, aliquots of the extracts were dissolved in Triton X-100 (20). Total cholesterol and triglycerides were determined using enzymatic reagent kits obtained from Merck. The concentration of total phospholipids in heart and liver was calculated from the amount of its esterified fatty acids (26). The concentrations of total cholesterol, triglycerides, and phospholipids in plasma were measured enzymatically using an autoanalyzer (model EPOS; Eppendorf, Hamburg, Germany) and commercial available kit reagents (Merck; and bioMerieux, Marcy-l'Etoile, France).

The osmotic fragility of erythrocytes was determined according to the method of O'Dell *et al.* (21). Aliquots (40 µL) of fresh blood were added to 2-mL portions of hypotonic sodium chloride solutions. The suspensions were incubated for 15 min at ambient temperaure and were then centrifuged at 1,100 × *g* for 5 min. The absorbance of the supernatants at 540 nm was determined as a measure of hemoglobin release from erythrocytes during incubation and was used for calculation of percentages of erythrocytes hemolyzed during incubation.

Statistics. For statistical evaluation, means of the two dietary treatments were compared by Student's *t*-test.

RESULTS

Data on feed intake, growth, and food conversion are shown in Table 4. Feed intake because of the restrictive feeding sys-

TABLE 4
Feed Intake and Weight Gain During the Study[a]

	Control oil	Oxidized oil
Period 1 (d 1–35)		
Feed intake (g)	598 ± 13	603 ± 3
Body weight gain (g)	243 ± 15[b]	227 ± 14[c]
Feed conversion ratio (g body weight gain/g feed)	0.406 ± 0.024[b]	0.376 ± 0.022[c]
Period 2 (d 36–51)		
Feed intake (g)	267 ± 1	267 ± 1
Body weight gain (g)	62.9 ± 10.0	61.6 ± 10.7
Feed conversion ratio (g body weight gain/g feed)	0.236 ± 0.038	0.231 ± 0.040
Whole period (d 1–51)		
Feed intake (g)	864 ± 13	870 ± 3
Body weight gain (g)	306 ± 23	289 ± 19
Feed conversion ratio (g body weight gain/g feed)	0.354 ± 0.026[b]	0.332 ± 0.021[c]

[a]Results are means ± SD with n = 10 for both groups. Means with different superscript letters (b, c) differ significantly by Student´s t-test ($P < 0.05$).

tem used was nearly identical in both groups. The small difference observed in the first period was caused by one animal in the control group which did not completely consume the diet administered. Body weight gain and feed conversion rate were only slightly different between the two groups. The rats fed the oxidized oil exhibited 6% lower body weight gain and feed conversion over the whole feeding period. This effect was mainly due to differences in the first period; in the second period, the growth rate was nearly identical between both groups.

Tocopherol concentrations in liver and plasma measured to assess the vitamin E status of the rats are shown in Table 5. Although the diets were equalized for their tocopherol concentrations, rats fed the oxidized oil exhibited markedly lower concentrations of tocopherols in liver and plasma than rats fed the fresh oil. In liver, feeding the oxidized oil reduced the concentration of total tocopherols by *ca.* 80%, and in plasma by *ca.* 60%. The concentration of γ-tocopherol was more strikingly reduced by the oxidized oil than was the concentration of α-tocopherol.

To assess the effects of a dietary oxidized oil on fatty acid desaturation, the fatty acid composition of liver and heart total lipids and phospholipids was determined. In liver total lipids, the main effect of the dietary oxidized oil was an increased level of saturated fatty acids (SFA) at the expense of monounsaturated fatty acids (MUFA) (Table 6). Thus, the ratio between SFA and MUFA was significantly reduced. Levels of PUFA in liver total lipids were not significantly influenced by feeding the oxidized oil. Levels of long-chain n-3 PUFA, which were determined to assess the desaturation of α-linolenic acid, were relatively low and were not different between both groups of rats. Hepatic phospholipids contained relatively low levels of MUFA; the levels of both SFA and MUFA were not considerably influenced by the oxidized oil. Thus, the ratio between SFA and MUFA was not altered in hepatic phospholipids. However, hepatic phospholipids exhibited altered levels of some individual PUFA. Rats fed the oxidized oil had higher levels of linoleic acid, α-linolenic acid, dihomo-γ-linolenic acid, and docosapentaenoic acid than rats fed the fresh oil. In contrast, levels of arachidonic acid and DHA were lower in the rats fed oxidized oil than in the rats fed the fresh oil. Thus, the ratios between precursors and products of fatty acid desaturation reactions were altered by the dietary oxidized oil (Table 7).

The fatty acid composition of heart total lipids and phospholipids is shown in Table 8. In total lipids, levels of SFA and MUFA were not different between both groups. Levels of total PUFA were also not different between both groups. However, the levels of some of the individual PUFA were changed by the dietary oxidized oil. The level of α-linolenic acid was increased in the rats fed the oxidized oil, and levels of EPA and DHA were reduced. Heart phospholipids of rats fed the oxidized oil had increased levels of SFA and MUFA at the expense of PUFA. Within the group of PUFA, levels of precursors of desaturation such as linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3) were slightly elevated in rats fed oxidized oil, and those of products such as docosapentaenoic acid and DHA were slightly reduced. Thus, the ratios between products and precursor of Δ4-, Δ5-, and Δ6-desaturase in heart phospholipids were reduced by the dietary oxidized oil (Table 7).

Feeding the oxidized oil tended to reduce lipid concentrations in plasma and liver (Table 9). In plasma, concentrations of triglycerides were significantly ($P < 0.05$) reduced and those of cholesterol and phospholipids were slightly ($P < 0.10$) reduced by feeding the oxidized oil. In the liver, concentrations of cholesterol ($P < 0.05$) and triglycerides ($P < 0.1$) were

TABLE 5
Tocopherol Concentrations in Liver and Plasma of the Rats[a]

	Control oil	Oxidized oil
Liver		
α-Tocopherol (nmol/g)	26.4 ± 6.6[b]	7.5 ± 1.5[c]
γ-Tocopherol (nmol/g)	15.1 ± 4.2[b]	1.5 ± 0.6[c]
Plasma		
α-Tocopherol (μmol/L)	9.6 ± 2.1[b]	4.2 ± 0.9[c]
γ-Tocopherol (μmol/L)	1.1 ± 0.5[b]	< 0.2[c]

[a]Results are means ± SD with n = 10 for both groups. Means with different superscript letters (b, c) differ significantly by Student´s t-test ($P < 0.05$).

TABLE 6
Fatty Acid Composition (moles/100 moles of fatty acids) of Liver Total Lipids and Phospholipids[a]

Fatty acid	Total lipids		Phospholipids	
	Control oil	Oxidized oil	Control oil	Oxidized oil
SFA, total	40.18 ± 3.00[c]	43.52 ± 2.04[b]	50.67 ± 1.26[b]	49.67 ± 0.60[c]
14:0	0.37 ± 0.12[b]	0.24 ± 0.10[c]	—[b]	—
16:0	20.82 ± 1.35[c]	23.04 ± 2.64[b]	17.73 ± 1.53[c]	20.46 ± 1.45[b]
18:0	18.73 ± 3.90	19.88 ± 1.44	32.94 ± 2.22[b]	29.21 ± 1.69[c]
22:0	0.26 ± 0.04[c]	0.37 ± 0.08[b]	—	—
MUFA, total	19.75 ± 3.80[b]	15.56 ± 1.52[b]	5.76 ± 0.48	6.22 ± 0.65
16:1	1.41 ± 0.52[b]	1.00 ± 0.18[c]	0.45 ± 0.20	0.32 ± 0.12
18:1	18.34 ± 3.36[b]	14.55 ± 1.39[c]	5.31 ± 0.55[c]	5.90 ± 0.66[b]
PUFA, total	40.06 ± 1.84	40.92 ± 1.72	43.57 ± 0.89	44.11 ± 0.58
18:2n-6	13.84 ± 1.14[c]	15.07 ± 1.16[b]	12.69 ± 1.24[c]	15.55 ± 1.50[b]
18:3n-3	5.07 ± 1.94	4.33 ± 0.91	0.42 ± 0.12[c]	0.56 ± 0.14[b]
20:2n-6	1.02 ± 0.17[b]	0.77 ± 0.12[c]	—	—
20:3n-6	0.66 ± 0.29	0.80 ± 0.62	0.97 ± 0.22[c]	1.51 ± 0.31[b]
20:4n-6	11.58 ± 2.53	11.88 ± 1.61	19.85 ± 1.14[b]	17.52 ± 1.49[c]
20:5n-3	2.54 ± 0.72	2.66 ± 0.77	2.89 ± 1.02	2.80 ± 0.78
22:5n-3	1.22 ± 0.32	1.49 ± 0.29	0.92 ± 0.28[c]	1.17 ± 0.17[b]
22:6n-3	4.13 ± 1.34	3.92 ± 1.27	5.82 ± 1.11[b]	5.00 ± 0.65[c]

[a]Results are means ± SD with $n = 10$ for both groups. Means with different superscript letters (b, c) differ significantly by Student´s *t*-test ($P < 0.05$).
[b]—, indicates that the amount of fatty acid was lower than 0.1 mole/100 moles of fatty acids). Abbreviations: MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids.

also reduced. However, rats fed the oxidized oil had a higher liver weight than rats fed the control oil; thus, amounts of triglycerides and cholesterol in the total liver were not different between both groups. In contrast, the total amount of phospholipids was 30% higher in rats fed oxidized oil than in rats fed the fresh control oil; the ratio between phospholipids and cholesterol in the liver was increased from 3.75 to 5.61 by the dietary oxidized oil. Heart weight was not significantly increased by feeding the oxidized oil, and concentrations of triglycerides and cholesterol in heart were also similar in rats of both groups. The concentration of phospholipids in the heart was slightly, though significantly, higher in the rats fed the oxidized oil; however, the total amount of phospholipids in the heart was not different between both groups.

The osmotic fragility of erythrocytes is shown in Table 10. In general, erythrocytes of rats fed the oxidized oil exhibited lower percentages of hemolysis during incubation in hypotonic solutions than erythrocytes of rats fed the fresh oil. Because of the relatively large standard deviations, significant differences were observed only at the incubation in the 0.34% sodium chloride solution.

DISCUSSION

The present study was performed to investigate the effects of a dietary oxidized oil on parameters of lipid metabolism in rats. The oxidized oil was prepared by heating a mixture of lard and safflower oil at a temperature of 150°C for 6 d. This treatment caused a large reduction of the PUFA concentration of the oil by free radical-mediated reactions. It is expected that under high temperature, the primary lipoxy radical is not stable, and thus predominately secondary products such as polymer compounds and carbonyls might have been formed during this treatment (22). This might be an explanation for the relatively low POV of the oxidized oil used in this study. Relative proportions of primary to secondary lipid peroxidation products are particularly significant for the discussion of the effects of an oxidized oil on animal metabolism. Primary

TABLE 7
Ratios Between Various Products and Precursors of Desaturation Reactions in Liver and Heart Phospholipids[a]

	Control oil	Oxidized oil
Liver		
20:4n-6/18:2n-6 (Δ5- and Δ6-desaturase)	1.58 ± 0.23[b]	1.14 ± 0.21[c]
20:5n-3/18:3n-3 (Δ5- and Δ6-desaturase)	7.02 ± 2.14	5.23 ± 1.67
20:4n-6/20:3n-6 (Δ5-desaturase)	21.5 ± 5.0[b]	12.1 ± 2.4[c]
22:6n-3/22:5n-3 (Δ4-desaturase)	7.00 ± 3.03[b]	4.39 ± 1.03[c]
Heart		
20:4n-6/18:2n-6 (Δ5-and Δ6-desaturase)	1.08 ± 0.09[b]	0.89 ± 0.13[c]
22:5n-3/18:3n-3 (Δ5- and Δ6-desaturase)	2.71 ± 0.33[b]	0.82 ± 0.23[c]
22:6n-3/18:3n-3 (Δ4-, Δ5- and Δ6-desaturase)	8.08 ± 0.93[b]	2.47 ± 0.61[c]

[a]Results are means ± SD with $n = 10$ for both groups. Means with different superscript letters (b, c) differ significantly by Student´s *t*-test ($P < 0.05$). See Table 6 for abbreviations.

TABLE 8
Fatty Acid Composition (moles/100 moles of fatty acids) of Heart Total Lipids and Phospholipids[a]

	Total lipids		Phospholipids	
Fatty acid	Control oil	Oxidized oil	Control oil	Oxidized oil
SFA, total	39.35 ± 1.29	38.72 ± 1.45	46.50 ± 1.32[b]	49.97 ± 2.16[c]
16:0	15.86 ± 0.95	15.89 ± 0.88	15.64 ± 0.79	17.36 ± 2.67
18:0	23.00 ± 2.07	22.30 ± 2.01	30.86 ± 0.82[b]	32.61 ± 1.55[c]
MUFA, total	15.36 ± 3.29	15.84 ± 2.72	7.57 ± 0.58[b]	8.33 ± 0.73[c]
16:1	1.04 ± 0.56	1.10 ± 0.32	—[b]	—
18:1	14.32 ± 2.82	14.74 ± 2.42	7.57 ± 0.58[b]	8.33 ± 0.73[c]
PUFA, total	45.29 ± 2.23	45.45 ± 2.01	45.93 ± 1.03[c]	41.70 ± 2.42[b]
18:2n-6	16.04 ± 1.34	16.98 ± 0.82	16.51 ± 1.33[b]	18.87 ± 1.45[c]
18:3n-3	3.03 ± 0.72[b]	4.71 ± 1.44[c]	0.99 ± 0.13[b]	1.43 ± 0.16[c]
20:4n-6	15.16 ± 1.32	15.02 ± 1.92	17.75 ± 0.76	16.68 ± 1.88
20:5n-3	0.50 ± 0.08[c]	0.39 ± 0.11[b]	—	—
22:5n-3	2.87 ± 0.66	2.50 ± 0.29	2.68 ± 0.62[c]	1.17 ± 0.23[b]
22:6n-3	7.70 ± 1.49[c]	5.84 ± 1.15[b]	8.00 ± 1.39[c]	3.54 ± 0.86[b]

[a]Results are means ± SD with $n = 10$ for both groups. Means with different superscript letters (b,c) differ significantly by Student´s *t*-test ($P < 0.05$).
[b]— indicates that the amount of fatty acid was lower than 0.1 mole/100 moles of fatty acids). For abbreviations see Table 6.

products of lipid oxidation such as peroxides and hydroperoxides are highly toxic when administered parenterally, whereas they are less toxic when orally administered, probably due to low absorbability (23,24). In contrast, secondary products, in particular aldehyde compounds, were shown to be highly toxic because of a relatively high digestibility (25). The heat treatment for preparing the oxidized oil increased POV and TBARS concentration to a similar extent, by a factor of *ca.* 30 compared with the fresh oil. Although TBARS concentration is not a very specific indicator of secondary lipid peroxidation products (26), it suggests that the rats fed the oxidized oil had a high ingestion of both primary and secondary lipid peroxidation products.

Although this experiment was carried out over nearly the whole growth period of the rats, there were no considerable differences in the growth of the groups of rats. This suggests that, although the dietary vitamin E supply was relatively low, the oxidized oil used in this study did not produce toxic effects in the animals. It is known that a high vitamin E supply reduces the toxicity of lipid peroxidation products (6). Tissue and plasma tocopherol concentrations were markedly reduced by feeding the oxidized oil, although control and oxidized oils

TABLE 9
Lipids in Plasma, Liver and Heart[a]

	Control oil	Oxidized oil
Plasma (mM/L)		
Triglycerides	0.89 ± 0.19[b]	0.61 ± 0.14[c]
Cholesterol	2.41 ± 0.55	2.09 ± 0.32
Phospholipids	2.05 ± 0.66	1.63 ± 0.27
Liver		
Weight (g)	12.7 ± 1.2[c]	13.8 ± 0.9[b]
% of body weight	3.08 ± 0.17[c]	3.50 ± 0.32[b]
Triglycerides (μM/g)	38.4 ± 12.2[b]	30.3 ± 7.6[c]
(μM/liver)	488 ± 166	417 ± 112
Cholesterol (μM/g)	8.65 ± 2.09[b]	6.90 ± 0.65[c]
(μM/liver)	111 ± 31	96 ± 13
Phospholipids (μM/g)	32.5 ± 2.5[c]	38.7 ± 1.2[b]
(μM/liver)	411 ± 38[c]	532 ± 36[b]
Heart		
Weight (g)	1.25 ± 0.15	1.27 ± 0.09
% of body weight	0.303 ± 0.03	0.320 ± 0.02
Triglycerides (μM/g)	11.5 ± 4.2	13.3 ± 4.3
(μM/heart)	14.4 ± 5.0	16.9 ± 4.7
Cholesterol (μM/g)	3.57 ± 0.13	3.51 ± 0.10
(μM/heart)	4.46 ± 0.62	4.45 ± 0.30
Phospholipids (μM/g)	20.4 ± 1.2[b]	18.4 ± 1.3[c]
(μM/heart)	25.5 ± 2.4	23.4 ± 1.5

[a]Results are means ± SD with $n = 10$ for both groups. Means with different superscript letters (b,c) differ significantly by Student´s *t*-test ($P < 0.05$).

TABLE 10
Osmotic Fragility of Erythrocytes[a] (% hemolyzed during incubation)

Concentration of NaCl solution (%)	Control oil	Oxidized oil
0.40	26 ± 14	17 ± 8
0.38	45 ± 21	34 ± 13
0.36	64 ± 18	54 ± 16
0.34	85 ± 11[b]	69 ± 16[c]
0.32	89 ± 8	83 ± 13

[a]Results are means ± SD with $n = 10$ for both groups. Means with different superscript letters (b, c) differ significantly by Student´s *t*-test ($P < 0.05$).

were equalized for their vitamin E activities. This result agrees with other studies demonstrating adverse effects of dietary lipid peroxidation products on the vitamin E status of animals, which might be the consequence of an enhanced turnover or catabolism of tocopherols (4,7,9,27). Chemical processes that consume tocopherols include the oxidation of tocopherols by lipid peroxyl radicals. The observation that γ-tocopherol concentrations were depressed more by dietary lipid peroxidation products than were α-tocopherol concentrations suggests that γ-tocopherol is the main initial target for lipid peroxyl radical reaction. Besides an enhanced consumption of tocopherols by radical-induced oxidation, a reduced absorption of tocopherols also could contribute to the reduced tissue tocopherol concentrations in rats fed the oxidized oils (27).

The desaturation of fatty acids is an important physiological reaction because highly unsaturated fatty acids derived from linoleic acid and α-linolenic acid function as critical constituents of cellular membranes, influencing their properties or acting as precursors of eicosanoids. This study aimed to assess fatty acid desaturation in rats fed oxidized oils by determining the fatty acid composition of liver and heart total lipids and phospholipids. It is well known that SFA and unsaturated fatty acids containing one to three double bonds in most part and highly unsaturated fatty acids in minor part are catabolized by mitochondrial β-oxidation (28). In spite of this, the ratio between precursor and product of desaturation in tissue lipids commonly was used as an indicator of fatty acid desaturation (29,30). Since highly unsaturated fatty acids are mainly incorporated into tissue phospholipids and MUFA are mainly incorporated into triglycerides, it seems plausible to assess the rate of desaturation of essential fatty acids from the fatty acid composition of isolated phospholipid fractions and that of desaturation of SFA from the fatty acid composition of neutral or total lipids (30). Considering liver total lipids and phospholipids and heart phospholipids, our study suggests an impaired desaturation of SFA by Δ9-desaturase and an impaired desaturation of linoleic and α-linolenic acid by Δ6-, Δ5-, and Δ4-desaturases (Table 7). Reduced levels of fatty acids derived from desaturation in general also could be the result of an enhanced degradation of those fatty acids by mitochondrial β-oxidation or of an increased loss of highly unsaturated fatty acids by autoxidation. If the rate of mitochondrial β-oxidation, however, would be increased by feeding oxidized lipids, it would be expected that levels of saturated, mono-, di-, and triunsaturated fatty acids would be mainly reduced because they are the main substrate of β-oxidation (28). If losses of PUFA by autoxidation would play an important role, it would be expected that the reduction of individual PUFA would increase with increasing number of double bonds. Such an effect, however, was not observed. For instance, in liver phospholipids, levels of arachidonic acid and DHA were reduced, that of EPA remained unchanged, and that of docosapentaenoic acid was even increased. Hence, changes of individual PUFA levels might be the result of an impaired desaturation rate rather than the result of an increased rate of β-oxidation or increased losses by autoxidation. The results of this study are in contrast with other studies suggesting an enhanced desaturation of linoleic acid in animals fed oxidized oils by observing increased levels of arachidonic acid in tissue phospholipids (1,4,7). The different results could be due to different nutritional conditions of the animals. It is well-established that Δ6- and Δ5-desaturase activities are highly dependent on several nutritional and hormonal factors, particularly on the ingestion of linoleic acid. The studies reporting increased tissue levels of arachidonic acid used oxidized oil preparations with markedly lower levels of PUFA compared with control oils. Thus, it cannot be ruled out that the increased rate of fatty acid desaturation in those studies was caused by a lower intake of linoleic acid rather than by dietary oxidation products. Interestingly, feeding cholesterol oxidation products in rats caused an enhanced Δ6-desaturation if casein was used as the source of protein, whereas the activity of Δ6-desaturase activity remained unchanged by dietary cholesterol oxidation products if soybean meal was used as source of protein (31). These results suggest that the effect of oxidized lipids on the lipid metabolism, i.e., fatty acid desaturation, might be modified by several nutritive factors.

It is known that the activities of fatty acid desaturases are directly controlled by the fluidity of microsomal membranes (32). Thus, effects of dietary oxidized oils on desaturase activities might be mediated by an altered structure of microsomal membranes. A recent study in rats fed an oxidized linoleic acid preparation reported an increased ratio between phospholipids and cholesterol and an increased fluidity of hepatic microsomal membrane (1). Reduced activities of desaturating enzymes leading to lower levels of highly unsaturated fatty acids, as observed in this study, could be a compensatory means to maintain the membrane fluidity at a constant level. Interestingly, in contrast to liver microsomal membranes, erythrocyte membranes exhibited a reduced fluidity in rats fed oxidized lipids (3). It was suggested that incorporation of lipid peroxidation products apart from alterations of membrane lipid composition could account for the altered membrane fluidity observed in animals fed oxidized oils (3). A recent study investigating the effects of cyclic fatty acid monomers reported a decreased lipid packing order of membrane bilayers of cultured aortic endothelial cells associated with a reduced activity of Ca^{2+} ATPase (33). These studies demonstrate that dietary treatment of animals with oxidized

oils as well as incubation of isolated cells with lipid peroxidation products has profound effects on membrane structure and function.

This study shows that dietary oxidized oils reduce triglyceride concentrations in plasma and liver. Recent studies demonstrated that the activities of glucose-6-phosphate dehydrogenase (8) and other enzymes involved in the synthesis of fatty acids in the liver (13) are reduced by dietary lipid peroxidation products. Thus, reduced concentrations of triglycerides in plasma and liver might be caused by a reduced rate of lipogenesis. On the other hand, this study as well as others (6,7,11,34) reported increased liver weights in animals fed oxidized oils. It has been shown that the enlargement of liver is largely due to a proliferation of endoplasmic reticulum, which is indicated by the induction of microsomal detoxifying enzymes (8,10). This also might explain the significant increase in phospholipid content in the liver and the increased ratio between phospholipids and cholesterol. It is well known that cell organelle membrane generally has a lower cholesterol/phospholipid ratio than the plasma membrane (35).

The concentration of cholesterol in plasma plays a particular role in the risk of coronary heart disease in humans. In this study, plasma cholesterol was slightly reduced by feeding an oxidized oil. This result agrees with that of another study (10); however, there are also studies reporting increased plasma cholesterol concentration in animals fed oxidized oils (1,2). A reduced concentration of cholesterol in plasma observed in this study could be due to enhanced fecal excretion of cholesterol which was found in rats fed an oxidized oil (1).

This study observed that the osmotic fragility of erythrocytes is reduced rather than increased by feeding an oxidized oil. A similar effect was recently observed in rats fed a moderately oxidized fat (12). This effect might be due to an altered structure of the erythrocyte membrane. A study of Hayam *et al.* (3) which demonstrated a reduced fluidity of erythrocyte membranes in rats fed oxidized oils suggested the incorporation of lipid peroxidation products into erythrocyte membranes. Lipid peroxidation products, in particular malondialdehyde, can react with free amino groups of proteins and lipids to form cross-linked products between various membrane components (36), thereby leading to increased membrane rigidity.

In conclusion, this study shows that feeding an oxidized oil causes several alterations of the lipid metabolism which might be of physiologic relevance. However, further studies are required to understand the mechanisms underlying those alterations.

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Absorption in Rats of Rapeseed, Soybean, and Sunflower Oils Before and Following Moderate Heating

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ABSTRACT: Rapeseed, soybean, and sunflower oil were heated for 15 min in a 5-mm oil layer in a pan at 180°C. The fatty acid composition was almost unaffected by heating, while the polymer content rose slightly and the tocopherol content decreased, except in soybean oil. The absorption of oils before and after heating was investigated in lymph-cannulated rats. Oils were administered as emulsions through a gastrostomy tube and lymph was collected during the next 24 h. The highest accumulated lymphatic transport of total fatty acids was observed after administration of rapeseed oil, and the lowest after heated sunflower oil. The accumulated transport was similar for all unheated oils. The transport of fatty acids was significantly lower in rats receiving heated oil compared to those receiving the corresponding unheated oil. Small increases in polymers may have contributed to the decreased lymphatic transport of oil following heating, although this probably does not fully explain the effect. The absorption of sunflower oil was more affected by heating than the absorption of soybean or rapeseed oil. Furthermore, the largest decrease in total activity of tocopherols following heating was observed in sunflower oil. Overall, these results demonstrate that the absorption of vegetable oils is affected by moderate heating.

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According to the current dietary recommendations from the American Heart Association (1), a low intake of saturated fat and a high intake of unsaturated fat balanced between monounsaturated and polyunsaturated fatty acids, combined with a low intake of total fat, are advised as ways of reducing the risk for lifestyle-related diseases, such as coronary heart disease. From this point of view, rapeseed oil (RO) is a nutritionally healthy oil with a high content of the monounsaturated fatty acid, oleic acid, and also high contents of the essential polyunsaturated fatty acids linoleic and α-linolenic acid. With its high content of oleic acid, RO resembles olive oil, but it has a much higher content of polyunsaturated fatty acids, although not as high as oils such as soybean oil (SOO), corn oil, and sunflower oil (SUO).

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Abbreviations: HPLC, high-performance liquid chromatography; RO, rapeseed oil; SOO, soybean oil; SUO, sunflower oil; α-TE, α-tocopherol equivalent.

The frying of fats, and especially highly unsaturated fats, results in thermoxidative and hydrolytic alterations. The deterioration of the lipid is correlated with the content of unsaturated fatty acids (2–5). Furthermore, the rate of oxidation depends on factors such as temperature, frying time, and antioxidant content (2,3,6–8). Heating causes several chemical alterations, e.g., elevations in the level of free fatty acids, changes in peroxide value, decreases in the degree of unsaturation and antioxidant content, and the formation of polymers resulting in a decreased nutritive value in the oils (4,9–12). The chemical changes are accompanied by changes in smell, taste, and color (3,6,13,14).

The potential toxicity of heated fats has been investigated in animal models. Most studies suggest that, in moderately heated oils, the suspected toxic components are not present in significant quantities, and combined with an otherwise adequate diet, these oils will have only limited or no detrimental effects when fed to animals (15–19).

Feeding experiments with rats have shown either no effect (20) or decreased absorbability of heated fats (16,17,21–23). In the present study, the aim was to investigate the stability of three vegetable oils during heating, with the use of a moderate heating procedure, thereby imitating pan-frying procedures in the kitchen (6,13). This was followed by a comparison of the lymphatic absorption of oils before and following heating in lymph-cannulated rats. The oils were a new Danish variety of low α-linolenic RO, SOO, and SUO, oils normally used in human nutrition. The physiological studies in rats were supplemented with extensive chemical and sensory analyses (to be reported elsewhere).

EXPERIMENTAL PROCEDURES

Animals. The following experiment was approved by the Danish Committee for Animal Experiments. Male Wistar rats (Møllegård Breeding Centre, Ll., Skensved, Denmark) weighing 250–290 g were anesthetized intramuscularly with a combination of zolazepam, xylazin, and butorphanol (Zoletil-mixture; 0.18 mL/350 g; The Royal Veterinary and Agricultural University, Denmark) and subjected to cannulation of the main mesenteric lymph duct with a clear vinyl tube (o.d. 0.8 mm, i.d. 0.5 mm; Critchley Electrical Products Pty. Ltd., New South Wales, Australia). A feeding silicone tube

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(o.d. 3.0 mm, i.d. 1.0 mm; Polystan, Værløse, Denmark) was inserted into the fundus region of the stomach and fixed with a purse-string suture. Following surgery, the rats were placed in individual restraining cages (24) with tap water freely available and a steady infusion of physiological saline (0.9% NaCl) at 2 mL/h through the feeding tube. Four to six hours after the operation, they received 0.2 mL of analgesic (Butorphanol; diluted 1:10 with sterile water, Fort Dodge). The rats were fasted until the following day.

Administration of oil and collection of lymph. The postoperative day, the experiment was started by collecting a baseline fraction of lymph from −1 to 0 h. At time "zero," a sonicated emulsion of 0.3 mL dietary fat and 0.3 mL of a solution containing 20 mM taurocholate (Sigma Chemical Company, St. Louis, MO) and 10 mg/mL choline (Sigma Chemical Company) in distilled water was injected through the feeding tube, followed by 0.6 mL saline. The infusion of saline was continued at 2 mL/h. Lymph was collected in tubes at 1-h intervals for the following 8 h, and a combined fraction was obtained from 8 to 23 h followed by a 1-h fraction from 23 to 24 h after the administration of fat. The tubes contained 100 µL of a 10% (wt/vol) Na_2-EDTA-solution (E. Merck, Darmstadt, Germany). They were frozen immediately after collection and kept at −20°C until analysis.

Oils and lipid analysis. The following oils were used for the experiment: a low α-linolenic RO (seed selected by DLF-Trifolium, St. Heddinge, Denmark; oil refined at the Department of Biotechnology, DTU, Lyngby), an SO, and an SUO (Aarhus Olie A/S, Aarhus, Denmark). The oils were heated in a stainless steel pan at 180°C in a 5-mm layer for 15 min. The unheated oils and corresponding heated oils were administered to 6 rats each as described above. The fatty acid composition of oils was determined by gas–liquid chromatography after methylation with KOH in methanol (25). Lipid was extracted from the lymph samples as described by Folch *et al.* (26) and methylated with KOH. The resulting fatty acid methyl esters were analyzed using a Hewlett-Packard 5880A chromatograph (Ingelsheim, Germany) with a SP2380 capillary column (30 m, i.d. 0.32 mm; Supelco, Bellefonte, PA), flame-ionization detection, and helium as the carrier gas. Initial oven temperature was 120°C, which was increased by 4°C/min until 160°C. This was maintained for 2 min, then increased by 8°C/min until 200°C, which was maintained for 10 min. The temperature was then raised to 225°C and maintained for 5 min. Peak areas were calculated using a Hewlett-Packard computing integrator and used to calculate the mol% of fatty acids in the oils and the lymphatic transport of fatty acids using internal standards (13:0 and 17:0 added as methyl esters before esterification), and based on response factors for actual standards (Nu-Chek-Prep, Elysian, MN).

Tocopherols. Tocopherols (α, γ, and δ) were determined by high-performance liquid chromatography (HPLC) using a Silica Chromsep 25231 column. The HPLC system consisted of the following equipment purchased from Waters Corporation, Milford, MA: a programmable 490 UV detector measuring at 292 nm (λ_{max} of α-tocopherol), an automated 717 sample injector, and a model 510 pump. HPLC-grade hexane with 0.03% ethanol was used as mobile phase at a flow rate of 1.0 mL/min. The sample preparations were performed in the dark. Two drops of oil (approximately 25–30 mg) were weighed precisely, diluted in 400 µL mobile phase, and mixed. Before injection of 10 µL into the HPLC, the sample was filtered (Chromafil filter, Type AO-45/3, Macherey-Nagel, Düren, Germany), mounted on 1-mL syringes, and transferred to colored vials. Quantification was based on peak area measurements using external standardization.

Polymers. Polymers were determined by high-performance size exclusion chromatography using the methods of Burkow and Henderson (27), Dobarganes and Márquez-Ruiz (28), and modified by Bo M. Jørgensen and R.G. Nielsen (unpublished). HPLC-grade tetrahydrofuran was used as dilution and elution solvent. The high-performance size exclusion chromatographic column (600 × 7.5 mm) was packed with 3-µm particle size PLgel of the mixed-E type (Polymer Labs, Aarhus, Denmark). A guard column (6 cm) packed with the same gel was mounted upstream the main column. An evaporative light-scattering detector (Cunow DDL 21) was used and operated with the following parameters: photomultiplier voltage at 400 V, temperature at 70°C, and filtered air flow rate at 4.0 L/min. The oil was diluted in tetrahydrofuran to a concentration of 50 mg/L in order to obtain detectable polymer peaks. This solution was further diluted 100 times to obtain the main peak of triacylglycerols. The injection volume was 20 µL, and the elution rate was 0.5 mL/min. All samples were analyzed consecutively at 15-min intervals. Polymer peak areas were calculated by commercial PC-software. Quantification was based on these areas and the dilution factor.

Statistical analyses. Results were expressed as mean ± SEM. Differences in lymphatic transport between the groups receiving different fats were tested using one-way analysis of variance after calculating the area under the curve using the trapezoidal rule (29) from 0 to 8 h, or by comparing selected time points. Student-Newman-Keuls method was used to determine the exact nature of the differences. All procedures were carried out using the Jandel SigmaStat statistical package (Jandel Corporation, Erkrath, Germany). The level of statistical significance was $P < 0.05$.

RESULTS

Changes in oils following heating. The main fatty acid in RO was oleic acid (18:1n-9, 58.4 mol%), while in SOO and SUO, linoleic acid (18:2n-6) was the dominating fatty acid, constituting 50.6 and 60.6 mol%, respectively (Table 1). No changes were observed in the fatty acid composition of the oils following heating, except for minor decreases in 18:2n-6 in SOO and SUO and concomitant increases in 16:0 and 18:1n-9. *Trans*-fatty acids were not detected following heating. The content of polymers in the oils was low (Table 2), especially in RO, with values below or close to 1 wt%. The differences between unheated and heated oils were small, al-

TABLE 1
Fatty Acid Composition of Oils[a]

Fatty acid (mol%)	Rapeseed	Heated rapeseed	Soybean	Heated soybean	Sunflower	Heated sunflower
16:0	4.7	4.8	12.3	12.9	7.7	7.7
16:1	0.3	0.2	N.D.[b]	N.D.	N.D.	0.1
18:0	1.7	1.8	4.2	4.4	4.2	4.1
18:1n-9	58.4	57.4	21.9	21.8	23.8	25.1
18:1n-7	3.0	3.0	1.5	1.5	0.8	0.8
18:2n-6	22.7	22.9	50.6	49.7	60.6	59.5
18:3n-3	6.2	6.5	7.8	7.9	1.3	1.1
20:0	0.6	0.6	0.5	0.5	0.4	0.3
20:1n-9	1.3	1.2	0.2	0.2	N.D.	0.2
22:0	0.3	0.2	0.3	0.3	0.5	0.5
22:1n-9	0.3	0.4	N.D.	N.D.	N.D.	N.D.
24:0	0.8	0.9	0.8	1.0	0.8	0.5
∑SAFA[c]	8.1	8.3	18.1	19.1	13.6	13.1
∑MUFA[d]	63.3	62.2	23.6	23.5	24.6	26.2
∑PUFA[e]	28.9	29.4	58.4	57.6	61.9	60.6

[a]The values represent the mean of three determinations.
[b]N.D., not detected.
[c]∑SAFA = ∑saturated fatty acids.
[d]∑MUFA = ∑monounsaturated fatty acids.
[e]∑PUFA = ∑polyunsaturated fatty acids.

though the values in heated oils were higher than the values in the corresponding unheated oils. The content of total tocopherols was highest in soybean oil (1105 µg/g oil) and lowest in rapeseed oil (701 µg/g oil), and it decreased in all three oils during heating, although it was insignificant in SOO. The most biologically active tocopherol is α-tocopherol. The activities of different tocopherols are expressed as α-tocopherol equivalents (α-TE), 1 mg of *RRR*-α-tocopherol being assigned 1 α-TE and the activity of γ- and δ-tocopherol to 0.1 and 0.03 α-TE, respectively (30). Calculating the total α-TE in heated and unheated oils gave the highest value for SUO and the lowest for SOO. The largest decrease in α-TE caused by heating was observed in SUO (9% lower after heating) followed by RO (6%), while SOO was unaffected.

Lymphatic transport. The lymphatic transport of total fatty acids showed marked differences between the unheated and corresponding heated oils (Fig. 1), with significantly higher

TABLE 2
The Content of Polymers and Tocopherols (T) in Oils[a] and the Calculated α-Tocopherol Equivalent Activity (α-TE)[b]

	Rapeseed	Heated rapeseed	Soybean	Heated soybean	Sunflower	Heated sunflower
Polymers (wt%)	0.03	0.05	0.15	0.24	0.78	1.03
T (µg/g oil)						
α-T	265	252	117	136	720	656
γ-T	341	290	632	605	N.D.[c]	N.D.
δ-T	95	87	356	347	73	83
Total T	701	629	1105	1088	793	739
α-TE	302	284	191	207	722	658

[a]The values represent the mean of two determinations.
[b]Calculated as described by Sheppard *et al.* (30).
[c]N.D., not detected.

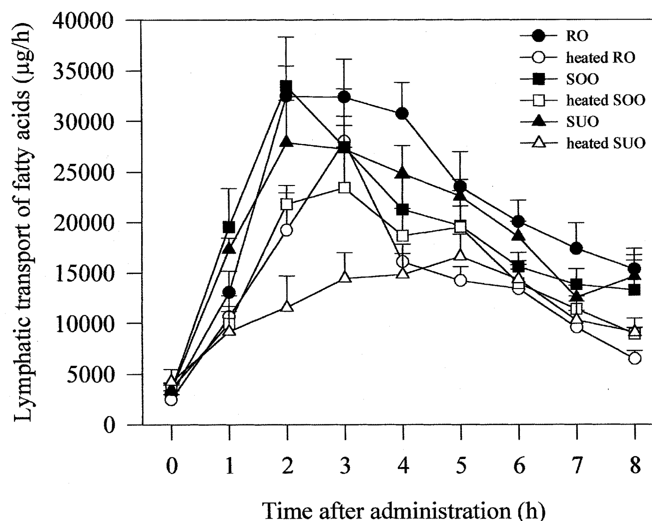

FIG. 1. Lymphatic transport (µg/h) of total fatty acids in rats after administration of dietary oils before and following heating. Values are means ± SEM ($n = 6$). RO, rapeseed oil; SOO, soybean oil; SUO, sunflower oil.

transport of the unheated oils ($P < 0.05$). The transport of heated SUO was significantly lower than all other oils ($P < 0.05$). No difference was observed in the lymphatic transport of the three unheated oils. Maximum transport was obtained 2 h after administration of the unheated oils, reaching values between 27.9 and 33.5 mg/h, and 3 h after heated SOO and RO (23.4 and 28.0 mg/h), while the transport of heated SUO (16.7 mg/h) did not peak until 5 h after administration. Figure 2 shows the accumulated transport of total fatty acids following administration of oils. The transport at 8 h was significantly higher for all unheated oils compared to all heated oils ($P < 0.05$). At 24 h the transport of all unheated oils was significantly higher than the transport of heated SUO ($P < 0.01$), and the transport of RO was higher than the transport of

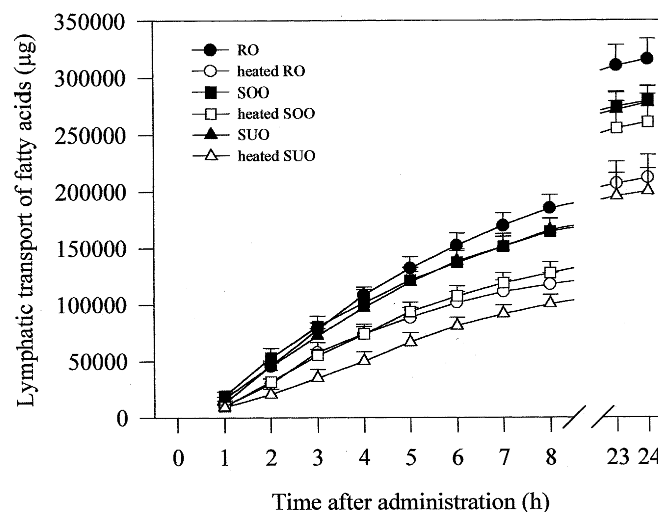

FIG. 2. Accumulated lymphatic transport (µg) of total fatty acids in rats after administration of dietary oils before and following heating. Values are means ± SEM ($n = 6$). See Figure 1 for abbreviations.

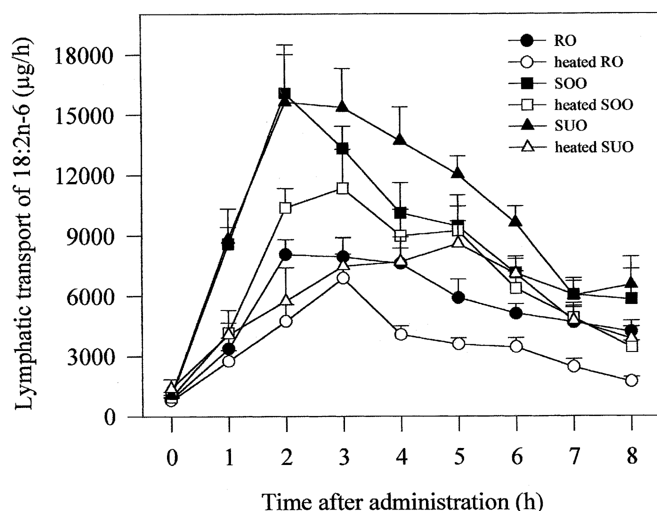

FIG. 3. Lymphatic transport (μg/h) of linoleic acid (18:2n-6) in rats after administration of dietary oils before and following heating. Values are means ± SEM (n = 6). See Figure 1 for abbreviations.

TABLE 3
The Ratio Between the Mean Area Under the Curve (AUC) of Unheated Oil and the Corresponding Mean AUC of Heated Oil for Lymphatic Transport of Individual and Total Fatty Acids[a]

	Rapeseed oil		Soybean oil		Sunflower oil	
Fatty acid	Ratio	SEM	Ratio	SEM	Ratio	SEM
16:0	1.9	0.2	1.3	0.1	2.1	0.4
18:0	1.7	0.2	1.5	0.2	1.9	0.4
18:1n-9	1.7	0.1	1.3	0.1	2.0	0.2
18:2n-6	1.8	0.1	1.3	0.1	2.1	0.2
18:3n-3	1.7	0.1	1.4	0.1	4.0	1.0
Total	1.7	0.1	1.3	0.1	2.1	0.2

[a]The areas under the curve are calculated as the mean of results from six rats each.

heated RO ($P < 0.01$). The highest accumulated transport was observed after RO administration (316 mg) and the lowest after administration of heated SUO (200 mg). Each rat received 0.3 mL of oil, corresponding to 270 mg fat, and this led to a recovery after RO at 117%, and at 74% after heated SUO.

The transport of 18:2n-6 and 18:3n-3 is shown in Figures 3 and 4, respectively. The transport of 18:2n-6 after administration of unheated oils was significantly higher than the transport of the corresponding heated oils ($P < 0.05$). Administration of SOO and SUO led to transport of 18:2n-6 significantly higher than after all other oils. The transport of 18:2n-6 after heated RO was significantly lower than after all other oils. Administration of SOO resulted in transport of 18:3n-3 significantly higher than after all other oils. RO led to higher transport of 18:3n-3 compared to heated RO. Similar differences between heated and unheated oils were observed for the saturated fatty acids (not shown).

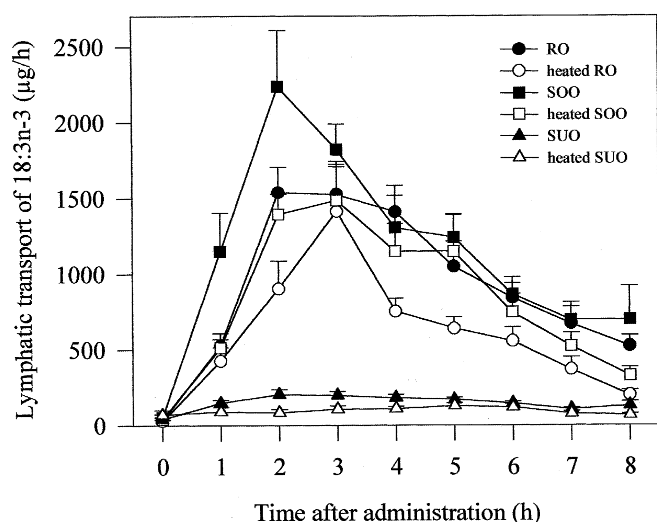

FIG. 4. Lymphatic transport (μg/h) of α-linolenic acid (18:3n-3) in rats after administration of dietary oils before and following heating. Values are means ± SEM (n = 6). See Figure 1 for abbreviations.

The ratio between the mean area under the curve from 0 to 8 h for lymphatic transport of unheated oil and the mean area under the curve for the corresponding heated oil was calculated for individual and total fatty acids (Table 3). The highest ratio was found for SUO (around 2.0) and the lowest ratio for SOO (around 1.3), with RO (around 1.8) in between. These ratios illustrate that the lymphatic transport of unheated SOO was only slightly higher than the transport of heated SOO, while the transport of unheated SUO was nearly twice as high as the transport of heated SUO. Thus the effect of heating on the lymphatic transport was most pronounced with SUO.

DISCUSSION

In this study, three vegetable oils, RO, SOO, and SUO, were heated in a pan for 15 min at 180°C. Heating resulted in insignificant changes in fatty acid composition, but a slightly increased content of polymers and decreased tocopherol contents. The unheated and corresponding heated oils were administered to lymph-cannulated rats, and the lymphatic absorption of fat was followed for 24 h. The lymphatic transport was significantly lower after feeding the heated oils compared to the unheated oils. This was observed for both total and individual fatty acids (Figs. 1–4), indicating that heating oils even for a short period of time at a moderate temperature, as with those conditions applied in the normal use of the oils, leads to changes affecting the absorptive process.

Several groups have investigated the effects of including heated fats in the diets fed to rats for different periods of time. Nolen *et al.* (17) and López-Varela *et al.* (20) observed decreased rate of body weight gain in groups of rats fed heated oils compared to those fed unheated oils. This is in contrast to the findings by Izaki *et al.* (15), Kok *et al.* (16), and Hageman *et al.* (18), in which no effect on body weight gain was observed. Nothing in the experimental conditions can explain the differences in these results. Although the weight gain was

unaffected in the study by Kok *et al.* (16), the food intake was increased, indicating a decreased feed efficiency of heated oils. In various studies, long-term feeding of heated oils led to increases in organ weights, especially in the liver (15,16,19), while shorter periods of feeding (4 wk) showed no changes in liver weights (18,20). Independent of the duration of the feeding period and the nature of the administered oils, changes in the activities of several liver enzymes, although sometimes inconsistent, have been observed after feeding heated oils. Izaki *et al.* (15) observed increases, while Kok *et al.* (16) found decreases in the activity of gluthatione peroxidase. Decreases in the activity of carnitine palmitoyl transferase-I, isocitrate dehydrogenase, and glucose-6-phosphate dehydrogenase and increases in NADPH-cytochrome P_{450} reductase were found by Lamboni and Perkins (31). From these studies, it appears that the heating of fat can lead to the development of possibly toxic substances affecting metabolism in the liver. But, as observed by Nolen *et al.* (17), in a 2-yr feeding study in rats with heated oils, the level of these substances and the degree of their toxicity are generally low, and if the diet is otherwise balanced and adequate, they have no practical dietary significance.

In several studies, the absorption of heated fats was lower than the absorption of unheated fats (16,17,21,22). Fat absorption was determined either by calculation of fat excreted in feces compared to that ingested or through lymph collection studies. The main factor responsible for this reduced fat absorption is the content of unabsorbable polymeric material formed during heating, as observed by many investigators (5,9,11,21,32). According to some investigators, both reduced hydrolysis by pancreatic lipase of the complex molecules formed during heating and poor absorption of the released complex fatty acids contribute to the reduced absorbability (22,32). Others have found that the *in vitro* hydrolysis by pancreas lipase is only influenced to a minor degree by the heating of oils, resulting in complete hydrolysis of oils with low polymer contents (5,33). In our experiment we observed a remarkable reduction in the lymphatic transport of fat following heating. This was observed even though the increases in polymer content were relatively low. These observations indicate that either this small increase in the content of polymers could be responsible for the reduced transport or the answer has to be searched for in other, yet unidentified components.

The absorption of the three vegetable oils was not affected to a similar degree by heating. The highest reduction in transport was observed after feeding heated SUO. This is illustrated by a ratio of around 2.0 for the transport of unheated oil compared to heated oil (Table 3), indicating that the transport is twice as fast after administration of unheated oil. The lowest ratio (1.3) was found after administration of SOO. Furthermore, the largest reduction in total tocopherol activity following heating was observed in SUO (Table 2). The activity of tocopherols in SOO was not affected by heating. Even though SUO had the highest total activity of tocopherols, it had the most pronounced decrease in activity following heating, resulting in diminished antioxidative protection. This might also influence the lymphatic transport. Yoshida and Alexander (32) compared the *in vitro* hydrolysis by pancreatic lipase of monomers and dimers originating from the heating of corn oil, SO, and SUO, but they did not observe any differences in hydrolytic activity in the three oils. Maybe answers to the observed differences in lymphatic transport of the heated oils have to be searched for in the differences in the absorption process, rather than in the digestion by pancreatic lipase. Cepinskas *et al.* (34) showed that luminal hydroperoxides, which may be present following intake of heated fats, could be injurious to the intestinal epithelium and thereby affect absorption, but this needs further investigation.

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Fatty Acid Composition of Brain Glycerophospholipids in Peroxisomal Disorders

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ABSTRACT: This paper shows for the first time the differential fatty acid composition of ethanolamine plasmalogens (EP) and phosphatidylethanolamine (PE) in the brains of 12 patients with disorders of peroxisomal biogenesis and compares the results to normal values for the age. Other important glycerophospholipids (GPL), such as phosphatidylserine (PS) and phosphatidylcholine (PC), are also included in this study. GPL were separated by two-dimensional thin-layer chromatography, and their fatty acid composition was determined by capillary column gas–liquid chromatography. Total brain GPL were slightly decreased in peroxisomal disorders (27.98 ± 2.95 µmol/g in the patients against 34.5 ± 6.21 µmol/g in age-matched controls, $P = 0.005$), and the distribution of the different GPL classes was much altered. In confirmation of known data, EP were very much decreased (2.18 ± 1.3 µmol/g in the patients against 6.9 ± 2.3 µmol/g in controls) at the expense of PE, which was increased (8.58 ± 2.17 µmol/g in the patients against 5.97 ± 0.58 µmol/g in controls, $P < 0.005$). PS and PC were both significantly decreased ($P = 0.0001$ and $P = 0.037$, respectively). The polyunsaturated fatty acid (PUFA) composition of all the GPL fractions was markedly abnormal. In absolute terms, docosahexaenoic acid (22:6n-3) was drastically decreased in all GPL classes (always at the $P < 0.0001$ level) while arachidonic acid (20:4n-6) was increased in PE and PS ($P < 0.001$ in both cases). In the alkenyl acyl form, EP, 22:6n-3, and 20:4n-6 were both very significantly decreased ($P < 0.0001$), although the former was always the most affected. The myelin PUFA adrenic acid (22:4n-6) was decreased in EP ($P < 0.0001$) and slightly increased in PS ($P < 0.05$). The changes found confirm that 22:6n-3 deficiency is a predominant defect in the brain in peroxisomal disorders.

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Zellweger's (cerebro-hepato-renal) syndrome (1) is a lethal genetic disease characterized by the absence of normally formed peroxisomes in the liver and proximal tubules of the kidneys (2). The deficiencies in enzymes known to be located in peroxisomes cause multiple biochemical abnormalities, mostly affecting lipid metabolism. Characteristically, there is an increase in very long chain fatty acids (fatty acids with more than 22 carbon atoms) (3), owing to a deficient peroxisomal β-oxidation (4), and a decrease in plasmalogen levels (5), owing to defects in the enzymes alkyldihydroxyacetone phosphate synthase and dihydroxyacetone phosphate acyltransferase (6). Defective α-oxidation of phytanic acid leads to accumulation of this ramified fatty acid in plasma (7). Bile acid synthesis is affected, with accumulation of abnormal metabolites (8). Most organs are involved, but the clinical picture is dominated by profound neurodevelopmental, sensorial, and hepatic damage. The disorder is rapidly progressive, and death occurs in the first few months of life. Postmortem examination reveals neuronal differentiation and migration defects, as well as white matter abnormalities with reduction in myelin volume, dysmyelination, or demyelination (9).

There are milder variants of Zellweger's syndrome (ZS), like neonatal adrenoleukodystrophy (10) and infantile Refsum's disease (11). Genetically, however, no clear distinction can be made between these variants (12), the whole group representing a disease continuum with different degrees of clinical severity. In all these disorders, peroxisomal biogenesis is defective and the enzymatic deficit generalized. Clinically, these diseases are constantly characterized by psychomotor retardation, progressive sensorineural deterioration with blindness and deafness, failure to thrive, and hepatomegaly.

The underlying mechanism leading to such a severe clinical picture is unknown. Much of the neurological damage is attributed to abnormal accumulation of very long chain fatty acids in brain lipid structures (9). The decrease in plasmalogen levels could also be a deleterious factor. Recently, a dramatic decrease of docosahexaenoic acid (DHA, 22:6n-3) in the brain, retina, liver, kidneys, and blood of peroxisomal-disorder patients was found (13–15). Interestingly, the DHA levels seemed to be specifically decreased in the brains of peroxisomal-disorder patients, while the other main polyunsaturated fatty acid (PUFA), arachidonic acid (20:4n-6), was generally spared, or even increased. The existence of such a specific deficiency in a PUFA known to be especially localized in brain synapse phospholipids and the photoreceptor cells of the retina (16,17) may be pathogenic and deserves fur-

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Abbreviations: DHA, docosahexaenoic acid; EP, ethanolamine plasmalogens; FAME, fatty acid methyl esters; GPL, glycerophospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PUFA, polyunsaturated fatty acid(s); TLC, thin-layer chromatography; ZS, Zellweger's syndrome.

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ther attention. Thus, after studying the total fatty acid composition of the brain in peroxisomal patients, we wanted to assess the PUFA patterns of the isolated glycerophospholipids (GPL). Since brain plasmalogen levels are much decreased in peroxisomal disorders and these lipids are important in myelin, we separated the alkenyl acyl from the diacyl form of ethanolamine GPL, paying special attention to the PUFA composition of ethanolamine plasmalogens (EP). This study shows for the first time the PUFA changes of EP in the brain of peroxisomal-disorder patients compared with those in diacyl phosphatidylethanolamine (PE) and other important GPL.

EXPERIMENTAL PROCEDURES

Brain tissue. Postmortem brain tissue from 12 patients with disorders of peroxisomal biogenesis and 20 controls was collected. The ages of the controls ranged from 26 prenatal wk to 8 postnatal yr, and their developmental phospholipid fatty acid profiles have been reported elsewhere (18). Although for this study the results of the patients were compared to the whole age span, for statistical analysis only the eight age-matched controls (from 1 mon to 2 yr of age) were used. All the controls died from acute diseases unrelated to the central nervous system. Roughly half of the cases were males and half females, and no sex differences were found in their fatty acid profiles. Of the peroxisomal brains, one belonged to the first Zellweger patient studied by the authors (13) and the other 11 were kindly provided by Dr. Moser's laboratory. All the patients were diagnosed with classic ZS or severe Zellweger variants, and their ages ranged from 1 to 31 mon. Seven of the patients died before 6 mon of age, four before 1 yr and one before 2 yr. All autopsies were performed after obtaining the signed informed consent from the parents. Human tissue was always obtained and used with permission from the corresponding hospital ethical committee. The bodies were transferred to the mortuary immediately after death and kept at 4°C until autopsy, which was performed within the first 48 h of death. Brain tissue was stored frozen at –80°C in a double bag of aluminum foil and polyethylene until analyzed. Although the time of storage of the brain samples varied widely, repeated analyses proved that the lipid and fatty acid composition was remarkably stable for years, including the levels of the most unsaturated fatty acids.

Biochemical methods. The lipids of 50–100 mg of wet brain tissue were extracted with 20 vol of chloroform/methanol/water (50:50:10, by vol). After centrifugation, the pellet was reextracted twice with 10 vol of chloroform/methanol/water (50:50:15, by vol). The combined lipid extracts were concentrated under a stream of N_2 and made up with chloroform/methanol (2:1) to a convenient small volume (generally 1 mL) for direct application to the thin-layer chromatography (TLC) plate. All solvents were of the highest commercial quality (Merck, Darmstadt, Germany), and contained 50 mg/L of butylhydroxytoluene (Fluka, Buchs, Switzerland) as an antioxidant. Individual lipids were separated by two-dimensional TLC according to Horrocks and Sun (19). Briefly, an aliquot of the total lipid extract corresponding to about 10 mg of wet brain tissue was spotted on the lower left corner of a TLC silica gel 60 F_{254}, 20 × 20 cm, plate (Merck). After one development in the first dimension with chloroform/meth-anol/ammonia (65:25:4, by vol), the plate was dried with a stream of N_2 and exposed to fumes of 12 N HCl for 5 min. This split plasmalogens into aldehydes and lysophospholipids. A second development in the other dimension, with chloroform/methanol/acetone/acetic acid/water (75:15:30:15: 7.5), separated ethanolamine GPL into acid-stable diacyl PE, aldehydes, and the lyso PE derived from the split EP.

The plate was dried once more with an N_2 stream, and the phospholipid spots were visualized by developing the plate with bromothymol blue. The fatty acid methyl esters (FAME) were obtained by direct transmethylation of the phospholipid spots with HCl/methanol, basically by the method of Lepage and Roy (20) with some modifications (21). After scraping and transferring the spots into methanolysis tubes (Corning, NY), 50 µL of methanol/benzene 4:1 (vol/vol) containing 25 µg of 13:0 and 25 µg of 23:0 was added. The volume was made up to 2 mL with the same solvent, and the tubes were vigorously vortexed (Rotamixer, Hook & Tucker, Ltd., England). While still vortexing, 0.2 mL of acetyl chloride was slowly added. This procedure avoided spillage of the hot mixture and contamination of the sample by any external stirrer. The tubes were tightly stoppered and transferred to a heating block (Thermolyne 4600 Dri-Bath, Dubuque, IA) at 100°C for 1 h. Losses by evaporation were checked by weighing before and after the reaction. After centrifugation at 3,000 rpm for 10 min, the upper phase was concentrated under a stream of N_2 and an aliquot (1–3 µL) was injected into a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard GmbH, Waldbronn, Germany), equipped with a 25 m, 0.25 mm i.d., BPX-70 column (SGE, Australia). The carrier gas was helium, at a constant flow of 1.1 mL/min. Samples were analyzed at a split ratio of about 25:1, and the column was programmed from 140 to 210°C at 3°C/min. Injector and detector temperatures were 250°C. Detector response linearity was checked in each chromatogram by comparing the areas of the two internal standards (13:0 and 23:0). Only when the two internal standard areas were the same (within a range better than ± 10%) were the results considered acceptable. Otherwise, the analyses were repeated until obtaining totally linear responses.

Peaks were measured with a Merck-Hitachi D-2500 computer-integrator (Merck) and identified by comparison of their retention times with those of pure standards and of mixtures with known fatty acid composition. When necessary, peak identification was confirmed by mass spectrometry of picolinyl esters (22) using a Hewlett-Packard 5970B mass selective detector. Spectra were obtained at an ionization potential of 70 eV. FAME areas were measured and computed with a D-2500 computer-integrator. Total amounts of phospholipids were obtained on the basis of the known FAME molarities, as detailed elsewhere (23). Statistical analysis consisted of unpaired t-tests of the patients' data (n = 12) against those of

age-matched controls (n = 8), using the statistics package StatView IV (Abacus Concepts, Inc., Berkeley, CA) and a Macintosh IIfx computer (Apple Computer Inc., Cupertino, CA).

RESULTS

Figure 1 compares the GPL composition of the brain in peroxisomal patients with that in age-matched controls. The distribution of the different GPL is markedly altered in the patients. Notably, the peroxisomal brain has much less plasmalogens and more diacyl PE than the normal brain at the same developmental stage, agreeing with data from other authors (5). To a lesser extent, the amount and proportion of PS are also decreased in the brains of peroxisomal patients. The proportion of phosphatidylcholine (PC) does not change much in the peroxisomal brain although its amount is less than normal.

Figures 2–5 show the absolute values of the main long-chain PUFA, 22:6n-3, 20:4n-6, and 22:4n-6, in the different brain GPL classes, in neurologically normal children compared to the peroxisomal patients studied. As Figure 2 shows, all three PUFA are decreased in EP in the brains of peroxisomal patients when compared with normal controls of the same age, although DHA is clearly the most affected PUFA. In PE, on the other hand, Figure 3 shows that the drastic decrease in the DHA levels is accompanied by a significant increase in 20:4n-6. So the increase in arachidonic acid partly accounts for the marked increase in PE found in the peroxisomal brain (Fig. 1). A more general decrease of all fatty acids in EP was to be expected since plasmalogen levels are known to be very low in the peroxisomal brain (5), as confirmed in Figure 1. However, as in the other GPL, DHA was clearly the most affected PUFA in EP, a fact that can more clearly be detected when looking at the percentage fatty acid patterns (Table 1).

Figure 4 shows that DHA is the specifically deficient PUFA also in PS. As in PE, arachidonic acid is significantly increased in PS in the peroxisomal brain. A slight but significant increase in adrenic acid was also observed in PS. Although the changes are less marked in PC, Figure 5 shows that, again, DHA is the most affected PUFA in this GPL. The other two fatty acids do not show significant changes, although there is a tendency toward higher 20:4n-6 levels in the peroxisomal-disorder brain.

To obtain a more complete view of all the fatty acid changes in the brain of peroxisomal patients, Tables 1 and 2 show the fatty acid patterns of the four brain GPL in the patients studied and controls of the same age. Tables 1 and 2 show that in percentage terms, as well as in absolute values, 22:6n-3 is consistently decreased in all four phospholipid classes, whereas 20:4n-6 is augmented in PE and PS and 22:5n-6 is decreased in all GPL. This is the rule in classic ZS and its most severe variants, like all the cases included in the present study. It must be pointed out, however, that in some patients that survive longer, 22:5n-6 may behave differently.

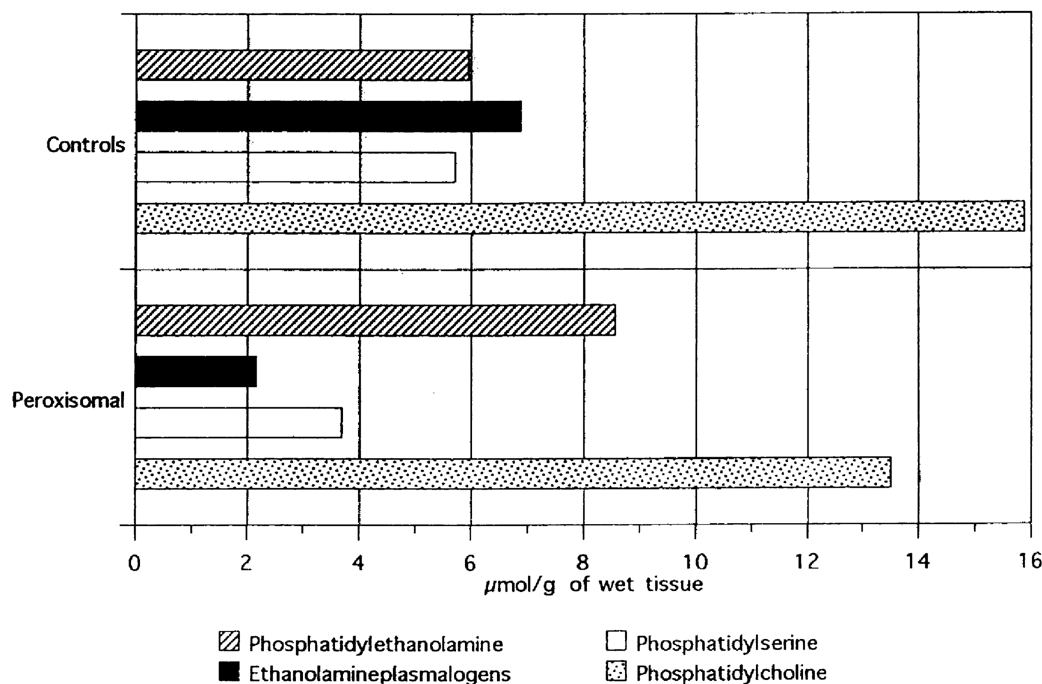

FIG. 1. Distribution of the main glycerophospholipids in the peroxisomal brain (n = 12) compared to that in the normal developing brain (n = 8). Both groups were matched for age (from 1 mon to roughly 2 yr). Although SD are not given here for clarity of the Figure, t-tests showed very significant statistical differences between patients and controls. While ethanolamine plasmalogen (EP) was much decreased in the Zellweger brain ($P < 0.0001$), phosphatidylethanolamine (PE) was increased ($P < 0.005$). Phosphatidylserine (PS) ($P = 0.0001$) and phosphatidylcholine (PC) ($P < 0.05$) were both decreased in the peroxisomal brain.

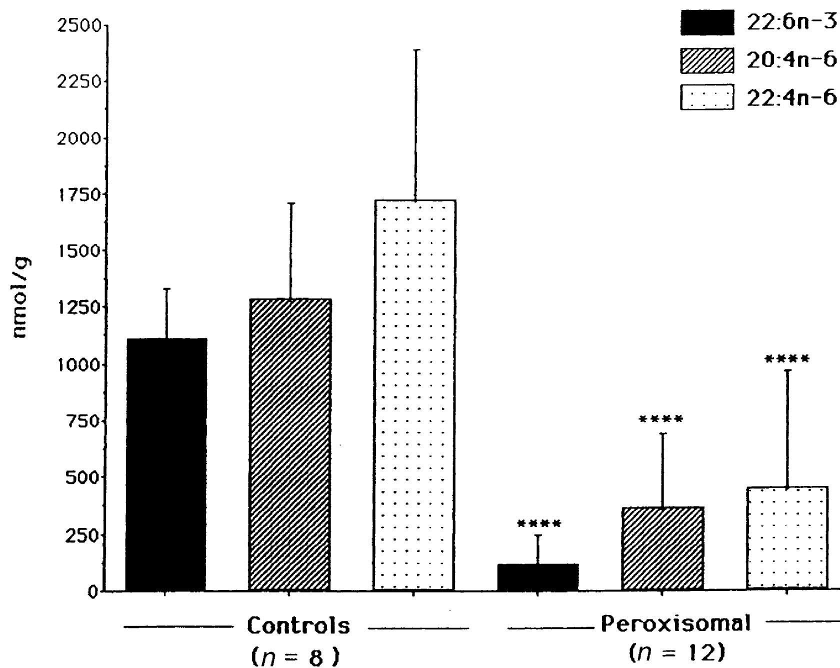

FIG. 2. Main polyunsaturated fatty acids (PUFA) of EP in the brains of peroxisomal disorder-patients compared to normal brains of the same age. All three PUFA are very significantly decreased in brain EP in peroxisomal patients, although 22:6n-3 is the most affected. Values are nmol/g of wet tissue; bars represent the means ±1 SD; ****, $P < 0.0001$. See Figure 1 for other abbreviation.

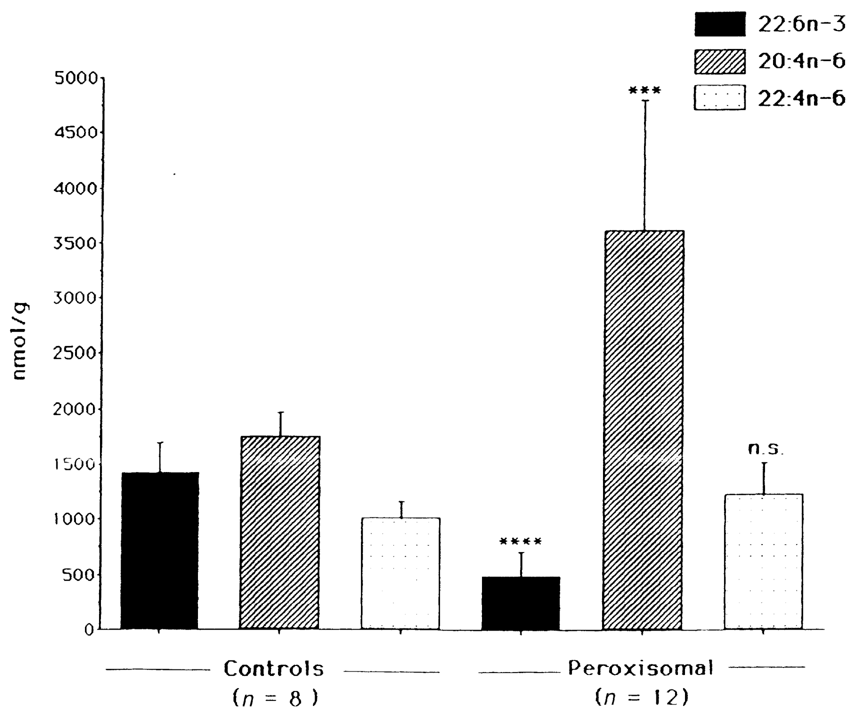

FIG. 3. Main PUFA of PE in the brains of peroxisomal disorder-patients compared to normal brains of the same age. Like in EP, 22:6n-3 is very significantly decreased in PE in the peroxisomal-disorder brain. In contrast to EP, however, 20:4n-6 is increased in PE in peroxisomal patients. Values are nmol/g of wet tissue; bars represent the means ±1 SD; ***, $P < 0.001$; ****, $P < 0.0001$, n.s., not significant. See Figures 1 and 2 for other abbreviations.

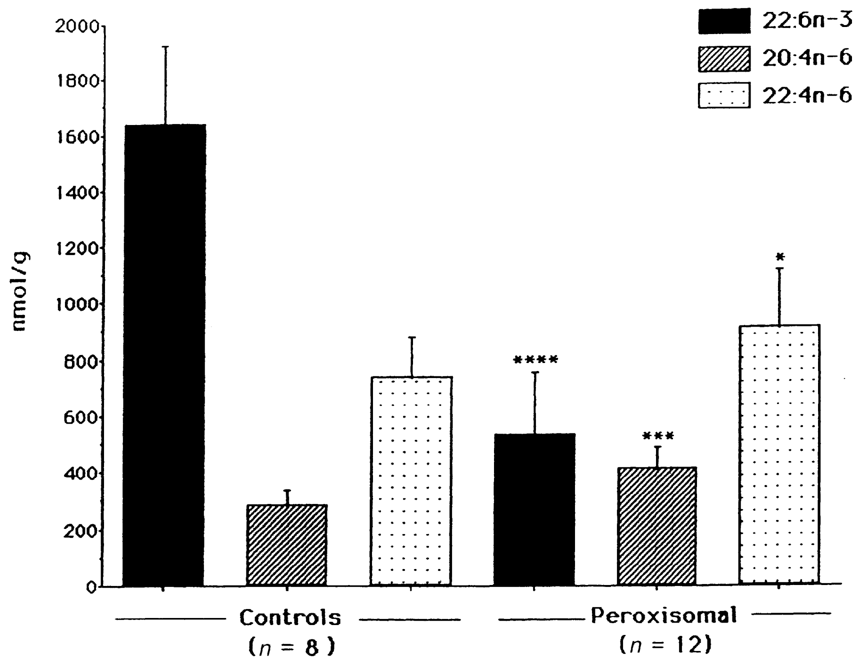

FIG. 4. Main PUFA of PS in the brains of peroxisomal disorder patients compared to normal brains of the same age. Again, 22:6n-3 is very significantly decreased in brain PS in peroxisomal disorders, while 20:4n-6 and 22:4n-6 are both significantly increased. Values are nmol/g of wet tissue; bars represent the means ±1 SD; *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$. See Figures 1 and 2 for abbreviations.

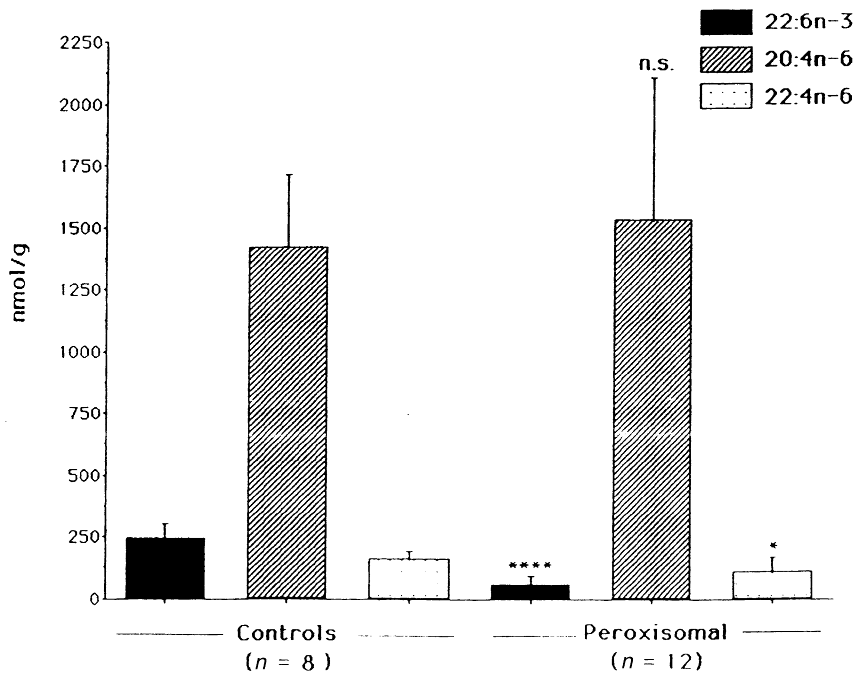

FIG. 5. Main PUFA of PC in the brains of peroxisomal disorder-patients compared to normal brains of the same age. In brain PC, 22:6n-3 is the only PUFA significantly decreased in peroxisomal patients. Values are nmol/g of wet tissue; bars represent the means ±1 SD; *, $P < 0.05$; ****, $P < 0.0001$; n.s., nonsignificant or $P > 0.05$. See Figures 1–3 for abbreviations.

TABLE 1
Fatty Acid Composition of Brain Ethanolamine Glycerophospholipids (1 mon–2 yr) in Zellweger's Syndrome (ZS) Compared with Age-Matched Controls[a]

	Phosphatidylethanolamine		Ethanolamine plasmalogens	
	ZS (n = 12)	Controls (n = 8)	ZS (n = 12)	Controls (n = 8)
14:0	0.40 ± 0.11	0.40 ± 0.11	3.47 ± 2.03**	0.74 ± 0.20
16:0	11.32 ± 1.45***	8.83 ± 0.52	17.05 ± 6.22***	6.46 ± 1.57
16:1n-9	0.27 ± 0.12	0.21 ± 0.08	1.07 ± 0.50*	0.59 ± 0.24
16:1n-7	0.48 ± 0.22**	0.23 ± 0.13	0.28 ± 0.43	0.34 ± 0.13
18:0	35.07 ± 3.83*	38.67 ± 2.12	24.81 ± 18.25**	4.50 ± 1.44
18:1n-9	8.90 ± 2.82	6.91 ± 1.04	5.51 ± 2.49****	13.51 ± 4.15
18:1n-7	3.13 ± 1.75	2.35 ± 0.34	1.22 ± 0.63	1.45 ± 0.39
18:2n-6	1.44 ± 0.44****	0.36 ± 0.17	1.01 ± 0.41***	0.29 ± 0.12
20:0	0.16 ± 0.05	0.12 ± 0.06	0.61 ± 0.58	0.34 ± 0.09
20:1n-9	0.70 ± 0.24**	0.37 ± 0.11	1.14 ± 0.48	1.46 ± 0.59
20:2n-9	0.23 ± 0.21	0.39 ± 0.08	0.24 ± 0.24	1.58 ± 0.50
20:2n-6	0.56 ± 0.23****	0.04 ± 0.04	0.41 ± 0.26**	0.10 ± 0.08
20:3n-9	0.78 ± 0.65	0.77 ± 0.46	0.58 ± 0.30	1.15 ± 0.57
20:3n-6	3.83 ± 1.02****	1.75 ± 0.21	1.88 ± 1.24	1.78 ± 0.45
20:4n-6	20.72 ± 2.73****	14.61 ± 1.17	14.91 ± 6.27	18.60 ± 1.23
22:4n-6	7.64 ± 2.86	8.57 ± 0.83	17.26 ± 11.49	24.67 ± 2.39
22:5n-6	0.93 ± 0.50****	3.12 ± 1.36	1.27 ± 0.93****	3.77 ± 1.20
22:5n-3	0.38 ± 0.22	0.33 ± 0.07	1.28 ± 0.93	0.96 ± 0.24
22:6n-3	2.83 ± 1.37****	11.82 ± 1.62	5.12 ± 2.96****	17.03 ± 4.47

[a]Values are molar percentages (means ± 1 SD). An unpaired t-test was performed for all fatty acids, between peroxisomal patients and controls. Statistical significance worse than $P < 0.05$ is not given. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$.

In some cases diagnosed with neonatal adrenoleukodystrophy, 22:5n-6 was found to be normal or even much increased (15), while 22:6n-3 is always decreased, in classic ZS as well as in the other phenotypes. Table 1 shows that, when only considering the percentage values, the changes of both 20:4n-6 and 22:4n-6 in EP are not significant, in contrast to the absolute values of these two PUFA. This stresses the importance of studying the data in the two forms, percentages as well as absolute amounts.

Apart from the long-chain PUFA, Table 1 shows some

TABLE 2
Fatty Acid Composition of Brain Phosphatidylserine (1 mon–2 yr) and Phosphatidylcholine (1 mon–2 yr) in ZS Compared with Age-Matched Controls[a]

	Phosphatidylserine		Phosphatidylcholine	
	ZS (n = 12)	Controls (n = 8)	ZS (n = 12)	Controls (n = 8)
14:0	0.71 ± 0.08****	0.38 ± 0.09	2.04 ± 0.58	1.86 ± 0.00
16:0	3.51 ± 0.58**	2.65 ± 0.57	55.64 ± 2.99***	49.94 ± 33.17
16:1n-9	0.32 ± 0.15	0.26 ± 0.12	1.27 ± 0.70*	2.00 ± 0.00
16:1n-7	0.12 ± 0.11	0.10 ± 0.04	1.21 ± 0.68	0.92 ± 0.55
18:0	47.20 ± 2.59	48.20 ± 1.76	7.91 ± 2.23***	12.31 ± 3.26
18:1n-9	7.15 ± 3.09**	13.36 ± 4.94	16.76 ± 1.91*	19.54 ± 0.71
18:1n-7	1.29 ± 0.62	1.63 ± 0.41	3.52 ± 1.28**	5.04 ± 0.24
18:2n-6	0.94 ± 0.47***	0.16 ± 0.07	2.10 ± 0.76****	0.62 ± 1.72
20:0	0.54 ± 0.24**	0.25 ± 0.03	0.08 ± 0.03	0.07 ± 2.49
20:1n-9	0.72 ± 0.29	0.62 ± 0.24	0.55 ± 0.15***	0.31 ± 0.39
20:2n-9	0.12 ± 0.10	0.73 ± 0.28	0.05 ± 0.03****	0.16 ± 0.26
20:2n-6	0.40 ± 0.14****	0.05 ± 0.03	0.61 ± 0.32***	0.06 ± 0.03
20:3n-9	0.28 ± 0.22	0.30 ± 0.14	0.12 ± 0.06	0.16 ± 0.02
20:3n-6	8.23 ± 2.25****	1.69 ± 0.39	1.54 ± 0.50*	0.83 ± 0.04
20:4n-6	5.65 ± 0.96****	2.63 ± 0.63	5.67 ± 2.03	4.48 ± 0.03
22:4n-6	12.43 ± 2.89***	6.77 ± 1.83	0.41 ± 0.23	0.52 ± 0.08
22:5n-6	2.25 ± 0.83****	5.00 ± 1.52	0.07 ± 0.04****	0.18 ± 0.15
22:5n-3	0.67 ± 0.30**	0.32 ± 0.11	0.05 ± 0.04	0.05 ± 0.53
22:6n-3	7.23 ± 2.92****	14.73 ± 2.86	0.21 ± 0.13****	0.79 ± 0.09

[a]Values are molar percentages (means ± 1 SD). An unpaired t-test was performed for all fatty acids, between peroxisomal patients and controls. Statistical significance worse than $P < 0.05$ is not given. * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$; **** = $P < 0.0001$. See Table 1 for abbreviation.

marked changes in other brain fatty acids, especially in EP in the peroxisomal-disorder patients. The proportion of 18:1n-9 is very much decreased in the Zellweger EP, its ratio to 18:0 being the opposite of normal. In general, in the brain of patients with disorders of peroxisomal biogenesis, EP are richer in saturated fatty acids (14:0, 16:0, 18:0) and markedly poorer in monounsaturated and PUFA than in the normal brain. This makes EP the only GPL in which 22:6n-3, 20:4n-6, and 22:4n-6 are all significantly decreased in absolute terms in the peroxisomal brain. In PS, on the other hand, the proportion of 20:4n-6, 22:4n-6, and 20:3n-6 is much higher in the Zellweger brain than in the normal brain. As in EP, the proportion of 18:1n-9 is lower than normal in PS. With the exception of PE, 18:1n-9 is lower than normal in all GPL. Such a decrease in a fatty acid particularly enriched in myelin correlates with the hypomyelination found in patients with peroxisomal biogenesis disorders.

A smaller but very significant change found in all GPL classes was the increase in linoleic acid (18:2n-6), a finding that could already be detected when studying the total fatty acid composition of the Zellweger brain (13). Other n-6 intermediates, such as 20:2n-6 and 20:3n-6, were also increased in brain GPL in the Zellweger patients. In contrast, 20:2n-9 (like the parent fatty acid 18:1n-9) was lower than normal in the brain of peroxisomal-disorder patients, although this change only reached statistical significance in PC.

DISCUSSION

This study complements previous findings and confirms that 22:6n-3 is dramatically decreased in all GPL fractions in the brain of patients with disorders of peroxisomal biogenesis. The differential fatty acid composition of the diacyl and alkenyl forms of brain ethanolamine GPL is described for the first time in peroxisomal biogenesis disorders. Clear differences are found in the main PUFA changes in these two phospholipid classes. While in the peroxisomal brain PE, the decrease in 22:6n-3 is accompanied by an increase in 20:4n-6, both PUFA are decreased in brain EP in the peroxisomal patients. However, in all four GPL, the decrease in 22:6n-3 is the most marked. In the normal brain, the PUFA composition is quite constant and 22:6n-3, in particular, is maintained within narrow limits (24,25). An important role of 22:6n-3 in signal transduction was lately demonstrated at the synapse (26). Thus, the disruption in phospholipid structure and function caused by such a specific 22:6n-3 deficiency could be deleterious in the Zellweger brain.

During normal brain development, EP are progressively enriched in adrenic and oleic acids (18), two fatty acids characteristic of myelin. In the ZS brain, however, EP are deficient in both 22:4n-6 and 18:1n-9. So, apart from their very low levels of 22:6n-3, brain plasmalogens in patients with ZS and its variants are also poor in other important unsaturated fatty acids. Thus, in the Zellweger brain, plasmalogens are not only quantitatively insufficient but probably also qualitatively inefficient, owing to their altered PUFA composition.

The possible relationship between 22:6n-3 and plasmalogens in the ZS is unknown. However, when correcting 22:6n-3 deficiency in patients with peroxisomal biogenesis disorders, significant increases in erythrocyte plasmalogens were consistently found (27). Treatment with DHA also produced an improvement in myelination in DHA-deficient peroxisomal patients (28), suggesting some possible relationship between 22:6n-3, on the one hand, and myelin and/or plasmalogens, on the other. Although EP have a high proportion of 22:6n-3 in the normal brain and a very low one in the Zellweger brain, the present study indicates that the 22:6n-3 deficiency present in ZS does not affect plasmalogens more than other GPL classes. On the contrary, EP are the only GPL in which the main long-chain PUFA (22:6n-3, 20:4n-6, and 22:4n-6) are quite proportionally reduced, a finding that can only be fully appreciated when considering the absolute values of these PUFA. Thus, whatever the effect that the 22:6n-3 deficiency may have on plasmalogen synthesis and myelinogenesis, it seems not to be simply compositional. Rather than acting as a mere substrate for plasmalogen synthesis, DHA might have some more basic role in myelinogenesis.

Another GPL rich in 22:6n-3 and related to myelination is PS. The changes in PS in the brain of patients with peroxisomal biogenesis disorders are most significant, and they are not limited to a reduction of all PUFA, as happens in EP. Indeed, although 22:6n-3 is very much decreased in PS, the other PUFA 20:4n-6, 20:3n-6, and 22:4n-6 are all increased. Such a disproportion between n-3 and n-6 PUFA in PS might have an unfavorable effect on myelination. In this context, it should be remembered that PS specifically activates galactosylceramidase (29), an enzyme related to cerebroside metabolism whose activity reaches maximal levels during myelinogenesis in the rat (30). Thus, it is tempting to hypothesize that normalizing the DHA levels in peroxisomal patients could partly correct PS composition and thus have a favorable effect on myelin turnover. In any case, the deficiency of DHA is so constant and involves so many structures, that it may play some role in the pathogenesis of peroxisomal disorders.

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Application of High-Resolution, Two-Dimensional ^{1}H and ^{13}C Nuclear Magnetic Resonance Techniques to the Characterization of Lipid Oxidation Products in Autoxidized Linoleoyl/Linolenoylglycerols

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ABSTRACT: Subjection of polyunsaturated fatty acid (PUFA)-rich culinary oils to standard frying episodes generates a range of lipid oxidation products (LOP), including saturated and α,β-unsaturated aldehydes which arise from the thermally induced fragmentation of conjugated hydroperoxydiene precursors. Since such LOP are damaging to human health, we have employed high-resolution, two-dimensional ^{1}H-^{1}H relayed coherence transfer, ^{1}H-^{1}H total correlation, ^{1}H-^{13}C heteronuclear multiple quantum correlation, and ^{1}H-^{1}H J-resolved nuclear magnetic resonance (NMR) spectroscopic techniques to further elucidate the molecular structures of these components present in (i) a model linoleoylglycerol compound (1,3-dilinolein) allowed to autoxidize at ambient temperature and (ii) PUFA-rich culinary oils subjected to repeated frying episodes. The above techniques readily facilitate the resolution of selected vinylic and aldehydic resonances of LOP which appear as complex overlapping patterns in conventional one-dimensional spectra, particularly when employed in combination with solvent-induced spectral shift modifications. Hence, much useful multicomponent information regarding the identity and/or classification of glycerol-bound conjugated hydroperoxydiene and hydroxydiene adducts, and saturated and α,β-unsaturated aldehydes, present in autoxidized PUFA matrices is provided by these NMR methods. Such molecular information is of much value to researchers investigating the deleterious health effects of LOP available in the diet.

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The autoxidation of polyunsaturated fatty acids (PUFA) is an autocatalytic, self-perpetuating chain reaction system that has been implicated in the pathogenesis of many human diseases, e.g., atherosclerosis and inflammatory joint diseases (1–3). PUFA are particularly susceptible to oxidative damage by virtue of the facile abstraction of one of their bis-allylic methylene group hydrogen atoms on exposure to light or radical species of sufficient reactivity, a process facilitated by the low bond dissociation energy of the methylene group C–H bonds. Subsequently, one major reaction pathway for the resulting resonance-stabilized carbon-centered pentadienyl lipid radical generated in this manner involves its interaction with molecular oxygen to produce a peroxyl radical which in turn can abstract a hydrogen atom from an adjacent PUFA to form a conjugated hydroperoxydiene (CHPD) and a further pentadienyl lipid radical species. In the absence of sufficient quantities of chain-terminating, lipid-soluble antioxidants such as vitamin E (α-tocopherol), the process is repeated many times. CHPD are subsequently degraded to a wide variety of secondary autoxidation products which include saturated and unsaturated aldehydes, di- and epoxyaldehydes, lactones, furans, ketones, oxo and hydroxy acids, and saturated and unsaturated hydrocarbons.

Thermal stressing of culinary oils according to standard frying/cooking practices (domestic or otherwise) gives rise to and/or perpetuates the radical-dependent autoxidation of PUFA therein. Indeed, a wide range of aldehydes arises from the thermally induced decomposition of CHPD *via* several processes, including the β-scission of pre-formed alkoxyl radicals. Such aldehydic fragments (*n*-alkanals, *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, 4-hydroxy-*trans*-2-alkenals, and malondialdehyde) have the capacity to exert a variety of toxicological effects in view of their extremely high reactivity with critical biomolecules [DNA base adducts, proteins such as the apolipoprotein B moiety of low density lipoprotein, peptides, free amino acids, endogenous thiols such as glutathione, etc. (4)]. Interaction of these aldehydes with DNA can give rise to genotoxic events and possibly cancer.

Our laboratory recently reported the detection and quantification of PUFA-derived autoxidation products (notably aldehydes and their conjugated hydroperoxy/hydroxydiene precursors) in culinary oils and fats by high-field proton (^{1}H) nuclear magnetic resonance (NMR) spectroscopy (5,6). Results obtained demonstrated that the thermally induced oxida-

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Abbreviations: CHPD, conjugated hydroperoxydiene; HMQC, heteronuclear multiple quantum coherence transfer; LOP, lipid oxidation products; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acids; RCT, relayed coherence transfer; RD, relaxation delay; TMS, tetramethylsilane; TOCSY, total correlation.

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tive stressing of PUFA-rich culinary oils produces much higher levels of cytoxic aldehydes than in corresponding samples of predominantly saturated fats (lard, drippings, etc.). The concentrations of aldehydes generated in culinary oils are critically dependent on PUFA content, the nature and capacity of the heating vessel employed, and the duration/conditions of heating and subsequent storage. More recently, we demonstrated that typical *trans*-2-alkenal compounds generated from the thermally induced autoxidation of PUFA are readily absorbed from the gut into the systemic circulation, metabolized (primarily *via* the addition of glutathione across their electrophilic carbon-carbon double bonds), and excreted in the urine as C-3 mercapturate conjugates in rats (7). Hence, such aldehydes have the ability to covalently modify lysine residues of the apolipoprotein B moiety of low density lipoprotein, rendering it susceptible to uptake by macrophages, a critical step in the production of foam cells *in vivo* (2). The hypothesis that dietary-derived lipid oxidation products (LOP) contribute significantly to the pathogenesis of atherosclerosis is further supported by reports that such agents can accelerate all three stages of the disease process, i.e., endothelial injury, accumulation of plaque, and thrombosis (8). Moreover, animal feeding studies showed that diets containing thermally-stressed [and therefore LOP-rich (5,6)] PUFA-laden culinary oils have a greater atherogenicity than those containing corresponding unheated oils (9). Indeed, Straprans *et al.* (10) recently examined the ability of oxidized dietary lipids to accelerate the development of atherosclerosis in New Zealand White rabbits and found that feeding with an oxidized lipid-rich diet gave rise to a 100% increase in fatty streak lesions in the aorta.

In view of the above observations, the detection and quantification of specific products arising from the autoxidation of culinary oil PUFA during standard frying practices are of paramount importance, and the multicomponent analytical ability of high-resolution ^{1}H NMR spectroscopy offers major advantages over alternative laboratory methods since it permits the rapid, simultaneous study of many primary and secondary LOP present in such samples (5,6). Indeed, the development of high-field NMR spectrometers with increased resolution, dynamic range, and sensitivity has given rise to rapid advances in the analysis of complex, multicomponent samples such as foodstuffs, human biofluids, and pharmaceutical formulations (11–14). The principles of this spectroscopic technique involve the absorption of energy from the radio frequency region of the electromagnetic spectrum to detect changes in the alignment of nuclear magnets during their exposure to a powerful external magnetic field. The absorption frequencies of such nuclei [for example, those of ubiquitous hydrogen nuclei (^{1}H)] present in the ^{1}H NMR spectrum of a particular chemical compound are critically dependent on their magnetic (and therefore, chemical) environment, and the appearance (multiplicity) of a resonance (signal) is influenced by neighboring ^{1}H nuclei in a well-characterized manner. Moreover, the intensity of each signal is directly proportional to the product of the number of magnetically equivalent nuclei in the structural/functional group responsible for it and the concentration of the molecule containing that group. Hence, much valuable molecular information regarding the nature and levels of a wide variety of compounds present in biological or biologically derived matrices can be obtained from high-field, high-resolution NMR investigations.

Although numerous prominent ^{1}H NMR signals ascribable to aldehydes and their CHPD precursors are observed in the less-crowded, higher-frequency regions of one-dimensional spectra acquired on such materials, there is a high degree of overlap between selected vinylic (isomeric CHPD and conjugated hydroxydienes, and α,β-unsaturated aldehydes) and aldehydic group proton resonances, even at operating frequencies as high as 600 MHz. However, the recent availability of spectrometers of operating frequencies ≥600 MHz has led to a synchronous increase in the development and application of both homonuclear and heteronuclear two-dimensional NMR methods in order to resolve the many overlapping multiplet resonances (many with higher-order coupling patterns) now detectable in very high field ^{1}H NMR spectra acquired on complex multicomponent samples. Indeed, the battery of such two-dimensional techniques employable facilitates the establishment of the molecular structures of specified organic compounds (15–19). Proton-proton and proton-carbon scalar couplings readily determine groups of resonances arising from individual components, and the unambiguous structural information derived therefrom is, in general, not obtainable from alternative analytical techniques.

Therefore, in this investigation we employed ^{1}H-^{1}H relayed coherence transfer (RCT), ^{1}H-^{1}H total correlation (TOCSY), ^{1}H-^{13}C heteronuclear multiple quantum coherence (HMQC), and ^{1}H-^{1}H *J*-resolved NMR spectroscopic techniques for the purpose of clarifying our spectral assignments and hence provide further useful information regarding the molecular structures of LOP present in thermally/oxidatively stressed culinary oils and model PUFA.

MATERIALS AND METHODS

Culinary oil and model linoleoylglycerol samples. Control (unheated) and repeatedly utilized culinary frying oil samples were kindly supplied by a fast-food retail establishment. Electronic integration of the bis-allylic-CH_2, ω-CH_3, and highly unsaturated fatty acid acyl chain terminal-CH_3 group ^{1}H resonances (δ = 2.76, 0.90, and 0.95 ppm, respectively) present in single-pulse 400 MHz proton NMR spectra of the control (unheated) sample acquired immediately after collection revealed that the material had a polyunsaturate content of 42 molar %, of which *ca.* one-quarter consisted of highly unsaturated fatty acids, i.e., those with ≥3 unconjugated double bonds (predominantly linolenoylglycerol adducts).

The above repeatedly used culinary oil samples were placed in glass vessels and stored in the dark at ambient temperature (22°C) for a period of 30 d prior to NMR analysis.

Sealed vials containing authentic samples of the model linoleoylglycerol species 1,3-dilinolein (Sigma-Aldrich

Chemical Co., Gillingham, Dorset, United Kingdom) were opened, and the compound therein allowed to autoxidize in the presence of atmospheric O_2 at an ambient temperature for a period of 2.0 h prior to subjecting it to NMR experiments.

Portions (300 g) of chipped potatoes and bacon were fried in 60- and 20-mL vol, respectively, of a commercially available sample of sunflower seed oil [PUFA content 62% (w/w)] in a 15-cm diameter shallow frying pan at 180°C for a period of 30 min in the presence of atmospheric O_2. A 60-mL volume of this culinary oil subjected to an identical thermal stressing episode in the absence of added food material, together with an unheated sample of this frying medium, served as controls. Subsequently, samples (1.00 mL) of oil were removed for ^{1}H NMR analysis.

Reagents. Tetramethylsilane (TMS) and the model autoxidized PUFA-derived aldehydes pentanal, hexanal, heptanal, octanal, *trans*-2-pentenal, *trans*-2-heptenal, *trans,trans*-nona-2-4-dienal, and *trans,trans*-deca-2,4-dienal were purchased from Sigma-Aldrich Chemical Co. Deuterated NMR solvents (C^2HCl_3 and ^2H_2O) were purchased from Goss Scientific Ltd. (Great Baddow, Essex, United Kingdom).

NMR measurements. NMR measurements on the above samples were conducted on Bruker AMX-600 (University of London Intercollegiate Research Services, Queen Mary and Westfield College Facility, University of London, United Kingdom) or Bruker AMX-400 (ULIRS; King's College Facility, University of London, United Kingdom) spectrometers, the former operating at frequencies of 600.13 (^{1}H) and 150.93 (^{13}C) MHz, the latter at 400.13 (^{1}H) MHz. ^{1}H-^{1}H *J*-resolved, ^{1}H-^{1}H TOCSY and ^{1}H-^{13}C transfer (HMQC) experiments were performed on the 600 MHz facility, while ^{1}H-^{1}H RCT experiments were performed on the 400 MHz spectrometer. The probe temperature was 298 K. Typically, 0.30-mL aliquots of culinary oil or 1,3-dilinolein samples were diluted to a volume of 0.90 mL with deuterated chloroform (C^2HCl_3) [or deuterated methanol (C^2H_3OH), where appropriate], which provided a field frequency lock, the solutions were thoroughly rotamixed and then were transferred to either 5- (one-dimensional ^{1}H, ^{13}C, ^{1}H-^{1}H RCT, ^{1}H-^{1}H TOCSY and ^{1}H-^{1}H *J*-resolved experiments) or 8-mm (^{1}H-^{13}C HMQC experiments) diameter NMR tubes.

Chemical shifts were referenced to internal TMS ($\delta = 0.00$ ppm) and/or the residual chloroform proton signal ($\delta = 7.262$ ppm), [and for samples in C^2H_3OH solution, the residual methanol methyl group signal ($\delta = 3.210$ ppm)]. For ^{13}C spectra recorded in C^2HCl_3 solution, chemical shifts were referenced to internal TMS ($\delta = 0.00$ ppm) and/or the chloroform carbon signal ($\delta = 77.00$ ppm). Resonances present in single-pulse ^{1}H NMR spectra of culinary oil and model PUFA compound samples were routinely assigned by a consideration of chemical shift values, coupling patterns, and coupling constants, where appropriate. The molecular nature of particular classes of aldehydes detectable was confirmed by performing standard additions of authentic, commercially available compounds [*n*-alkanals, *trans*-2-alkenals, and *trans,trans*-alka-2,4-dienals, the latter also containing trace (although ^{1}H NMR-detectable) levels of corresponding *cis,trans*-isomer impurities].

^{1}H-^{1}H RCT experiments employed the standard sequence 90°-t_1-90°-τ-180°-τ-90°-ACQ (20,21), 90° on the AMX-400 spectrometer being equivalent to 9.5 µs. Acquisition parameters were: 256 t_1 increments, each of magnitude 1,024 data points; spectral width 4,902 Hz in each dimension; 64 transients in each case; 4 dummy scans; relaxation delay (RD) 2.0 s; acquisition time 0.21 s. Unshifted sine-bell window functions were applied in each dimension, with zero-filling twice in f_1, prior to transformation of the matrix of size 1,024 × 1,024 data points. The delay period τ (0.05 s) was optimized for the appropriate vicinal coupling constants and rotameric equilibria existing in the expected constituent glyceride moieties.

^{1}H-^{1}H TOCSY spectra were recorded using the pulse sequence RD-(90°-t_1-spin lock)-ACQ (22), 90° on the AMX-600 spectrometer for that experiment being equivalent to 8.4 µs. The spin lock employed the MLEV-17 sequence (23), with a typical mixing time of 70 ms. For experiments where a restriction of carbon chain correlations was required, this mixing time was only 20 ms. Acquisition parameters were: 256 t_1 increments, each of magnitude 2,048 data points; spectral width 12,019 Hz in each dimension; 64 transients in each case; 4 dummy scans; RD 3 s; acquisition time 0.17 s. Sine-bell squared window functions shifted by π/2 (i.e., a cosine squared function) were applied in each dimension, with zero-filling twice in f_1, prior to transformation of the matrix of size 2,048 × 1,024 data points. The standard phase-sensitive (time-proportional phase incrementation) method (24) for optimal detection along the second dimension was utilized in such experiments.

^{1}H-^{1}H *J*-resolved experiments employed the standard sequence 90°-t_1/2-180°-t_1/2-ACQ (16), 90° on the AMX-600 spectrometer for that experiment being equivalent to 7.9 µs. Acquisition parameters were: 128 t_1 increments, each of magnitude 2,048 data points; spectral width 8,621 Hz in f_2 and 100 Hz in f_1; 64 transients in each case; 4 dummy scans; RD 2.0 s; acquisition time 0.24 s. Unshifted sine-bell window functions were applied in each dimension before transformation of the matrix of eventual size 2,048 × 256 data points.

The time-proportional phase incrementation phase-sensitive method was also utilized in the ^{1}H-^{13}C HMQC (25) experiments for optimal detection of ^{13}C frequencies in the second dimension, ^{1}H detection being achieved through the employment of a dedicated 8-mm inverse geometry probe in order to maximize sample information. Acquisition parameters were: 200 t_1 increments each of magnitude 2,048 data points; spectral width 13,228 Hz in f_2 and 63,381 Hz in f_1; 960 transients in each case; 4 dummy scans; RD 1.0 s. A delay equivalent to 3.7 ms (1/2 $^1J_{CH}$) was utilized for optimal refocusing of heteronuclear information, and composite pulse broadband ^{13}C decoupling (GARP) (26) was employed during the ^{1}H acquisition period. A sine-bell-squared function, shifted by π/2 (i.e., a cosine squared function) was applied in each dimension, with forward complex linear prediction from

200 to 400 data points and subsequent zero-filling to 1,024 data points in f_1, prior to transformation of the matrix of size 2,048 × 1,024 data points.

Confirmation of NMR assignments using computational methods. ^{1}H NMR assignments and appropriate chemical shift values and coupling constants of resonances of LOP possessing complex spin systems were confirmed with the HNMR prediction software (version 3.0) from the Advanced Chemistry Development (ACD Inc., Toronto, Canada) software suite. This software was employed to generate a theoretical spectrum which did not incorporate an allowance for solvent-mediated shielding effects, and the ^{1}H chemical shift and ^{1}H-^{1}H spin-spin coupling constant values generated therefrom were then adjusted to reflect those obtained from the experimental spectrum *via* a first-order analysis. The theoretical spectrum was then recalculated, a correct first-order analysis giving rise to an exact match between the experimentally and theoretically derived spectra. Calculations also made an allowance for the concentration of each LOP within sample mixtures. The ^{13}C facility of the ACD suite of programs (ACD Inc.), CNMR, was employed for predicting spectra of LOP expected to be contributing to ^{13}C spectra of autoxidized culinary oils and model PUFA compounds exhibiting a high degree of resonance overlap. For example, the ^{1}H simulations were based around an internal database containing data for >81,000 experimental ^{1}H spectra, the associated algorithms employing intramolecular interaction parameters for >300 structural fragments, the associated subalgorithms estimating initial values for unique structural fragments. Fragment lists are handled with a modified HOSE (Hierarchical Organization of Shells Expert)-code which allowed for explicit substituent charge and stereo bond conventions, optimizing to the maximal number of spheres. The subsequent quantum mechanical shielding calculations allowed for the number of these codes found in the internal database search, as well as the number of those sought. Calculational errors were determined as the standard deviations of the experimental values found within the database. Typically, predicted ^{1}H chemical shifts were accurate to within 0.05 ppm, and predicted coupling constants were accurate to within 0.2 Hz. Predicted ^{13}C chemical shifts were accurate to within 3 ppm.

RESULTS

Figure 1A shows the 0.5–6.8 ppm region of a 400 MHz ^{1}H-^{1}H RCT spectrum of a sample of the model linoleoylglycerol compound 1,3-dilinolein which was allowed to autoxidize in the presence of atmospheric oxygen as described in the Materials and Methods section, and subsequently dissolved in C^2HCl_3 solution. CHPD vinylic proton multiplets are present in the 5.4–6.6 ppm chemical shift range, whereas the 9- and/or 13-position proton bonded to the hydroperoxy group-bearing carbon has a chemical shift value of 4.38 and 4.30 ppm for the *cis,trans*- and *trans,trans*-isomers, respectively. Cross-peaks refer to protons **a–j** in Figure 2, the chemical shift values of which are listed in Table 1. Figure 1B shows the 0.40–6.60 ppm region of a 600 MHz ^{1}H-^{1}H TOCSY spectrum of the same sample, this time dissolved in C^2H_3OH solution. The selection of a comparatively short mixing time in this experiment yields a two-dimensional spectrum that is equivalent to that generated with the RCT method. Solvent-induced spectral shifts of the appropriate protons encountered in the spectrum served as a convenient means of improving the resolution of overlapping signals observed in spectra acquired in C^2HCl_3 solution. These shift changes are indicated in parentheses within the table. Such two-dimensional spectra afford the complete, unambiguous (and to our knowledge, the first) indexing of all vinylic, allylic, and neighboring aliphatic protons of both *cis,trans*- and *trans,trans*-isomers of 9- and 13-hydroperoxy-octadecadienoylglycerol species. Utilization of the RCT and short mixing time TOCSY methods in this case is particularly suited to the ^{1}H NMR study of these primary LOP derived from the peroxidation of PUFA, furnishing spin system information that is largely restricted to protons separated by five bonds, and thus generating less-crowded spectra. A predicted spectrum (ACD Inc.), based on spectral parameters derived from the *cis,trans*- and *trans,trans*-hydroperoxydiene assignments in the chloroform solution (corroborated with the further resolution of overlapped signal information in the methanol solution), was in excellent agreement with typical experimentally acquired spectra (Fig. 3).

A number of broad hydroperoxide-OO<u>H</u> group resonances were detectable in the 8.1–8.9 ppm chemical shift range of spectra acquired on autoxidized 1,3-dilinolein (data not shown). As expected, these signals were completely removed from spectra following treatment of samples with ^2H_2O (0.030 mL was added to C^2HCl_3 solutions, the mixture rotamixed, equilibrated at ambient temperature for 30 min, centrifuged, and the predominant, lower C^2HCl_3 phase removed for further ^{1}H NMR analysis).

A consideration of spectral parameters for the CHPD components derived from autoxidation of the above model dilinoleoylglycerol compound facilitated assignment of such LOP resonances present in two-dimensional ^{1}H-^{1}H NMR spectra acquired on thermally stressed culinary oil samples. Figure 4A exhibits the 5.0–10.0 ppm region of the 600 MHz ^{1}H-^{1}H TOCSY spectrum of a typical sample of repeatedly utilized frying oil obtained from a fast-food restaurant. The cross-peak signals indicated confirm the presence of the CHPD LOP components in this sample together with signals concordant with the spin systems expected for *trans*-2-alkenals and 13-hydroxy-*cis*-9,*trans*-11 and 9-hydroxy-*trans*-10,*cis*-12 hydroxydiene derivatives, their ^{1}H chemical shift values being consistent with previously published data (27). The confirmation of the presence of such aldehydes is of particular clinical importance since they, rather than the conjugated hydroperoxydienes, are absorbed from the gut into the systemic circulation (*in vivo*) (7), where they have the capacity to exert a range of deleterious health effects. Other correlations involving the aldehydic proton region highlighted the presence of two further alkenal species represented by spin systems with chemical shift values of 9.57, 6.83 and 6.35 ppm, and 9.55,

FIG. 1. (A) 0.50–6.80 ppm region of a 400 MHz ^{1}H-^{1}H relayed coherence transfer (RCT) spectrum of an autoxidized sample of 1,3-dilinolein in C^2HCl_3 solution, and (B) 0.40–6.60 ppm region of a 600 MHz ^{1}H-^{1}H total correlation (TOCSY) spectrum of the same sample in C^2H_3OH solution, the latter acquired with a short mixing time (20 ms). Typical spectra are shown. *Cis,trans-* and *trans,trans*-conjugated hydroperoxydiene [or more specifically 9-hydroperoxy-*trans*-10,*cis*-12- (and, in an analogous manner 13-hydroperoxy-*cis*-9,*trans*-11-) and 9-hydroperoxy-*trans*-10,*trans*-12- (and, in an analogous manner 13-hydroperoxy-*trans*-9,*trans*-11-) octadecadienoylglycerol isomers] cross-peak assignments are indicated as connectivities involving protons **a–j** in the accompanying molecular structures.

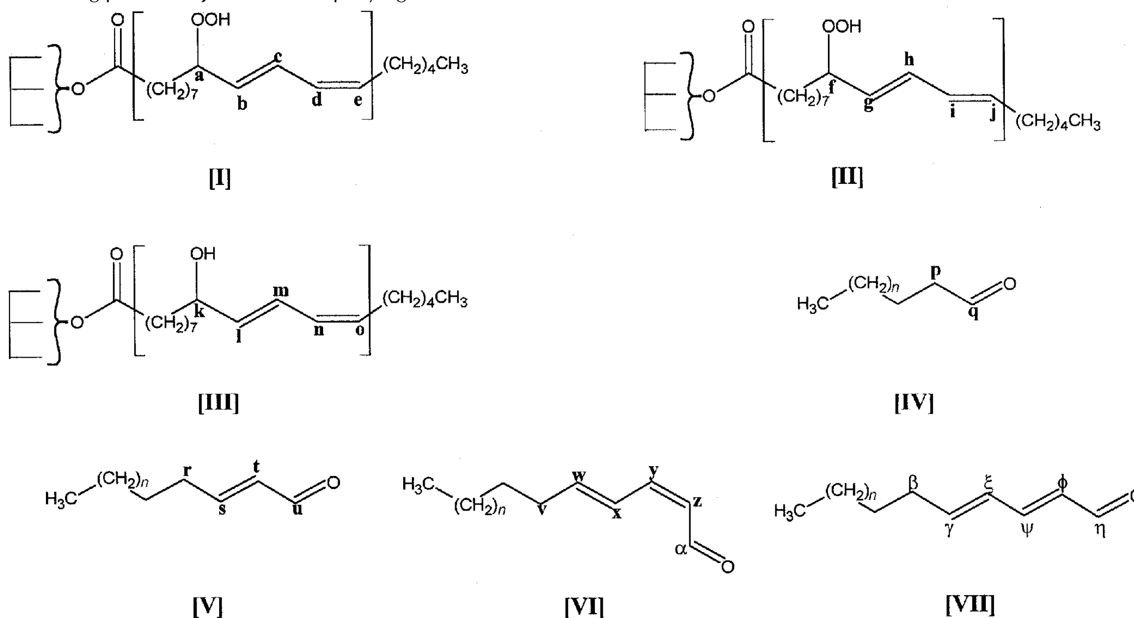


FIG. 2. Structural units of lipid oxidation products (LOP) detectable in peroxidized polyunsaturated fatty acids (PUFA) by high-resolution nuclear magnetic resonance (NMR) spectroscopy. The lower case letter labels correspond to ^{1}H (and/or) ^{13}C nuclei distinguishable by the two-dimensional techniques employed in this investigation. Key: **[I]**, 9-hydroperoxy-*trans*-10,*cis*-12-octadecadienoylglycerol adduct; **[II]**, 9-hydroperoxy-*trans*-10,*trans*-12-octadecadienoylglycerol adduct; **[III]**, 9-hydroxy-*trans*-10,*cis*-12-octadecadienoylglycerol adduct; **[IV]**, *n*-alkanals; **[V]**, *trans*-2-alkenals; **[VI]**, *cis,trans*-alka-2,4-dienals; **[VII]**, *trans,trans*-alka-2,4-dienals.

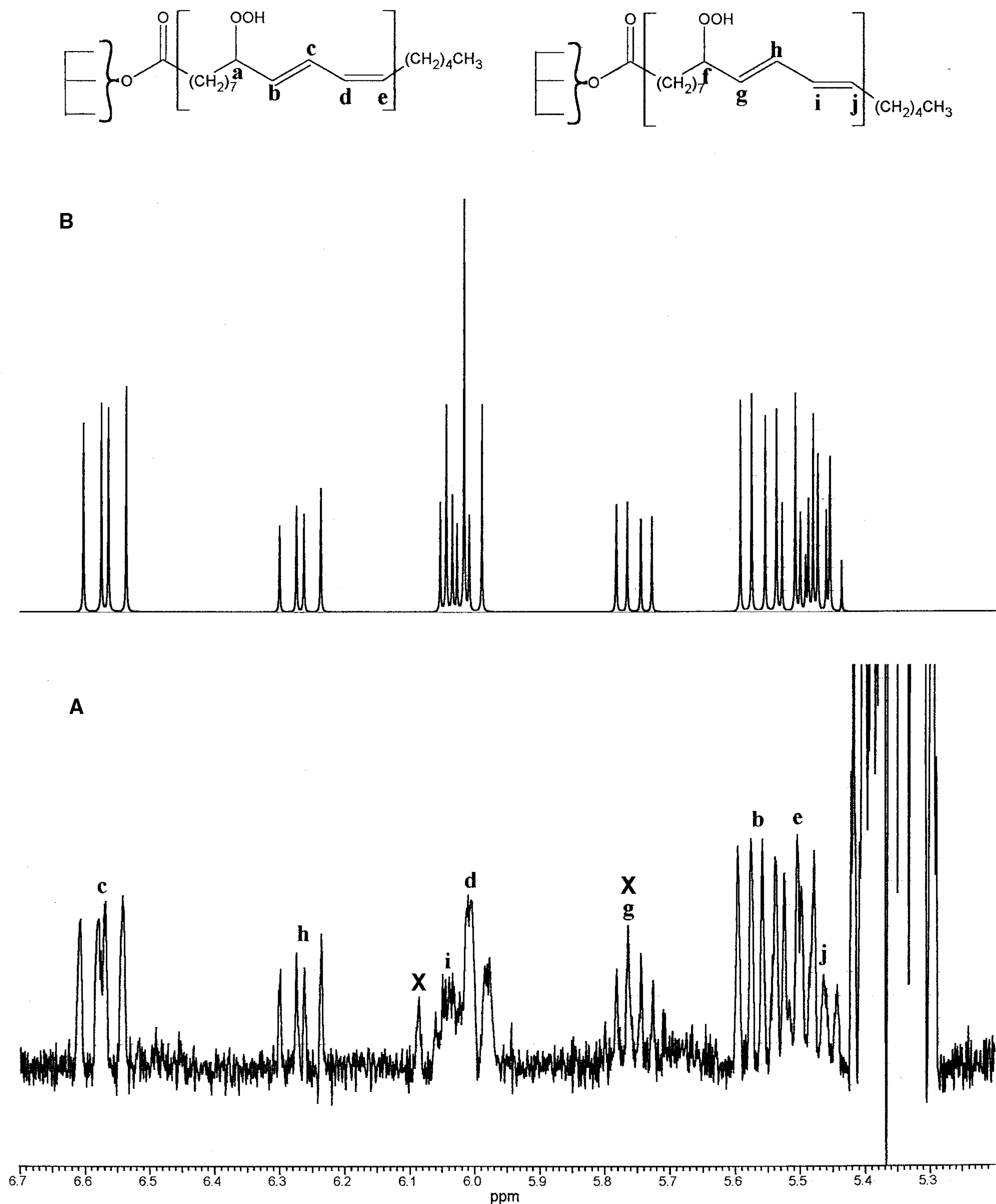


FIG. 3. Expanded 5.20–6.70 ppm regions of (A) an experimental 400 MHz one-dimensional ^{1}H NMR spectrum of an autoxidized sample of 1,3-dilinolein (in $C^{2}HCl_3$ solution), and (B) a corresponding computer-simulated spectrum generated using the software suite described in the Materials and Methods section, a system which employed spectral parameters obtained from all LOP assignments. A typical spectrum/simulation is shown. Abbreviations: ^{1}H nucleus labels of isomeric conjugated hydroperoxydienes (CHPD) correspond to those given in the molecular structures depicted above the spectra. Signals labeled as "X" indicate spectral artifacts. See Figure 2 for other abbreviations.

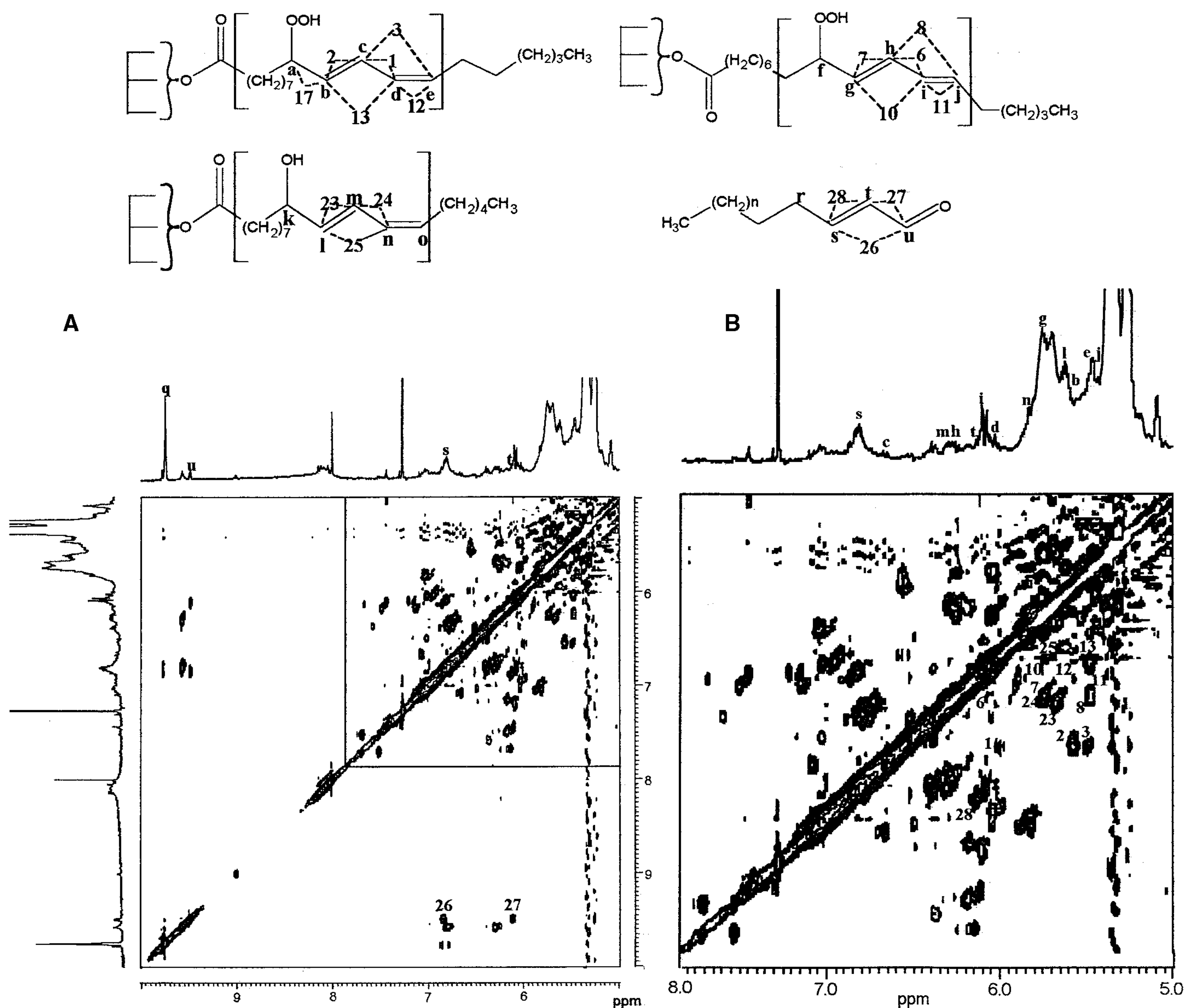
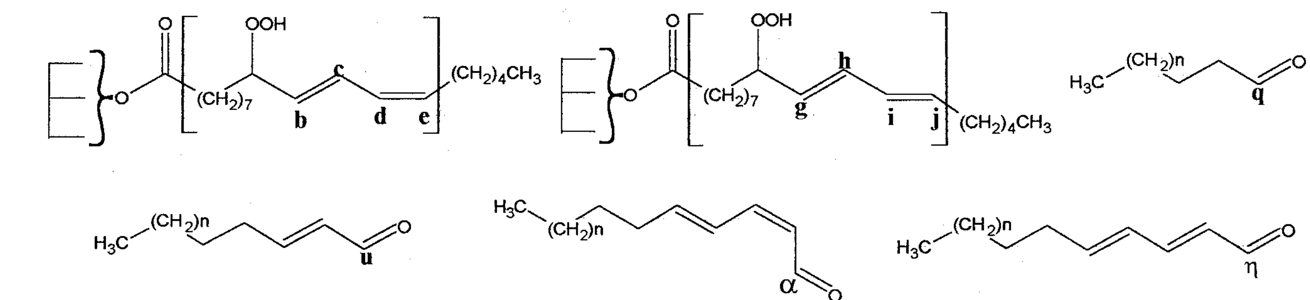


FIG. 4. (A) 5.0–10.0 ppm region of a 600 MHz ^{1}H-^{1}H TOCSY spectrum of a sample of repeatedly used culinary frying oil obtained from a fast-food retail outlet in C^2HCl_3 solution, acquired with a short mixing time (20 ms). The expanded 5.0–7.9 ppm region of this spectrum is shown in (B). A typical spectrum is shown. Abbreviations: ^{1}H nucleus labels of isomeric CHPD, 9-hydroxy-*trans*-10, *cis*-12-octadecadienoylglycerol adducts, and saturated and α,β-unsaturated aldehydes correspond to labeled protons in the accompanying molecular structures. See Figures 1 and 3 for abbreviations.

6.80 and 6.25 ppm. Moreover, the expanded 5.0–8.0 ppm region of this spectrum (Fig. 4B) revealed a complex range of additional overlapping signals, none of which corresponded to those expected for other known LOP such as *trans*-4,5-epoxyhept-*trans*-2-enal (28), various epoxides, hydroxyepoxides, 5- and 6-membered hydroperoxyepidioxides of linolenoylglycerol species [e.g., 5.40, 5.62, 6.00, and 6.64 ppm for the conjugated diene vinylic protons and characteristic 4.45 and 4.75 ppm multiplets for the methine group protons of the epidioxide ring of *cis,trans*-9- and 16-hydroperoxy epidioxides (29)], and conjugated oxodienes (30–32). This observation indicates that such LOP (important precursors of volatile components which reflect the oxidative degradation of linolenoylglycerols) are readily fragmented to peroxidation end products when exposed to a source of oxidative stress (i.e., prolonged thermal stressing episodes). Further experiments to establish the identities of these resonances are currently in progress.

Figure 5 exhibits complete and expanded regions of the ^{1}H-^{13}C HMQC spectrum of a further repeatedly utilized culinary oil sample (also obtained from a fast-food retail outlet), acquired in C^2HCl_3 solution, the full spectrum in (Fig. 5A) highlighting signals expected for glycerol backbones and their esterified fatty acid units. Consideration of the nature of the vinylic carbon ^{13}C signals and the average chain length as deduced from the single-pulse ^{1}H spectrum confirmed that



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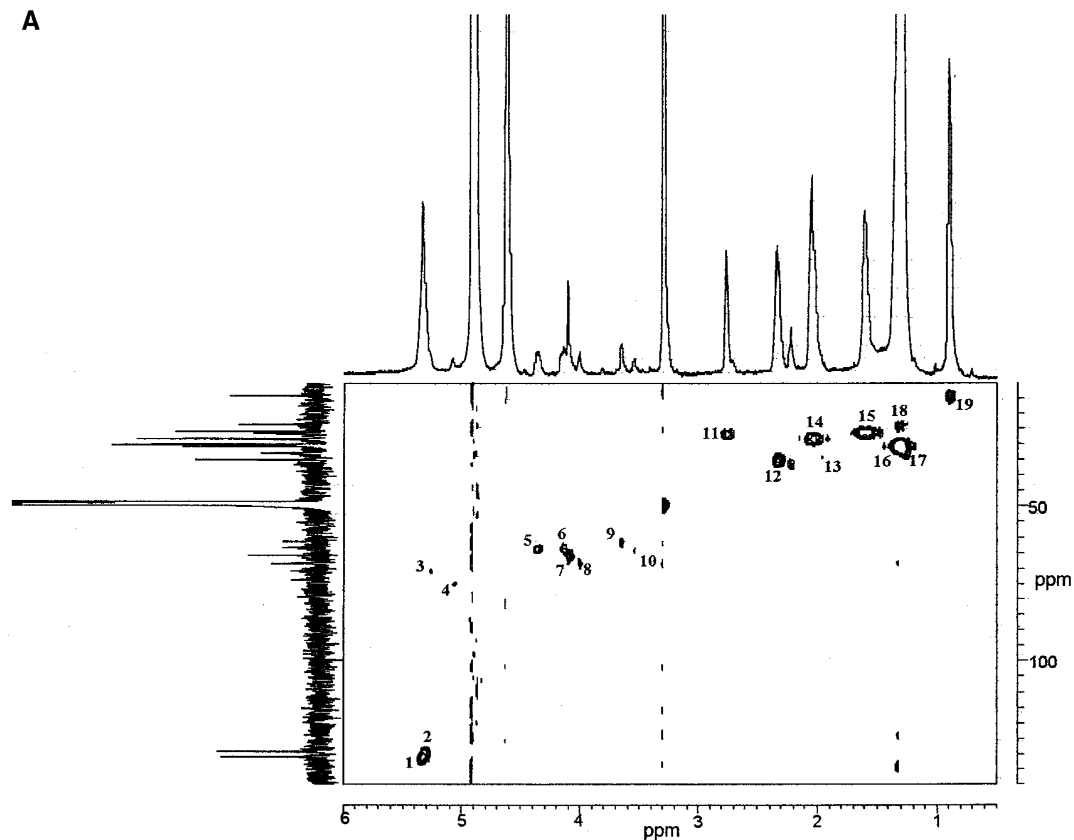

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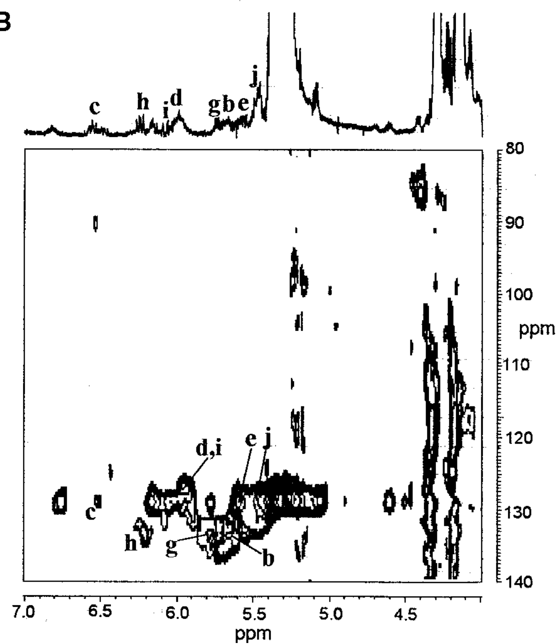

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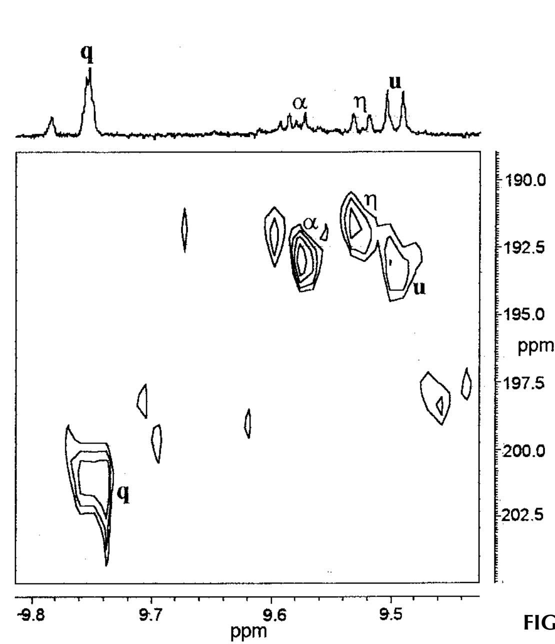


FIG. 5

glycerol-bound linoleate was the major PUFA constituent present in this material. Resolution of all glyceride acyl signal correlations provides information concerning the nature and distribution of glyceride esterification. The ^{1}H-^{13}C chemical shift correlations observed for 1-, 1,2-, 1,3- and 1,2,3-substituted glycerol backbone units were verified utilizing either previously reported data (33,34), or from predicitive software (ACD Inc.). Knowledge of the distribution of these species is of much importance since the nature of glyceride substitution was shown to significantly influence the rate of PUFA autoxidation. Indeed, dilenilenoyl-linoleoylglycerols are slightly less resistant to oxidation when linolenate is in the 1,2-rather than the 1,3-triacylglycerol positions, and dilinoleoyl-linolenoylglycerols are less resistant to oxidation when linoleate is in the 1,3- rather than the 1,2-triacylglycerol positions (35).

Glycerol, an end-product arising from the hydrolysis of triacylglycerols (a process occurring during standard frying practices), was undetectable in the HMQC spectra acquired. This observation is presumably a consequence of its volatilization during thermal stressing episodes since its boiling point (182°C) (36) is very similar to the temperature recommended for this process (180°C).

Comparisons of the one-dimensional ^{1}H NMR spectra of repeatedly-utilized frying oils with that of a corresponding control (unheated) material provided evidence consistent with the thermally-induced autoxidation of glycerol-bound PUFA therein. Indeed, the ratios of the intensities of the *bis*-allylic-$C\underline{H}_2$ group and the total unsaturated fatty acid vinylic- $-C\underline{H}=C\underline{H}-$ proton resonances (δ = 2.76 and 5.38 ppm, respectively) to that of the acyl chain terminal-$C\underline{H}_3$ group protons (*t*, δ = 0.90 ppm) were markedly lower in repeatedly-employed samples. Moreover, the low-field-shifted highly unsaturated fatty acid acyl chain terminal-$C\underline{H}_3$ group triplet resonance located at 0.95 ppm was found to almost completely disappear from spectra following subjection of the culinary oil to repeated frying episodes (together with perpetuation of the autoxidation process *via* storage in the manner described in the Materials and Methods section), an observation concordant with the fact that linolelenoylglycerols autoxidize at a faster rate than linoleoylglycerols (35).

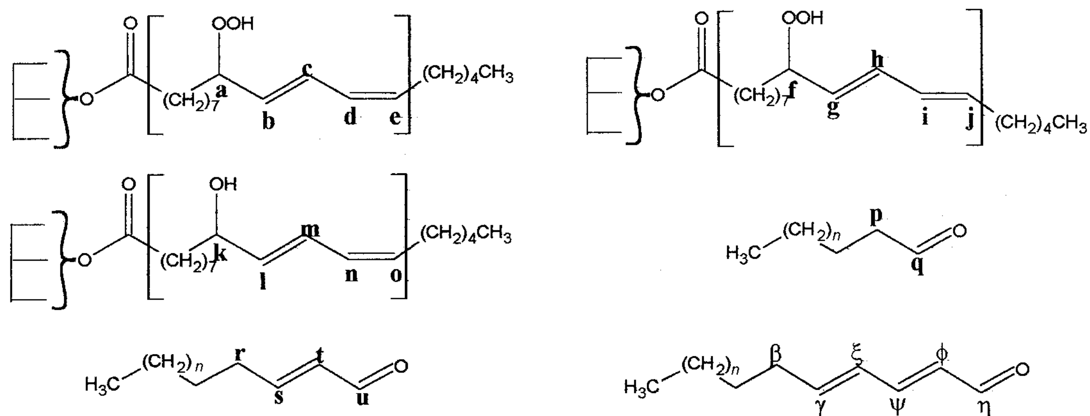
Figure 5B in particular demonstrates the ability of the HMQC method to resolve overlapping signals, the use of the 8-mm ^{13}C probe in this case undoubtedly facilitating the detection and identification of many low-level LOP components. Corroboration of previous ^{1}H assignments (5) with the appropriate ^{13}C NMR data is also important for the purpose

TABLE 1
^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR) Assignments for the Lipid Oxidation Products (LOP) in C^2HCl_3 Solution[a]

LOP nucleus positional code	^{1}H chemical shift (ppm)	^{13}C chemical shift (ppm)
a	4.38 (4.15)	86.0
b	5.57 (5.61)	132.5
c	6.57 (6.49)	131.0
d	6.02 (5.97)	130.0
e	5.48 (5.43)	132.0
f	4.30 (4.02)	87.5
g	5.76 (5.68)	132.5
h	6.27 (6.14)	133.0
I	6.05 (6.03)	130.0
j	5.46 (5.52)	131.0
k	4.09	
l	5.64	
m	6.28	
n	5.83	
o	5.30	
p	2.44	
q	9.74	201.0
r	2.33	
s	6.85	
t	6.10	
u	9.48	193.2
v	2.25	
w	6.20	
x	6.30	
y	7.39	
z	6.15	
α	9.63	192.8
β	2.25	
γ	6.20	
ξ	6.30	
ψ	7.07	
ϕ	6.04	
η	9.52	191.5

[a]Where appropriate, corresponding ^{1}H assignments in C^2H_3OH solution are indicated in parentheses. The LOP nucleus (nuclei) positional labels correspond to those indicated in Figure 2. ^{13}C assignments for 13- and/or 9-hydroxy-substituted octadecadienoylglycerol adducts (the *cis*-9,*trans*-11 and *trans*-10,*cis*-12 isomers, respectively) are absent since the very low levels of this LOP present in autoxidized model polyunsaturated fatty acids (PUFA)/thermally stressed PUFA-containing culinary oil samples preclude their detection (i.e., their ^{13}C features are unreceptive to NMR analysis under such conditions). Vinylic and bulk chain $-CH_2-$ group ^{13}C resonance assignments for each class of aldehyde were unobtainable in view of a high degree of overlap with those of their primary LOP precursors and PUFA substrates, together with further glycerol-bound fatty acids.

FIG. 5. (See previous page) (A) Complete 600 MHz ^{1}H-^{13}C heteronuclear multiple quantum coherence transfer (HMQC) spectrum of a sample of repeatedly-used culinary frying oil obtained from a fast-food restaurant outlet. In (B), the spectral display was pre-set to emphasize ^{1}H-^{13}C correlations of lower intensity (i.e., those of LOP). (C) Expanded aldehydic group region of the above ^{1}H-^{13}C HMQC spectrum showing clear correlations between the ^{1}H and ^{13}C nuclei of the –CHO groups of saturated and α,β-unsaturated aldehydes. A typical spectrum is shown. Abbreviations for (A) (C_A, C_B, and C_C refer to substituted/unsubstituted glycerol carbon centers as indicated, and the indices 1,2 or 1,2,3 indicate substitution mode, i.e., 1,2 represents a diacylglycerol with fatty acids esterified at the 1- and 2-glycerol backbone positions, and 1,2,3 represents a triacylglycerol): 1, olefinic –CH = CH–; 2, as 1; 3, C_B 1,2,3; 4, C_B 1,2; 5, C_A/C_C 1,2,3; 6, as 5; 7, C_A 1,2; 8, C_A/C_C 1,3; 9, C_C 1,2; 10, C_A/C_C 1/3 (i.e., 1 or 3 positions unsubstituted); 11, bis-allylic-$C\underline{H}_2$ groups of PUFA; 12, fatty acid C-2; 13, fatty acid C-16; 14, allylic-$C\underline{H}_2$ (i.e., $-CH = CH-C\underline{H}_2$) group of unsaturated fatty acids ; 15, fatty acid C-3; 16, fatty acid bulk chain C-4 to C-15 $-(C\underline{H}_2)_n-$ groups ; 17, as 16; 18, fatty acid C-17; 19, fatty acid terminal $-CH_3$. ^{1}H-^{13}C correlated signal labels in (B) and (C) correspond to those indicated on the molecular structures displayed above the spectra. See Figure 2 for abbreviations.



A

-OOH

q η u ψ s c ξ m h γ t i ϕ d n l b g e j o

B

q η u -OOH ψ s c ξ m h γ t i ϕ d n l b g e j

C

q η u -OOH ψ s c ξ m h γ t ϕ i d n g l b e j

-10 0 Hz 10

FIG. 6 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 ppm FIG. 6

of confirming the identity of particular LOP known to exert adverse toxicological (e.g., proatherogenic and proinflammatory) properties (1–3,37). The ^{13}C assignments and related HMQC correlated signals were confirmed with the aid of known chemical shifts for the appropriate model compounds (33,34) and predicted spectra (ACD Inc.). The conjugated hydroperoxydiene unsaturated carbon ^{13}C signals appear in the virtually exclusive 130–140 ppm region, their chemical shift values being in good agreement with those obtained in previous investigations (38). Correlated signals found in the region around 200 ppm serve to offer a useful confirmation of our earlier ^{1}H NMR assignments of different classes of aldehyde, i.e., *n*-alkanals, *trans*-2-alkenals, *cis,trans*- and *trans,trans*-alka-2,4-dienals (5) [the identity of each of these linked signals was verified by a combination of simulated (ACD Inc.) and reference (36) data]. None of the ^{1}H-^{13}C HMQC spectra acquired demonstrated the presence of the epoxide, hydroxyepoxide, hydroperoxyepidioxide, and oxodiene species described above, although there remains some unavoidable coincidence of ^{13}C resonances of similar frequencies.

^{1}H-^{1}H *J*-resolved NMR spectroscopy is a further important technique which can be employed for the purpose of precisely determining the chemical shift values of overlapping ^{1}H resonances (ideally in the absence of second-order coupling effects) through utilization of the "skyline" f_2 projection, and Figure 6 shows the expanded 5.0–10.0 ppm region of the 600 MHz ^{1}H-^{1}H *J*-resolved spectrum (f_2 "skyline" projection and corresponding contour plot of the two-dimensional matrix) of an additional repeatedly used culinary frying oil sample (the contour plot depicts the separation of signal splittings into the second dimension). The corresponding one-dimensional spectrum, also exhibited in Figure 6, illustrates the generation of "homonuclear proton-decoupled" signals in the f_2 "skyline" projection. With the exception of resonances ascribable to *cis,trans*-alka-2,4-dienals, all LOP signals were readily visible. After making appropriate allowances for the differing concentrations of each LOP, the computer-predicted spectrum of this particular mixture (Fig. 6A) has a high level of agreement with the experimentally obtained one-dimensional spectrum, demonstrating the applicability of the spectral simulation program employed as an aid to the analysis of complex, multicomponent LOP matrices.

The NMR techniques outlined above are, of course, also readily applicable to the analysis of LOP present in culinary oils employed in further food preparation methods, e.g., domestic frying episodes. Figure 7A and B display the 9.00–10.00 ppm aldehydic group proton regions of one-dimensional ^{1}H NMR spectra acquired on a sample of sunflower seed oil [polyunsaturate and monounsaturate contents 63 and 12% (w/w), respectively] both prior and subsequent, respectively, to heating at a temperature of 180°C for a period of 30 min. Corresponding spectra acquired on samples of this culinary oil utilized for the purpose of frying potato chips and bacon (30 min at 180°C) are illustrated in Figure 7C and D, respectively. Clearly, resonances ascribable to the aldehydic group protons of *trans*-2-alkenals, *trans,trans*- and *cis,trans*-alka-2,4-dienals, and *n*-alkanals are clearly visible in these spectra, demonstrating the ready generation of these cytotoxic LOP from the autoxidative deterioration of PUFA during standard frying practices. The ^{1}H-^{1}H TOCSY spectrum of the oil sample employed for frying potato chips is exhibited in Figure 7E. This two-dimensional spectrum displays clear connectivities between (i) ^{1}H resonances of the conjugated diene systems of CHPDs, (ii) the aldehydic, olefinic, and local bulk alkyl chain (–CH_2–) group resonances of α,β-unsaturated aldehydes, and (iii) the aldehydic and adjacent bulk *n*-alkyl chain (–CH_2–) group signals.

DISCUSSION

Employment of a combination of two-dimensional NMR spectroscopic methods for the analysis of products arising from the oxidative degradation of glycerol-bound PUFA in culinary oils (thermally stressed or otherwise) provides much valuable molecular information which is an essential requirement for future evaluations of the toxicological hazards putatively associated with the dietary consumption of LOP. Moreover, the identification of specific LOP also highlights particular reaction pathways which can be related to the degree of PUFA unsaturation, together with the nature of glyceride substitution.

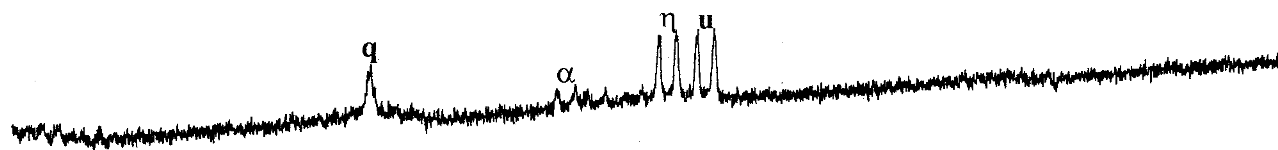
The RCT and TOCSY spectroscopic techniques permit the transfer of magnetization from an ^{1}H nucleus [or magnetically equivalent group (2 or 3) of such nuclei] bonded to one specific carbon atom (C_1), (the former through a relayed spin), to one or more magnetically distinct nuclei located two or more carbon positions further along a molecular chain (C_3, C_4, C_5 position, etc.), i.e., the latter nucleus/nuclei is/are not directly coupled to the C_1-bearing ^{1}H nucleus. Hence, the technique is extremely useful for the identification of LOP, particularly isomeric CHPD which have complex overlapping vinylic resonance patterns in their one-dimensional ^{1}H NMR spectra.

Direct observation of ^{13}C nuclei is poorly sensitive in view of its low natural abundance (1.11%), especially for relatively low concentrations of components such as LOP present in

FIG. 6. (See previous page) (A) Computer-simulated one-dimensional spectrum generated by the software suite described in the Materials and Methods section, utilizing spectral parameters derived from the assignment of all the detected lipid oxidation products and making allowance for their relative concentrations within the mixture. (B) One-dimensional ^{1}H NMR spectrum of a repeatedly utilized culinary frying oil sample collected from a fast-food restaurant, and (C) corresponding "skyline" f_2 projection and two-dimensional matrix contour plot of a 600 MHz ^{1}H-^{1}H *J*-resolved spectrum. Typical spectra are shown. Abbreviations: ^{1}H nucleus labels of isomeric CHPD, conjugated hydroxydienes, and saturated and α,β-unsaturated aldehydes correspond to labeled protons in the accompanying molecular structures. The label –OO<u>H</u> represents hydroperoxide-OO<u>H</u> group proton resonances. See Figures 2 and 3 for abbreviatons.

D

C



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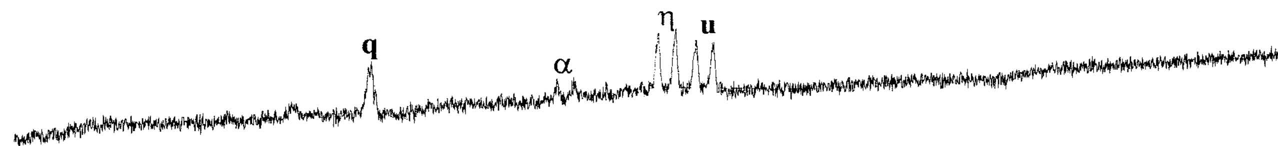

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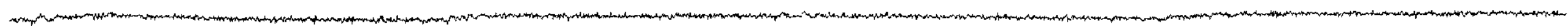

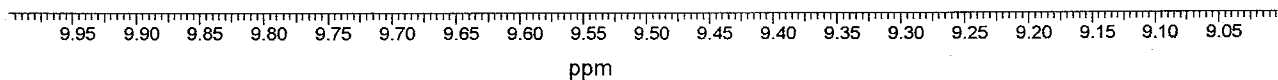

FIG. 7. Expanded 9.0–10.0 ppm regions of experimental 600 MHz one-dimensional ^{1}H NMR spectra of C^2HCl_3 solutions of (A) control (unheated) sunflower seed oil, (B) the above sample heated at a temperature of 180°C for a period of 30 min, (C) a sample of this culinary oil that had been utilized for the purpose of frying potato chips at 180°C for a period of 30 min (the Materials and Methods section) and (D) as (C), but employed for the purpose of frying bacon under the conditions outlined in the Materials and Methods section (30 min at 180°C), (E) 5.0–10.0 ppm region of a corresponding 600 MHz ^{1}H-^{1}H TOCSY spectrum of the sunflower seed oil sample giving rise to the one-dimensional spectrum shown in (C) above. Typical spectra are shown. Abbreviations: ^{1}H nucleus labels of saturated and α,β-unsaturated aldehydes correspond to labeled protons in the molecular structures depicted above the spectra. See Figure 2 for abbreviation.

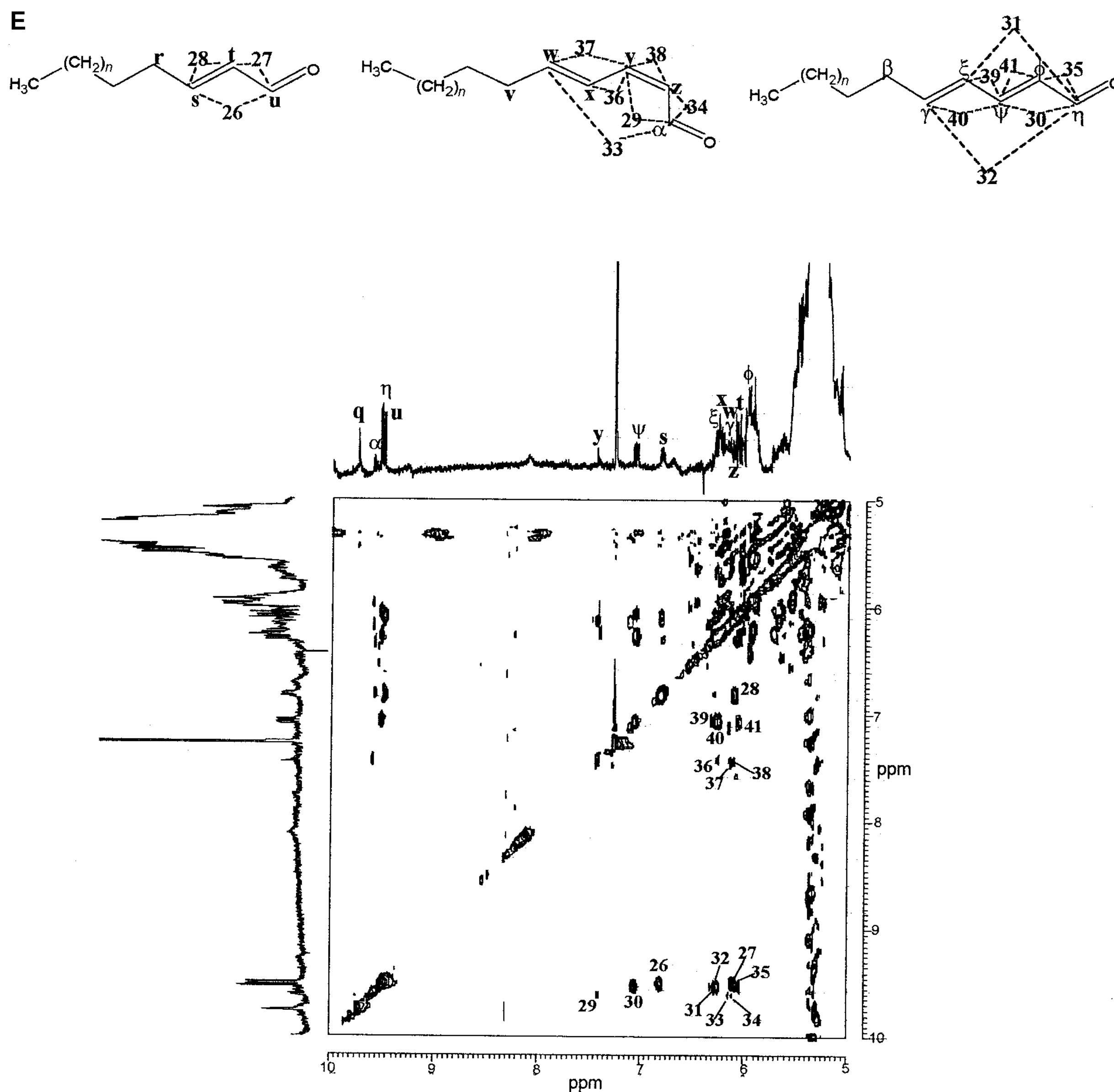

FIG. 7. (continued)

peroxidized triacylglycerol mixtures (the one-dimensional ^{13}C spectrum depicted as the f_1 projection of the HMQC profile displayed in Figure 5A required *ca.* 5,000 scans). However, the recent development of inverse-geometry probes and relevant accompanying pulse sequences has largely overcome this problem since HMQC spectroscopy and related techniques offer a substantial gain in sensitivity over conventional, one-dimensional spectroscopy. Hence, the HMQC technique is a particularly attractive one which readily facilitates investigations of the structure and dynamics of LOP.

Furthermore, application of the ^{1}H-^{1}H *J*-resolved spectroscopic method also serves to clarify the molecular nature of LOP present in autoxidized PUFA mixtures. Indeed, the technique is especially advantageous for the purpose of making resonance assignments in crowded spectral regions and enables the accurate determination of chemical shift and coupling constant values.

A high percentage of humans are frequently and continually exposed to LOP in the diet (arising, for example, from the shallow- or deep-frying of PUFA-rich culinary oils), and the possibility that regular consumption of such agents may be deleterious to human health has recently attracted much

interest. The short-term feeding of heated and/or oxidized oils and fats to experimental animals can give rise to loss of appetite, diarrhea, cardiomyopathy, hepatomegaly, hemolytic anemia, growth retardation, and an apparent accumulation of peroxides in adipose tissue (highly oxidized cod liver oil) (39). Moreover, cellular damage in various organs, elevated liver and kidney weights, and a modified fatty acid composition of tissue lipids in rats were shown to result from the short-term feeding of oils and fats subjected to the heat and oxidation associated with normal usage (40,41), and one long-term study revealed that the consumption of mildly oxidized culinary oils by rats throughout their lifespans produced an increased frequency of cardiac fibrotic and hepatic bile-duct lesions (42). Further toxicological investigations concerning thermally stressed oils and fats focused on their mutagenic properties (43,44). However, the precise molecular nature of mutagens formed during frying processes and their metabolic fate in humans were not considered by these researchers.

Although there is much epidemiological and experimental evidence available regarding the influence of the dietary consumption of saturated or PUFA to the development and/or progression of human diseases, the precise autoxidation status of the culinary oils and fats ingested (i.e., the structure and concentrations of CHPD, conjugated hydroxydienes, aldehydes, etc., therein) has not hitherto been sufficiently considered, and the two-dimensional NMR techniques employed here serve to provide a multicomponent profile of PUFA-derived autoxidation products, information not readily attainable from alternative analytical methods which generally require much prior information regarding the molecular nature of such LOP.

CHPD are acutely toxic to rodents when administered systemically, but such effects are much less severe when given orally. Indeed, a single intravenous (i.v.) dose of methyl linoleate hydroperoxide administered to rats gave rise to a high mortality within 24 h (animals dying from severe lung damage), whereas an oral dose of *ca.* 200 mg/kg was without effect (45). Holman and Greenberg (46) demonstrated that although the intravenous LD_{50} value for ethyl linoleate hydroperoxide in mice was 12 mg/kg, similar dose levels administered orally were nonlethal, an observation supported by the subsequent investigations of Olcott and Dolev (47). Furthermore, Bergen and Draper (48) obtained indirect evidence indicating that the lack of effect of orally administered CHPD is explicable by their failure to be absorbed across the gastric or intestinal epithelium.

Notwithstanding, CHPD have the capacity to exert damage to the gastrointestinal epithelium, and Jayaraj *et al.* (49) showed that oral administration of the aldehydic lipid oxidation end product 4-hydroxy-*trans*-2-nonenal to rats at a dose level of only 2.6×10^{-7} mol·dm^{-3}, a concentration similar to that found in healthy human blood plasma, induced peptic ulcers.

As noted in the introduction, aldehydic LOP have been implicated in the induction, development, and progression of atherosclerosis (2) (and therefore its associated pathological sequelae such as ischemic heart disease and peripheral vascular disease), and additional investigations established that these cytotoxic agents exert genotoxicological (50) and proinflammatory (51) actions.

The above two-dimensional NMR techniques are also applicable to the analysis of appropriate lipidic extracts of biofluids and tissues obtained from patients with clinical conditions in which the *in vivo* oxidation of PUFA (and/or the dietary ingestion of chemically reactive aldehydes) has been implicated (1–3,7–10), and experiments to investigate this are currently in progress.

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COMMUNICATION

Recognition and Quantitation of *cis*-Vaccenic and Eicosenoic Fatty Acids in Olive Oils by ^{13}C Nuclear Magnetic Resonance Spectroscopy

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ABSTRACT: The presence of 11-*cis* monoenoic fatty acids was detected in olive oil samples by means of ^{13}C nuclear magnetic resonance spectroscopy, and the positional isomery on the glycerol backbone was derived. The 11-*cis* vaccenic and eicosenoic fatty acid resonances were recognized and the amounts of the fatty acids quantified. For comparison purposes, a quantitative analysis was also made by gas chromatography.

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Numerous investigation methods, based mainly on chromatographic and spectroscopic techniques, were developed to characterize the molecular composition of the vegetable oils. Among these, the high-resolution ^{1}H and ^{13}C nuclear magnetic resonance (NMR) spectroscopies are giving detailed insight on both quantitative and qualitative aspects of the oil composition (1–4); particularly the simultaneous presence of mono-, di- and triacylglycerols, together with the fatty acid position on the glycerol backbone, was easily determined (5–6).

Among the minor components of the fatty acids, the *cis*-vaccenic (11*c*-18:1) acid and eicosenoic (11*c*-20:1) acid were identified in plant sources and in some vegetable oils (4,7–11), while recently their occurrence in extra-virgin olive oils was also detected by gas chromatography (GC) (2,12).

Here we report the results of a preliminary ^{13}C NMR investigation on a number of olive oil samples from different Mediterranean regions which show that *cis*-11 vaccenic and eicosenoic fatty acids can be identified *via* their carbonyl and olefinic resonances.

EXPERIMENTAL PROCEDURES

Solutions in $CDCl_3$ (50% vol/vol) of the olive oil samples were prepared and then filtered. The ^{13}C NMR spectra were run on a Varian VXR-300 spectrometer (Palo Alto, CA), operating at the frequency of 75.4 MHz, equipped with a 10-mm broad-band probe using the NOE (nuclear Overhauser effect)-suppressed, inverse-gated proton decoupled technique. The free induction decay of each oil sample was acquired at 30°C with a 4.8-s acquisition time, using a sweep width of 13 KHz. Typically, 2,000 scans were collected using a 90° excitation pulse and a 35-s delay.

Fatty acid methyl esters (FAME) were prepared as described by Morrison and Smith (13) and, dissolved in *n*-hexane, were analyzed by GC with a Hewlett-Packard HP-6890 gas chromatograph equipped with a flame-ionization detector. A capillary column of cyanopropyl-methylxyloxane HP-23 FAME (30 m × 0.32 mm × 0.25 μm) was used. Nitrogen was used as carrier at a flow rate of 2 mL/min. The oven temperature was set at 175°C, injector temperature 250°C, detector temperature at 300°C.

The tri-11 eicosenoin, the deuterated chloroform, and the standard compounds used to identify the FAME were purchased from Sigma Chemical Co. (St. Louis, MO). All the other reagents and chemicals were purchased from Carlo Erba (Milano, Italy).

RESULTS AND DISCUSSION

One peculiar aspect of the ^{13}C NMR spectra of olive oils is the occurrence of some resonances in the carbonyl and olefinic regions not yet exhaustively identified (Figs. 1A, 2A). In the region of the *sn*-1,3 carbonyl resonances of the fatty acids (Fig. 1A), among the already known absorption signals, a small peak appears at 172.82 ppm (peak 2) which has not been identified as yet. Also unidentified are the two peaks at 129.59 (peak 6) and 129.68 ppm (peak 5) in the olefinic region (Fig. 2A).

The general rules that apply to the resonances of triacylglycerols in vegetable oils and animal fats and the assignments reported in the literature (1,3,4,9,14,15) on similar systems suggest that these peaks belong to monoenoic *cis*-11 fatty acids, mainly in *sn*-1,3 position. This hypothesis was verified by the addition of a small amount of eicosenoic tri-

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Abbreviations: FAME, fatty acid methyl esters; GC, gas chromatography; NMR, nuclear magnetic resonance.

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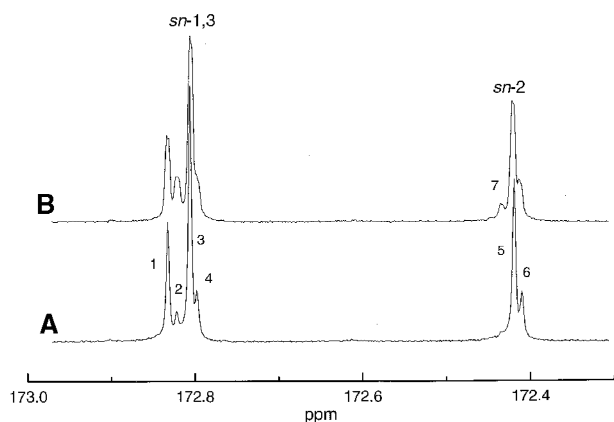

FIG. 1. (A) 75.4 MHz ^{13}C nuclear magnetic resonance (NMR) spectrum of the carbonyl region of triacylglycerols in extra-virgin olive oil in $CDCl_3$ at 30°C. Peak 1 belongs to saturates acyl group; peaks 3 and 5 belong to oleic (18:1,[*cis*]-9) and palmitoleic (16:1,[*cis*]-9); peaks 4 and 6 belong to linoleic (18:2,[*cis,cis*]-9,12) and linolenic (18:3,[*cis,cis,cis*]-9,12,15) acyl group; peak 2 belongs to *cis*-11 monoenoic group. (B) Same as Figure 1A after the addition of trieicosenoin. Peak 7 belongs to eicosenoin acyl group.

acylglycerol to one of the olive oil samples. In Figures 1B and 2B we show the resulting carbonyl and olefinic regions. The comparison of the two sets of spectra in the carbonyl region clearly shows that the enhancement of peak 2 is accompanied by the appearance of a small peak at 172.43 ppm (peak 7) in *sn*-2 position. Notably, although obscured by the noise, a corresponding peak seems to occur also in the neat sample. A similar comparison in the olefinic region shows the enhancement of the peaks 4, 5, and 6 and the simultaneous arising of peak 9 at 129.57 ppm. Again we note that probably a peak corresponding to the latter occurs also in the neat sample.

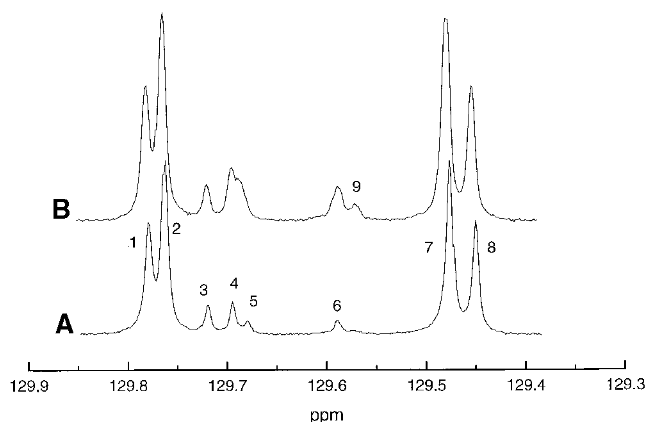

FIG. 2. (A) 75.4 MHz ^{13}C NMR spectrum of a portion of the olefinic region of triacylglycerols in extra-virgin olive oil in $CDCl_3$ at 30°C. Peaks 1 and 2 belong to C10 of oleic acyl group in *sn*-2 and *sn*-1,3 positions, respectively; peaks 3 and 4 belong to C9 of linoleic in *sn*-1,3 and *sn*-2 positions, respectively; peaks 7 and 8 belong to C9 of oleic in *sn*-1,3 and *sn*-2 positions respectively; peaks 5 and 6 belong to C12 and C11 of *cis*-11 monoenoic, respectively. (B) Same as Figure 2A after the addition of trieicosenoin. Peak 9 belongs to C11 of eicosenoin in *sn*-2 position. See Figure 1 for abbreviation.

TABLE 1
Fatty Acid Composition (% of the total fatty acids) of the *cis*-11 Monoenes in Olive Oils[a]

	Gas chromatography			Nuclear magnetic resonance
Source	*cis*-vaccenic ± SE	Eicosenoic ± SE	Total ± SE	Total ± SE
Puglia (Italy)	0.80 ± 0.01	0.43 ± 0.01	1.23 ± 0.01	1.66 ± 0.09
Spoleto (Italy)	1.61 ± 0.01	0.54 ± 0.01	2.15 ± 0.02	2.20 ± 0.06
Greece	1.42 ± 0.01	0.45 ± 0.01	1.87 ± 0.01	2.30 ± 0.11
Egypt	2.75	0.45	3.20	3.09

[a]The means and the standard errors were estimated from sets of at least six samples each. No statistics for the Egyptian olive oil could be obtained due to unavailability of a sufficient number of samples.

From the integrated peak areas of the olive oil samples, the amount of the eicosenoic fatty acid was found to be 1–3% of the total content of fatty acids, while the literature data quote this quantity as less than 0.6% (2,11,12).

Since the eicosenoic and *cis*-vaccenic fatty acids have quite close olefinic and carbonyl chemical shifts (1,4,9), a substantial signal overlapping should occur, and therefore these signals might comprise the *cis*-vaccenic fatty acid also. In fact the GC analysis revealed the presence of these two *cis*-11 fatty acids in amounts that add up to a value close to that found by NMR spectroscopy (see Table 1).

The extensive literature dealing with the comparison between NMR and GC sensitivity and errors indicates that the latter method is by all means the technique of choice in quantitating the composition of the vegetable oils. These remarks are also substantiated by the values of the relative standard errors reported in Table 1. However, it must be pointed out that the NMR analysis, although inherently much less sensitive and thus much more time-consuming, does not require the extensive chemical manipulations as in the GC methodology so that the overall experiment times of the two techniques are very similar. Furthermore, the NMR technique, as opposed to the GC analysis, allows within a single experiment the determination of combined *cis*-11 fatty acids composition and the positional distribution of these fatty acids on the glycerol backbone (although in each of the olive oil samples examined, *cis*-11 fatty acids are only detectable in the *sn*-1,3 position). However, it must be remarked that while the GC method gives separate measures of the two *cis*-11 fatty acids, the NMR technique only measures the sum of the two.

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REVIEW

Olestra Formulation and the Gastrointestinal Tract

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ABSTRACT: Olestra is a mixture of compounds comprising sucrose esterified with 6–8 long-chain fatty acids. It is not hydrolyzed by pancreatic lipase and as a result is not absorbed from the small intestine. Olestra in general has physical properties similar to those of a triacylglycerol with the same fatty acid composition. Foods made with olestra are virtually identical in taste and texture to those made with typical triacylglycerols. Olestra consumption does not generate hydrolytic products in the small intestine and, therefore, does not generate some of the signals that alter motility in the gastrointestinal tract. A reduction in gastroesophageal reflux with olestra, in contrast to triacylglycerols, is consistent with a lack of effect on stomach emptying. Unlike triacylglycerols that are absorbed in the proximal small intestine, olestra is distributed throughout the small intestine during transit and passes into the colon. In the colon, olestra's effects depend on its physical properties. Liquid nondigestible lipids result in separation of oil from the fecal matrix. Olestra formulations made with specific fatty acid compositions, particularly those containing a solid sucrose polyester component including behenic acid, possess appropriate rheology to hinder separation of oil from the rest of the fecal matrix, thereby reducing gastrointestinal symptoms.

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Recent studies have shown that olestra consumed in a free-choice manner in prepared snacks does not alter the frequency of reports of common gastrointestinal symptoms (1,2). At this time, however, products that contain the zero-calorie fat substitute olestra are required to have a label that states, "Olestra may cause abdominal cramping and loose stools." This label reflects symptoms that were elicited during the history of olestra development in which fluid prototypes of the nonabsorbable fat substitute were studied, and in studies in which subjects consumed olestra as part of a fixed diet for many consecutive meals. A recent clinical study has demonstrated that consumption of olestra does not cause diarrhea but can result in a predictable, gradual, dose-responsive stool softening effect similar to that of dietary fiber (3). In the discussion that follows, we examine how the chemical structure of olestra influences its properties and effects during transit from the mouth to the colon.

Although olestra is a sucrose polyester (sucrose esterified with six or more fatty acids), the product that is sold commercially is a mixture of sucrose polyesters of specific fatty acids that was developed both to optimize the texture sensed in the mouth and minimize separation of oil in the gastrointestinal tract from other components in the large bowel. We discuss below how the rheology and optimized fatty acid composition relate to these benefits; this progress has resulted from an understanding of the relationship between the physical properties and chemical composition of sucrose polyesters.

WHAT IS OLESTRA AND WHAT CAN WE PREDICT ABOUT ITS EFFECTS?

Olean® is the Procter & Gamble Company brand name for a fat substitute made from sucrose and vegetable oil fatty acids. Its common and usual name is "olestra" and in its development stage it was referred to as "sucrose polyester" to denote its chemical composition, that is, sucrose multiply-esterified with long-chain fatty acids. In January 1996, olestra was approved by the U.S. Food and Drug Administration for use in the commercial preparation of savory snack foods, such as potato chips and crackers (4).

Olestra is a subset of many possible sucrose polyester molecules and is defined by specific chemical measures, such as fatty acid composition and ester distribution. In addition, olestra is a semisolid at room and body temperature and is characterized by a specific rheological measure of stiffness to be discussed in detail later. Its semisolid consistency distinguishes olestra from early sucrose polyester compositions that were liquid at body temperature. In this review, these early compositions are referred to as "liquid sucrose polyester." A listing of olestra's chemical and rheological properties as specified in the Food and Drug Administration approval can be found in the olestra food additive regulation as summarized in Table 1 (4).

Olestra that is ingested as a replacement for typical dietary fat does not provide the body with utilizable energy by virtue of its unique resistance to intestinal lipases (5). Because it is not hydrolyzed into absorbable products, such as the fatty acids and monoacylglycerols resulting from normal fat digestion, olestra is not absorbed into the lymphatic or blood circulations (6,7). Olestra therefore passes into the large intestine chemically unchanged.

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Abbreviation: CCK, cholecystokinin.

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TABLE 1
Specifications for Olestra[a]

Property	Specification
Definition	Mixture of octa-, hepta-, and hexaesters of sucrose with fatty acids derived from edible fats and oils
Degree of esterification	Total content of octa-, hepta-, and hexaesters is not less than 97%. The content of octaester is not less than 70%, hexaester not more than 1%, and pentaester not more than 0.5%
Fatty acid composition	The unsaturated fatty acid content is not less than 25% and not more than 83%. The content of C_{12} and C_{14} acids is each not more than 1%, and the content of fatty acids with 20 or more carbon atoms is not more than 20%. C_{16} and C_{18} fatty acids make up the remainder, not less than 78%
Free fatty acids	Less than 0.5%
Stiffness	Not less than 50 kPa/s

[a]From Reference 4.

Humans, like most higher mammals, have an extremely efficient fat digestion process. Estimates of the excess capacity of pancreatic lipase range from 10 to 20 times that needed to digest and absorb a typical 100-g daily fat intake. Support for this excess capacity is given by a study in which 13 subjects were administered exaggerated levels of fat (200–639 g/d) for 4–45 d (8). Although fat absorption varied among individuals, it was nearly normal for most with a range of 82–98% of that ingested. One subject absorbed 98% of the ingested amount of 639 g/d, equivalent to a daily intake of seven sticks of butter.

The typical disposition of dietary fat is hydrolysis and absorption in the jejunum; however, it has been shown that uptake into the ileal enterocytes will take place if fat should arrive there (9). Fecal fat content typically accounts for less than 5% of ingested triacylglycerols (8), and this value also reflects the efficient process by which the body utilizes dietary fat. It should be noted that some of the fecal fat is undoubtedly of endogenous origin, resulting from sources such as sloughed intestinal cells or biliary lipid.

Based on these observations, a typical Western diet will deliver approximately 5 g of triacylglycerol to the colon on a daily basis. Dietary olestra, being neither hydrolyzed nor absorbed, is all transported into the colon and subsequently excreted unchanged in the feces (10). Any ingested olestra will therefore add to the normal flow of hydrophobic material in the colon.

The biochemical inertness of olestra suggests that it might behave in a manner that differs from that of the normal load of fat in the colon. This review discusses the current understanding of events that occur in the gastrointestinal tract after the ingestion of olestra. For a reference point it also covers the gastrointestinal transit of typical triacylglycerols, including their effects in the colon. We will discuss both olestra and triacylglycerols during transit from mouth to excretion. The topics covered in this discussion include oral sensory perception, stomach emptying, gastroesophageal reflux, gallbladder contraction, emulsification in the small intestine, movement through the colon, combination with fecal matter, effects on colonic microflora, and fecal excretion. The effects of olestra on fecal rheology and mass are discussed in relation to other dietary constituents.

PHYSICAL PROPERTIES OF OLESTRA

Although olestra is biochemically inert, its physical properties contribute to its gastrointestinal effects. As discussed below, the physical form of olestra, including its phase behavior and rheology, has been shown to influence the form of the contents of the colon and feces.

Early studies with rats included both liquid and semisolid sucrose polyester compositions (11). It was observed that feeding elevated levels of the liquid sucrose polyester, typically made from safflower oil fatty acids (90% linoleic and oleic acids), resulted in poorly formed fecal pellets with separation of an oil phase from the rest of the fecal matter. This oil frequently accumulated on the tails and fur of the animals during the studies. It was found that this separation of the sucrose polyester oil phase occurred above a threshold of 300 mg of dietary liquid sucrose polyester per day in rats that weighed 250–300 g (11).

It was also found that separation of an oil phase from the other fecal matter did not occur when rats were fed semisolid sucrose polyester synthesized from partially hydrogenated cottonseed and soybean oils at dietary levels of 8% by weight (Jandacek, R.J., unpublished observation). A typical fatty acid composition of sucrose polyester made from a blend of the methyl esters derived from these partially hydrogenated oils constituted 18% palmitic acid, 38% stearic acid, 11% linoleic acid, and 31% oleic acid. This composition melted in a range from body temperature to 42°C.

Although the olestra made from the hydrogenated oils appeared to be solid, its consistency was similar to that of lard or solid shortenings, and it contained both liquid and solid components. Dilatometry, which was used to determine solid and liquid fat content by measurement of differences in the change of volume with temperature (12), showed olestra made from a blend of partially and completely hydrogenated soybean oil to consist of 84% liquid and 16% solid sucrose polyester components at body temperature (37.0°C) (10).

Different gastrointestinal effects associated with consumption of liquid and semisolid sucrose polyester formulations have also been observed in humans. Consumption of 25 g/d of liquid sucrose polyester was found to cause separation of a fecal oil phase (13). A semisolid composition, on the other hand, did not result in oil separation even when fed at 50 g/d (10). The experience with liquid and semisolid sucrose polyester compositions indicated that the melting point or melting

range of a nondigestible hydrophobic compound is a determinant of its separation from feces. A nondigestible lipid with a high melting point (above body temperature) is less likely to flow and separate from other fecal matter during gastrointestinal transit in the large intestine.

In light of this relationship between physical properties of nondigestible lipids and the gastrointestinal effects elicited, higher-melting compositions would seem more appropriate for use in foods. However, the successful substitution of a nondigestible lipid for triacylglycerol in the diet will result only if the substitution yields highly palatable food products. High-melting sucrose polyester compositions containing a high level of solids at mouth temperature can be perceived as waxy or excessively greasy in the mouth and may impart the sensation of a residue of fat on the palate and/or lips. The development of a fat substitute is therefore subject to the dual constraints of providing both satisfactory taste and texture and of minimizing the potential discomfort of fecal oil separation in the gastrointestinal tract. Low-melting nondigestible lipids, such as liquid sucrose polyesters (see Table 2 for representative fatty acid compositions), provide very desirable taste performance but run the risk of oil separation from the fecal matrix above threshold dietary levels, which vary from individual to individual. Higher-melting compositions can reduce or eliminate the problems in the gastrointestinal tract, but their perceived waxiness may limit sensory acceptance.

As with dietary triacylglycerols, the physical properties of olestra are related to the properties of the constituent fatty acids. Fatty acids with high melting points yield high-melting triacylglycerols and sucrose polyesters. The use of a solid or semisolid sucrose polyester composition as a substitute for a high-melting dietary triacylglycerol may appear to be relatively straightforward; however, similar substitition for liquid dietary oils is a challenge because of the need to balance sensory performance with the potential gastrointestinal effects discussed above. Much of the effort in developing and optimizing olestra has focused on finding the appropriate compositions that provide both good taste performance and appropriate consistency in the gastrointestinal tract. The progress that has been made in developing olestra compositions with continually improved taste performance, while delivering acceptable performance in the gastrointestinal tract, is illustrated in the following discussion comparing three different types of olestra.

One class of olestra compositions described in the patent literature is synthesized from a blend of methyl esters derived from partially and fully hydrogenated vegetable oil fatty acids (14,15). The iodine value of this type of olestra typically ranges between about 20 and 55, depending upon the degree of hardening of the partially hydrogenated source oil and the molar ratio of the two methyl ester feed stocks. A representative fatty acid composition is shown in Table 2. This type of olestra has a semisolid consistency, and the solids level at body temperature (37.0°C) may range between about 5–20%, again depending upon the specific fatty acid composition and molar ratio of the source oils used. While effective for the control of fecal oil separation, these olestra formulations may provide less than optimal taste performance when used in certain food products as a full replacement for liquid triacylglycerol. The reason for this is evident upon examination of a representative solid fat content profile, shown as olestra composition A in Figure 1. Olestra composition A has a relatively steep solid fat content profile over the temperature range of room to body temperature (21.1–37.0°C). Consequently, there is a relatively high solids level at and below mouth temperature (33.3°C), which may result in a greasy or waxy tex-

TABLE 2
Fatty Acid Composition of Selected Sucrose Polyesters[a,b]

Form of sucrose polyester	Fatty acid composition (wt%)								
	14:0	16:0	18:0	18:1	18:2	18:3	20:0	22:0	24:0
Liquid (IV = 100; synthesized from partially hydrogenated cottonseed oil)	0.5	20.4	4.3	32.6	40.9	0.2	0.4	0.0	0.0
Liquid (IV = 90; synthesized from partially hydrogenated soybean oil)	0.0	9.7	5.9	64.5	18.9	0.2	0.3	0.2	0.0
Semisolid (IV = 41; synthesized from blend of methyl esters of partially and fully hydrogenated soybean oil fatty acids)	0.0	10.8	47.7	33.0	7.2	0.0	0.3	0.1	0.2
Solid (IV = 2; synthesized from fully hydrogenated soybean oil)	0.2	12.6	84.8	1.7	0.2	0.0	0.3	0.0	0.0
Solid (IV = 22; synthesized from C_{20}–C_{24} chain-length saturated and C_{18} chain-length unsaturated fatty acids)	0.0	1.2	4.6	3.7	10.9	0.0	4.6	71.7	2.8

[a]Data from References 14, 16, 17, and 19.
[b]Fatty acid compositions were determined according to AOCS Official Method Ce 1c-89 (21). IV, iodine value.

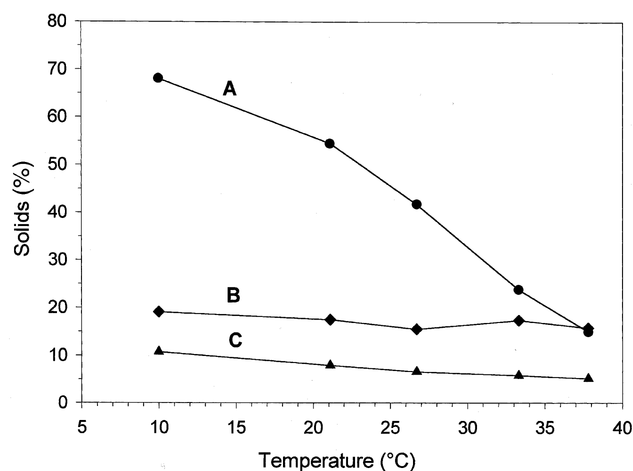

FIG. 1. Solid fat content profiles of three olestra formulations (Kester, J.J., and Wehmeier, T.J., unpublished data). (A) Olestra formulation synthesized from a blend of methyl esters of partially and fully hydrogenated soybean oil fatty acids; (B) olestra formulation comprising 82% liquid sucrose polyester (synthesized from partially hydrogenated soybean oil) and 18% solid sucrose polyester synthesized from fully hydrogenated soybean oil fatty acids; (C) olestra formulation comprising 92.5% liquid sucrose polyester (synthesized from partially hydrogenated soybean oil) and 7.5% solid sucrose polyester synthesized from C_{20}–C_{24} chain-length saturated and C_{18} chain-length unsaturated fatty acids. The solid fat contents were measured by pulsed nuclear magnetic resonance spectroscopy (22).

tural perception when composition A is used to completely replace liquid triacylglycerol. The U.S. patent issued to Bernhardt and Taylor (15), in fact, requires that a triacylglycerol be blended with this type of olestra in order to act as a solvent for the olestra solids, thereby helping to minimize the greasy or waxy mouth feel. A relatively steep solids profile is characteristic of olestra formulations synthesized from a blend of methyl esters derived from partially and fully hydrogenated vegetable oils. This profile is due to the extremely heterogeneous mixture of sucrose polyester molecules in the resulting olestra composition.

A second type of olestra is based on the physical blend of a liquid sucrose polyester and a high-melting, solid sucrose polyester derived from fully hydrogenated vegetable oil (see Table 2 for a representative fatty acid composition) (16). When solid sucrose polyester comprising primarily long-chain, saturated fatty acids is dissolved in liquid sucrose polyester by heating, spherulitic crystals are typically generated when the solution is allowed to cool (17). Sucrose polyester solids with a spherulitic crystal morphology have a relatively low surface area-to-mass ratio and hence are not particularly effective gelling agents for a liquid sucrose polyester oil phase. Nevertheless, if the level of the solid component is high enough, fecal oil separation can be controlled. Using an *in-vitro* fecal model to be discussed later, we have observed that ~18–20% solid sucrose polyester synthesized from fully hydrogenated soybean oil, when blended with liquid sucrose polyester and crystallized from a complete melt, will yield the appropriate rheology in the final olestra blend to control oil separation (Kester, J.J., unpublished observation). Measurement of the solid fat content profile for this olestra formulation (Fig. 1, composition B) reveals a relatively flat profile across the temperature range of room to body temperature (21.1–37.0°C). Compared to the previously discussed olestra formulation synthesized from a mixture of partially and fully hydrogenated fatty acid methyl esters (composition A in Fig. 1), olestra composition B would predictably display somewhat better taste performance when used as a full replacement for a liquid triacylglycerol (i.e., less greasy/waxy perception), owing to a lower solids level at and below mouth temperature (see Fig. 1).

The third olestra formulation to be discussed is also a physical blend of liquid sucrose polyester and a particular solid sucrose polyester component with unique crystallization behavior. The solid sucrose polyester was originally discovered by J.C. Letton and coworkers (18,19), who synthesized sucrose polyesters from fatty acids of disparate melting points. These solid sucrose polyesters are based on high-melting, long-chain saturated fatty acids (e.g., arachidic, 20:0, behenic, 22:0, and/or lignoceric, 24:0, acids) (18,19), combined at specific molar ratios with low-melting, fluid fatty acid chains. Typical molar ratios of the long-chain saturated fatty acids to the low-melting acyl chains are ~3:5–7:1 (19). The low-melting fatty acids may be short-chain, saturated (e.g., octanoic acid, 8:0, or lauric acid, 12:0) or long-chain, unsaturated fatty acids (e.g., oleic acid, 18:1, or linoleic acid, 18:2). A representative fatty acid composition for this type of solid sucrose polyester is shown in Table 2, in which the low-melting acyl chains are C_{18} chain-length, unsaturated fatty acids. When a solid sucrose polyester comprising fatty acids of disparate melting points is dissolved in liquid oil by heating and then the resulting solution allowed to cool and crystallize from a complete melt, thin platelet-shaped crystals are formed with a high surface area-to-mass ratio (17). The presence of the low-melting, fluid acyl chain in the solid sucrose polyester molecule is believed to be important for promoting formation of the platelet crystal morphology, presumbably by interfering with unrestrained three-dimensional crystal growth and thus promoting crystal growth, principally in two dimensions.

The platelet crystals of solid sucrose polyester constituting C_{20}–C_{24} chain-length saturated fatty acids and low-melting fatty acyl chains are highly effective gelling agents for liquid oils when cooled and crystallized from a complete melt (17,19). The efficient gelling performance is believed to arise from the high solid–liquid interfacial area. Depending upon the specific fatty acid composition of the solid, *in vitro* modeling studies and human clinical investigations have shown that ~5–10% of this solid type blended with liquid sucrose polyester will produce a semisolid gel with a stiffness sufficient to control fecal oil separation and passive oil loss.

Shortening and margarine prototypes can be made with mixtures of solid sucrose polyesters containing behenic acid and low-melting acyl chains, with either liquid sucrose polyester or triacylglycerol (18,19). The resulting products are extremely stable against phase separation. Therefore, these sucrose polyester solids are satisfactory replacements for β′ tri-

acylglycerol solids typically used in these food applications, such as those derived from fully hydrogenated palm or cottonseed oils.

Most importantly, however, olestra formulations containing sucrose polyester solids derived from fatty acids with disparate melting points display an optimized balance between taste performance and gastrointestinal effects. This balance is primarily a consequence of the relatively low level of solids required to provide a consistency sufficient to control fecal oil separation (i.e., these solids are efficient gelling agents). The low usage level of this solid sucrose polyester, combined with a flat solid fat content profile between room and body temperature (see Fig. 1, olestra composition C), results in a low solids level at mouth temperature that helps minimize greasy and/or waxy textural perception in finished food products. As a result, these olestra formulations provide optimal taste performance when used as fat substitutes in food products containing liquid triacylglycerols, such as in savory snack foods (20).

An important aspect of the work conducted to optimize the balance between olestra's taste properties and gastrointestinal effects involved developing an understanding of the relationship between the rheology of sucrose polyester compositions and fecal oil separation in the large bowel. This understanding was necessary in order to tailor semisolid olestra formulations to meet the required consistency for control of fecal oil separation, without incorporating excessive solids that negatively impact taste and texture. Rheological and fecal oil separation modeling studies of various sucrose polyester compositions were carried out and some of that work is summarized below.

Experience from animal studies previously discussed (11) led to the working hypothesis that separation of an unabsorbed lipid from the fecal matrix in the large intestine is a prerequisite to the occurrence of passive or uncontrolled oil loss through the anal sphincter. Furthermore, it is logical to assume that the greater the amount of fecal oil separation for any given unabsorbed lipid, the higher the risk that oil loss will occur. The same animal studies also provided evidence that oil separation from the fecal matrix and subsequent oil loss can be controlled by formulating olestra to have a semisolid consistency at body temperature (11). A semisolid consistency presumably allows an unabsorbed lipid to resist the thermodynamic tendency for oil separation from the hydrophilic fecal matrix, thereby remaining well dispersed within the stool. The objective of the following work was to define a rheological measurement of semisolid olestra that is predictive of the degree of fecal oil separation and hence the risk of oil loss. Definition and validation of an appropriate rheological measurement allowed subsequent human clinical testing to be conducted in order to set a minimal olestra rheological or stiffness specification that ensures the control of oil loss.

Definition of the olestra rheological measurement first required development of an *in vitro* fecal model that could be used as a quantitative tool to assess fecal oil separation of various olestra compositions. Use of an *in vitro* model allowed for more efficient screening of multiple olestra formulations than could have been accomplished through human clinical testing. A detailed description of the *in vitro* fecal model and methodology used in this work is provided in Elsen *et al.* (17). The objective of the fecal model was to simulate the peristaltic movement and dehydration of the contents in the large bowel, followed by measurement of the amount of free oil separation from the simulated fecal matrix that is elicited by any given nondigestible lipid. An aqueous dispersion of 9.4% simulated fecal solids (Feclone FPS-2 solids; Silicone Studios, Valley Forge, PA) was prepared by blending with hot (~100°C) distilled, deionized water. The dispersion was equilibrated at 37.8°C, after which 276.7 g of the dispersion was blended with a 26-g load of the sucrose polyester composition. Each lipid sample had been crystallized by cooling from a complete melt (~121°C) to approximately room temperature over ~3 min following the standard cooling profile described in Elsen *et al.* (17). The aqueous dispersion of simulated fecal solids (blended with the sucrose polyester composition of interest) was then added to dialysis tubing that allowed the passage of water vapor. After sealing both ends, the dialysis tubing and its contents were maintained at 37.8°C and subjected to a mild rolling mechanical action intended to simulate perstaltic movement in the colon (see Reference 17 for a further description of the apparatus used to induce the mechanical action). Over the course of approximately 20 h, water evaporation through the dialysis tubing led to partial dehydration of the simulated fecal matrix until the moisture content was reduced to ~75%, which is within the typical moisture content range for human feces (23). Dehydration of the simulated fecal matrix resulted in a change in consistency from a watery fluid to a stiffened consistency approximating that of a soft stool. Following completion of the dehydration step, the dialysis tubing was opened and the contents quantitatively transferred to a centrifuge bottle, after which the material was subjected to a mild centrifugation at 37.8°C (30 min at 115 × g) and the quantity of free oil that separated from the simulated fecal matrix was measured. This *in vitro* fecal model was validated by correlating oil separation for specific olestra formulations to reports of *in vivo* fecal oil separation from human subjects (as measured by oil droplets in the toilet after defecation) participating in clinical trials (Kester, J.J., unpublished data).

A variety of sucrose polyester formulations was assessed for level of oil separation in the *in vitro* fecal model, including the three olestra types previously discussed (see Fig.1). The sucrose polyester formulations included multiple solid crystal morphologies (e.g., spherulite, platelet) with expected variability in their efficiency of oil binding and gelation of the liquid sucrose polyester phase. Oil separation data were then statistically correlated to various rheological measurements made on the same sucrose polyester formulations crystallized from a melted state under an identical cooling regimen. The rheological measure that best correlated with oil separation data from the *in vitro* fecal model was the thixotropic area obtained from a shear rate vs. stress flow

curve (see Figs. 2 and 3). The flow curve was generated at 37.8°C using a controlled rate rheometer equipped with a cone and plate configuration. Shear rate was first increased from 0 to 800 s^{-1} over 7.5 min and then decreased back to 0 s^{-1} over 7.5 min, while shear stress was monitored. Thixotropic area, measured in units of kPa/s, is the area of hysteresis bounded by the ascending and descending portions of the flow curve (Fig. 3). The term thixotropy refers to the decrease of apparent viscosity of a material with time under shear and the subsequent partial or full recovery of apparent viscosity when the shear is discontinued (24). Colloidal dispersions, such as a dispersion of solid lipid crystals in a liquid oil phase, often display thixotropic behavior. This behavior arises from particle associations that exist in the absence of flow and, in the case of a gel, these associations give rise to a particulate network (24,25). Under increasing shear, these particle associations are broken and, consequently, the descending portion of the flow curve for a thixotropic material is displaced below the ascending portion of the curve (Fig. 3). The magnitude of thixotropic area enclosed by the ascending and descending portions of the flow curve can be expressed in terms of the energy per unit volume of sample being sheared, which indicates that thixotropic area is related to the energy required to break down the particulate network structure of the material being sheared (25,26). Therefore, thixotropic area may be interpreted as a relative measure of the solid-like structural strength or stiffness of a material that exists prior to shearing. This interpretation is valid as long as the flow curve is generated under a standardized testing protocol (e.g., sample handling, temperature, shear rate ramp, etc.) (26). A comparison of thixotropic areas measured for semisolid olestra and liquid sucrose polyester is shown in Figure 3.

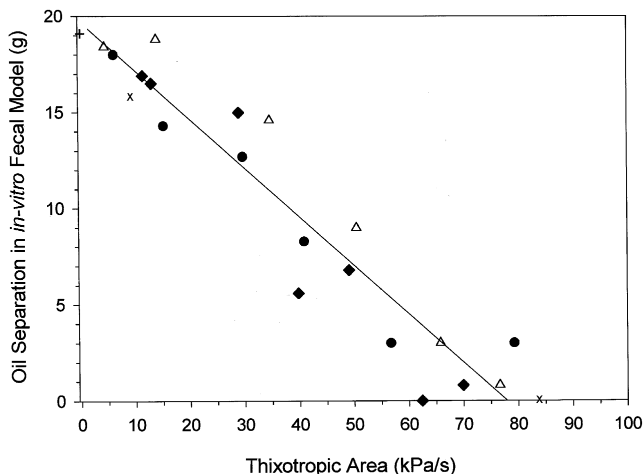

FIG. 2. Relationship of thixotropic area measured at 37.8°C of various sucrose polyester formulations to oil separation in the *in vitro* fecal model (Kester, J.J., and Wehmeier, T.J., unpublished data). Each data point is the average of duplicate measurements. The sucrose polyester formulations were as follows: Liquid sucrose polyester synthesized from partially hydrogenated cottonseed oil (+); semisolid sucrose polyester synthesized from a blend of methyl esters of partially and fully hydrogenated soybean oil fatty acids (×); physical blends of liquid sucrose polyester with 5, 10, 15, and 20% solid sucrose polyester synthesized from fully hydrogenated vegetable oil (●); physical blends of liquid sucrose polyester with 2, 4, 6, and 8% solid sucrose polyester synthesized from C_{20}–C_{24} chain-length saturated and C_{18} chain-length unsaturated fatty acids (◆); physical blends of liquid sucrose polyester with 2, 4, 8, 10, 12, and 14% solid sucrose polyester synthesized from C_{20}–C_{24} chain-length saturated fatty acids and lauric acid, 12:0 (△).

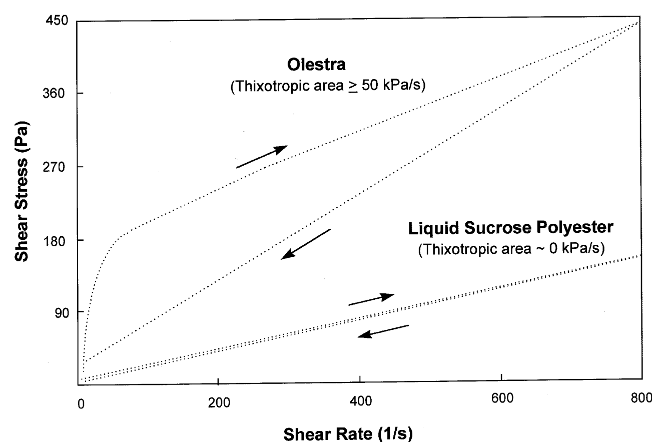

FIG. 3. Representative flow curves at 37.8°C for liquid sucrose polyester and semisolid olestra, both of which were derived from partially hydrogenated cottonseed oil. The flow curves were generated on a controlled rate rheometer with cone and plate configuration (model #RH115, Contraves AG, Zurich, Switzerland). The shear rate was increased from 0 s^{-1} to 800 s^{-1} in 7.5 min and then decreased back to 0 s^{-1} in 7.5 min. The lipid samples were cooled from a complete melt (~121°C) to approximately room temperature over a time period of ~3 min according to the cooling profile in Elsen *et al.* (17). Thixotropic area is the area of hysteresis between the ascending and descending portions of the flow curve.

Nondigestible lipids that display little or no thixotropic area have minimal solid-like structure or stiffness and therefore have a high propensity to separate from the fecal matrix in the large bowel. Consequently, if consumed at relatively high levels, these materials have a high risk of separation from the fecal matrix in the colon and subsequent leakage through the anal sphincter. Liquid sucrose polyester exhibits an extreme example of this phenomenon. Liquid sucrose polyester displays Newtonian flow behavior; consequently the ascending and descending portions of the flow curve are superimposed, yielding a thixotropic area that is essentially zero (Fig. 3). When liquid sucrose polyester was tested in the *in vitro* fecal model, over 70% of the lipid load separated from the simulated fecal matrix; i.e., ~19 g of the 26-g load (Fig. 2) (17).

Olestra formulations that are semisolid at body temperature display a measurable thixotropic area. As thixotropic area of the sucrose polyester formulations evaluated in this study increased, the amount of oil separation measured in the fecal model decreased in a linear manner (Fig. 2). Complete control of oil separation in the *in vitro* fecal model was achieved at a thixotropic area of ~80 kPa/s.

The relationship of the thixotropic area of various olestra formulations to the occurrence of oil droplets in the toilet (an indication of fecal oil separation) and underwear oil staining in humans was supported in a subsequent clinical investiga-

tion to be discussed in detail later. This investigation provided the basis for setting the current olestra rheological specification (termed "stiffness" in the olestra food additive regulation) that ensures control of oil leakage; i.e., thixotropic area greater than or equal to 50 kPa/s (see Table 1). Further details regarding the method for measuring thixotropic area of olestra can be found in Elsen *et al*. (17) and in the olestra food additive regulation (4).

OLESTRA INGESTION AND GASTROINTESTINAL TRANSIT

Mouth. The physical properties of olestra are important determinants of its effects in the mouth. Although lingual lipases are present in the mouth during mastication of a fat-containing food (27), the short residence time and low enzyme activity result in negligible hydrolysis of triacylglycerol in the oral cavity. In addition, since there is no evidence of olestra's absorption in the gastrointestinal tract (28,29), we may also conclude that olestra is not hydrolyzed by lingual lipase since absorbable products would be produced by hydrolysis.

The flavor of olestra is very similar to that of the triacylglycerols containing the same fatty acids from which it was derived. However, depending on the specific food application and the melting behavior of the triacylglycerol being replaced, there is potential for olestra to display differences in textural perception in the mouth. The perception of fat in the mouth depends on its melting point, viscosity, and interfacial tension with the aqueous phase. The oil/water interfacial tension of olestra that comprises predominantly octaesters is similar to that of analogous triacylglycerols (30), as might be predicted since the ratio of lipophilic to hydrophilic portions of the olestra and triacylglyerol molecules are similar. Sucrose polyester compositions can be made with a variety of melting points and apparent viscosities, depending on the fatty acids used in its production (30). However, as previously discussed, olestra for use in foods is formulated to possess a semisolid consistency at body temperature in order to control fecal oil separation. Therefore, when olestra is used as a replacement for liquid triacylglycerols (e.g., in the preparation of fried savory snacks), this physical property constraint necessitates careful control by the food manufacturer of the total olestra level and location in the finished food in order to yield optimal taste properties.

Stomach. There are measurable differences between the effects of olestra and triacylglycerols in the stomach. Although this differentiation may result from both local effects and different feedback signals from the small intestine, the effects manifest in the stomach will be discussed first.

(i) Gastric emptying. A fundamental question about olestra's effects on gastric motility was addressed in an early study by Cortot and coworkers (31). The rate of emptying of food components was measured in five healthy subjects by the use of a multilumen duodeno-jejunal tube and a double lumen gastric sump tube, along with nonabsorbable markers. ^{14}C-Labeled sucrose octaoleate and ^{3}H-labeled glyceryltriether were used as lipid markers, and polyethylene glycol 4000 and phenolsulfonphthalein as markers of the aqueous phase. Phenolsulfonphthalein and glyceryltriether were continuously infused into the duodeno-jejunal tube, and the other markers were fed with the test meal of steak, bread, orange sherbet, 20 g of sucrose octaoleate, and 210 mL of water containing 15 g of polyethylene glycol 4000. The intestinal contents were aspirated at sites 20 and 30 cm below the duodenal infusion site, with an occluding balloon placed at a point just past the most distal sampling site. The intestinal samples were continuously aspirated and the stomach samples were aspirated every 10 min.

This study showed the rates of stomach emptying of sucrose octaoleate and of polyethylene glycol 4000 to be similar. Virtually all of the sucrose octaoleate had left the stomach within 2 h after the test meal was consumed. The authors noted that this time was markedly shorter than that which they had observed for butter's exit from the stomach. They concluded that the difference was not due to a more rapid exit of the entire meal from the stomach as determined by food particles in the aspirate. The data were consistent with the slow rate of gastric emptying of dietary fat resulting from its ultimate hydrolysis and more rapid emptying of a nonhydrolyzable fat. Presumably feedback signals elicited by the hydrolytic products in the small intestine influenced the rate of emptying. The authors noted that gastric emptying has been observed to be faster in patients with impaired lipolysis. The physical properties of the fat, including viscosity and density, did not determine the rate of stomach emptying since the physical properties of sucrose octaoleate were similar to those of typical triacylglycerol fats that were observed to leave the stomach slowly.

In a study of the effect of olestra on the intestinal transit of food, Aggarwal and coworkers (32) followed regional movement of radiolabeled solid polystyrene pellets, which showed the transit of solid residue. They compared the effects of four different meals that contained 45 g of lipid, of which olestra accounted for 0, 7.5, 15, or 30 g. In addition, they included a low-fat (15 g) meal with no olestra. There were no differences between subjects consuming the different meals in the length of time to first entry of solid into the small intestine (lag phase) or in the rate of the solids leaving the stomach after the lag phase (postlag slope). This study, in which the transit of meal solids was followed, showed therefore that stomach emptying was not influenced by levels of olestra in the meals between 0 and 30 g. These results do not contradict those of Cortot *et al*. (31), since the latter workers followed the movement of a liquid oil form of sucrose polyester (sucrose octaoleate) from the intubated stomach, rather than solid pellets followed noninvasively.

(ii) Esophageal reflux and gastric acid. The results of a study of the effect of olestra on postprandial esophageal acid exposure are consistent with a difference in the behavior of olestra and triacylglycerols in the stomach (33). In this study, 19 subjects received in random order potato chips prepared either with olestra or vegetable oil (cottonseed oil) on two

consecutive days. An esophageal pH probe measured intraesophageal pH for 30 min prior to the meal and for 3 h after the consumption of the chips. Subjects were upright during the measurements. The postprandial gastroesophageal reflux, which was defined as a decrease in pH below 4.0, was measured in terms of cumulative minutes of duration. The number of times the pH decreased below 4.0 was also determined. The chips prepared in vegetable oil resulted in a significantly greater duration of esophageal acid exposure, 27 min, compared with 2 min after the olestra chips ($P < 0.05$). Similarly, the number of episodes over the 3-h study period was significantly greater with the triacylglycerol chips (25 vs. 9; $P < 0.05$) . The investigators suggested that the results are consistent with a lower production of serum cholecystokinin (CCK) after the olestra meal, since CCK is one signal involved in decreasing lower esophageal sphincter pressure.

A study by Maas *et al*. (34) examined the effect of sucrose polyester (presumably similar to marketed olestra; rheological and melting parameters not specified) on the secretion of gastric acid and the release of CCK. As discussed above, although the release of CCK is an event mediated by intestinal receptors, it affects gastric emptying. These investigators found that sucrose polyester differed from typical triacylglycerols in that it did not stimulate CCK release. This observation is consistent with the finding of Cortot *et al.* (31) that triacylglycerols left the stomach more slowly than sucrose octaoleate, presumably because the hydrolysis products of the triacylglycerol slowed stomach emptying by CCK-based feedback.

Maas and coworkers (34) also found that sucrose polyester did not reduce gastric acid secretion in the manner of triacylglycerols, and it is possible that the absence of feedback by free fatty acids also was involved in this effect. These authors' concerns about the risk of increased acid production with ingestion of a nonhydrolyzable lipid, thereby leading to more gastroesophageal reflux, were not borne out by the results in the prior paper by Just *et al*. (33). These results actually showed a decrease in esophageal acid reflux after ingestion of olestra.

The activity of gastric lipase on fat in the stomach can catalyze formation of some diacylglycerols and free fatty acids. This predigestion step is of more importance in neonates with pancreatic insufficiency than in normal adults (35). Since there is no evidence of olestra's absorption (28,29), we may conclude that hydrolysis of olestra is not catalyzed by gastric lipase.

Small intestine. (i) Pancreatic lipase. The most important distinctions between olestra and triacylglycerol are evident in the small intestine. Whereas, after emulsification, triacylglycerols are rapidly hydrolyzed by pancreatic lipase-colipase, olestra is biochemically inert in the presence of this enzyme complex. The presence of olestra in the proximal small intestine would not be predicted to cause any untoward effects since this region regularly accommodates unhydrolyzed fat.

The jejunal contents during the digestion of a fatty meal include droplets of unhydrolyzed triacylglycerol (36) that may differ from olestra in rate of emulsification. Oil/aqueous interfacial tension measurements of pure olestra and triacylglycerol are similar (30), but the influence of different concentrations of fatty acids associated with the oil droplets emptying from the stomach into the small intestine could result in different rates of emulsification. The emulsification of triacylglycerols may occur more rapidly in the small intestine relative to olestra since fatty acids produced by gastric lipase would be expected to associate with the triacylglycerol oil droplets that exit the stomach into the duodenum. Although intestinal fatty acids conceivably could diffuse to the olestra–water interface, the fatty acid (or soap) concentration at the triacylglycerol-aqueous interface will presumably be higher because of the local production of free fatty acids and monoacylglycerol by lipase at the triacylglycerol oil droplet surface. In addition, the semisolid consistency of olestra may tend to limit emulsification in the small intestine, since oils are more efficiently emulsified than solid or semisolid fats.

Bile salts readily emulsify sucrose polyesters as shown by *in vitro* preparations that require ultracentrifugation to separate sucrose polyester and bile salt micellar phases (37). Therefore, the complete absence of hydrolysis of olestra by pancreatic lipase cannot be attributed to reduced emulsification in the small intestine.

The ingestion of typical quantities of dietary fat allows the near completion of hydrolysis and absorption of fat in the jejunum, so that only small amounts reach the ileal region (9). The presence of fat in the distal small intestine is not an abnormal situation, however, and fat absorption takes place in this region if fat is delivered there (9). There do not seem to be untoward effects associated with the presence of unhydrolyzed fat in this part of the gastrointestinal tract.

In spite of the inability of pancreatic enzymes to split olestra into hydrolytic products, the pancreas does not "overproduce" to compensate for incomplete digestion as it does in response to a high level of absorbable fat in the diet (38). A study by Hager and Schneeman (39) compared pancreatic enzyme secretion in rats fed olestra with those fed triacylglycerol and found no differences. Therefore, olestra does not induce signals to the pancreas to increase lipase production in response to the presence of nondigestible lipid in the small intestine.

(ii) Bile acids. An effect of olestra that could hypothetically introduce a gastrointestinal change would be if it interfered with bile acid absorption. Reduced reabsorption of bile acids would introduce a higher than normal level of bile acids into the colon with accompanying irritant effects (40). A study in humans showed that bile acid excretion was not increased with moderate to high levels (8 to 50 g/d) of dietary olestra (41). In another trial with humans consuming 50 g/d of olestra, a modest increase in bile acid excretion was observed (from 225 ± 121 to 376 ± 222 mg/d; $P < 0.05$) (42). Studies with experimental animals showed no increase in the excretion of either total bile acid or ^{14}C-labeled chenodeoxycholic acid (a relatively lipophilic, dihydroxy bile acid) when olestra was included in the diet (43). These observations are

consistent with the relatively hydrophilic nature of the bile salts. Even one of the more lipophilic bile salts, chenodeoxycholic acid, has little affinity for olestra relative to an intestinal micellar phase.

(iii) Nutrient absorption. It is possible that olestra could interfere with the absorption of nutrients in the small intestine and subsequently carry them into the large intestine. Nutritional studies in pigs with olestra have shown that it does not alter the absorption of macronutrients (44). The study of excreted fat from humans fed sucrose polyester also showed no effect on the excretion of lipids from dietary triacylglycerol (10). In the latter study there was no difference in plasma triacylglycerol between the basal period and the end of the 20-d period in which 50 g/d of olestra was consumed. There was, however, a reduction in plasma low density lipoprotein cholesterol in the normocholesterolemic subjects. The absence of an effect of olestra on fat absorption was confirmed by Daher *et al*. (45) in a study of the absorption of ^{14}C-triolein, in which absorption was reduced by only 1.2% by 32 g/d of olestra. From these data, and from the biochemical inertness of olestra, we may conclude that the ingestion of olestra does not introduce macronutrients into the large intestine. Dietary olestra therefore does not add substances to the colon contents that cause irritation or reactions that produce irritant chemicals as in the fermentation of fiber.

Studies of the effects of olestra on lipophilic micronutrients have repeatedly shown that olestra can reduce the absorption of these materials. The probability of interference increases with the lipophilicity of the nutrient and the mass of ingested olestra (43,46). The absorption of cholesterol, the fat-soluble vitamins, and carotenoids is lowered by the presence of olestra, but that of water-soluble vitamins is not affected. Because the amount of these lipophilic micronutrients is small (approximately 500 mg/d for cholesterol; several milligrams or micrograms/d for the vitamins), and because their retention in the olestra phase reduces their aqueous chemical activity, they do not alter normal patterns of fecal consistency or gastrointestinal motility.

Large Intestine. (i) Comparison of malabsorbed triacylglycerol with olestra. Normal digestion and absorption removes virtually all dietary triacylglycerol from the gastrointestinal tract by the completion of transit through the small intestine. As discussed earlier, dietary loads of 600 g/d, approximately six times normal levels, can be digested and absorbed (8), giving evidence of a large excess capacity of the fat digestion/absorption enzymes and structures in the small intestine. This complete absorption results in the colon typically containing 3–5 g of unabsorbed fat each day. The abnormally high excretion of triacylglycerol or products of its hydrolysis is clinically considered to reflect fat malabsorption and is referred to as steatorrhea. As discussed below, the reactions of this fat in the colon are of clinical importance.

Fat malabsorption can result from a number of causes. Inadequate emulsification accompanying bile obstruction hinders the hydrolysis of fat in the intestine. Incomplete hydrolysis also occurs because of pancreatic insufficiency in diseases such as cystic fibrosis or sprue. Unabsorbed, unhydrolyzed triacylglycerols pass from the ileum to the colon in these cases. Fat absorption does not take place in the large intestine, and most of the fat that exits from malabsorbers is in the form of fatty acid soaps (47). The hydrolysis of fat in the colon is known to result from the lipases associated with colonic bacteria.

The symptoms of steatorrhea presumably reflect the effects of hydrolyzed fat in the large intestine, since most excreted fat is in the form of fatty acids and soaps, and since fatty acids are known to induce fluid secretion and epithelial cell damage in the colon (48). These symptoms would not be expected from an inert, nonhydrolyzed lipid, such as olestra. Olestra is completely insoluble in aqueous systems and, therefore, its chemical activity is quite low. In contrast, the products of triacylglycerols hydrolyzed by bacterial lipases, particularly soaps, have significant aqueous phase activity and cause irritant and osmotic effects in the colon.

Further support for the inertness of olestra in the colon is the study by Aggarwal *et al.* (32), in which colonic transit was studied with ^{111}In pellets. In six subjects who received up to 30 g of olestra in a meal, there was no alteration of colonic transit by the substitution of olestra for triacylglycerols. The absence of an effect of olestra on aqueous fluid secretion was shown in a study of 93 subjects who ate 24 g/d of olestra for 36 d (49). Dietary olestra decreased fecal water concentration, and this effect was attributed to its dilution of the fecal mass rather than an alteration of fluid secretion.

(ii) The effect of olestra on the fecal matrix. Much understanding of the effects of a chemically inert lipid in the colon was achieved in the studies of olestra in animals and in clinical trials. The phenomena that occur during transit in the colon may be divided into those that are dependent on the rheology of the olestra phase (e.g., liquid or semisolid), and those that are dependent on the mass of ingested olestra. A study that addresses these two types of effects is discussed below.

Olestra rheology. The relationship between rheology of sucrose polyester formulations and fecal oil separation in an *in vitro* model has been discussed. The *in vitro* modeling work was subsequently followed by a human clinical investigation to evaluate the effect of varying rheology of sucrose polyester formulations on the characteristics of the stool (4). Following is a summary of the study design and results.

The primary objective of the study was to determine how variation in stiffness (i.e., thixotropic area) of sucrose polyester formulations affects subjects' assessment of oil loss and other gastrointestinal effects when consuming a high daily intake of olestra in savory snacks. These results were then used to set the rheological specification for olestra to ensure control of oil leakage. The study was conducted in multiple cities and was a parallel, double-blind, and placebo-controlled design. The subjects were 1,228 healthy males and females 18–44 yr in age. For five consecutive days, subjects consumed 4 ounces of potato chips that had been fried in either triacylglycerol (cottonseed oil placebo) or one of several different sucrose polyester formulations (derived from cottonseed oil)

as part of their normal diet. The potato chips provided 34 g/d of the placebo or test lipid. The sucrose polyester formulations were of varying stiffness, ranging from 18 to 103 kPa/s thixotropic area (see previous discussion for details regarding measurement of stiffness). After the 5-d consumption period, subjects completed a questionnaire regarding any underwear staining experiences resulting from oil loss (yellow-orange oil stains), the observation of oil droplets in the toilet following defecation, and other gastrointestinal effects.

Oil loss caused by the consumption of the various sucrose polyester formulations was measured by the subjects' reports of underwear oil staining. There was a marked decrease in the percentage of subjects who experienced staining for the sucrose polyester formulations of stiffness ≥50 kPa/s, relative to the lower stiffness formulations (Table 3). The percentage of subjects who reported oil staining when consuming sucrose polyester formulations with stiffness ≥50 kPa/s was not significantly different from the triacylglycerol placebo group (2.7 vs. 1.2%, respectively; $P < 0.05$). These data provided the basis for setting the current rheological specification in the olestra food additive regulation; i.e., olestra is defined as having a stiffness at 37.8°C not less than 50 kPa/s (see Table 1).

The incidence of oil droplets in the toilet after defecation was also affected by stiffness of the sucrose polyester formulations. The most fluid formulation (stiffness = 18 kPa/s thixotropic area) yielded the highest incidence of oil droplets in the toilet, 32.0% of the subjects. Formulations of stiffness ≥50 kPa/s yielded significantly fewer reports of oil droplets in the toilet (15.8% of subjects; $P < 0.05$). However, this incidence was significantly higher than the placebo group (4.7%; $P < 0.05$).

The percentage of subjects reporting other gastrointestinal effects for the placebo and olestra (stiffness ≥ 50 kPa/s) treatment groups are presented in Table 4. There was no significant difference between the placebo and olestra groups in reports of diarrhea-like stools or bloating ($P < 0.05$). Reports of certain other gastrointestinal effects were, however, significantly higher for the olestra group relative to the placebo (e.g., soft and loose stools, flatulence, urgency, abdominal pain or cramps).

Consideration of the mass of olestra. While the incidence of oil droplets in the toilet and certain other gastrointestinal effects in this study (Table 4) were higher for the olestra treatment group relative to the placebo, it is important to keep in mind that the primary objective of this study was to define the olestra rheological specification that ensures control of oil leakage. Therefore, daily olestra consumption was mandatory and purposely set at a high intake in order to stress the technology; 34 g/d is equivalent to ~3–4 times the 90th percentile chronic daily intake of lipid from snack foods for the age group tested (50). When considering the high daily consumption level, the increase in percentage of subjects who reported various gastrointestinal effects on the olestra treatment group is rather modest; i.e., 6–10 percentage points higher vs. the placebo group (Table 4). As mentioned at the beginning of this review, when olestra savory snacks are consumed *ad libitum*, the frequency of reports of these same gastrointestinal effects is no different from placebo (1,2).

TABLE 3
Incidence of Oil Loss, as Measured by Percentage of Subjects Who Reported Yellow-Orange Oil Staining of Underwear, When Consuming Placebo (cottonseed oil) and Sucrose Polyester Formulations of Varying Stiffness (thixotropic area)[a]

Treatment group	# Subjects	Incidence of oil loss[b] (% subjects who reported oil staining)
Placebo (triacylglycerol)	172	1.2[a]
Sucrose polyester formulations		
Stiffness < 50 kPa/s	347	6.9[a,b]
Stiffness ≥ 50 kPa/s	709	2.7[b]

[a]Subjects consumed 4 ounces of potato chips per day, providing 34 g/d of lipid, for five consecutive days (4).
[b]Values with the same superscript roman letter are significantly different ($P < 0.05$).

TABLE 4
Incidence of Selected Gastrointestinal (GI) Effects Reported by Subjects Consuming Placebo (cottonseed oil) and Olestra (stiffness ≥ 50 kPa/s)[a]

GI effect	Subjects reporting GI effect in response to direct questions[b] (%) Placebo group (n = 172)	Olestra group (n = 709)	
Diarrhea-like stools	16	20	
Soft stools	51	60	s
Loose stools	28	35	s
Bloating	29	32	
Flatulence	63	70	s
Urgency	30	40	s
Abdominal pain or cramps	22	28	s

[a]Subjects consumed 4 ounces of potato chips per day, providing 34 g/d of lipid, for five consecutive days (4).
[b]s = values in that row are significantly different ($P < 0.05$).

(iii) Olestra in patients with inflammatory bowel disease. Patients with ulcerative colitis and Crohn's disease were studied to determine whether olestra would have any deleterious effects in subjects with inflammatory bowel disease (51). Eighty-nine patients with known inflammatory bowel disease of at least 2 yr of mild-to-moderate ulcerative colitis (53 subjects) or Crohn's disease (46 subjects) entered a double-blind, placebo-controlled, parallel trial at 13 sites. All subjects were in remission at time of entry. The olestra group ate potato chips and cookies that contained olestra with a daily dose of 20 g/d while the placebo group ate the same foods prepared with normal fats. The duration of the diet-phase of the study was 4 wk, and a 4-wk follow-up period of observation concluded the study. There was no significant difference between the two groups in the worsening of symptoms during either part of the 8 wk of study (3 ulcerative colitis and 3 Crohn's patients in the olestra group; 1 ulcerative colitis and 3 Crohn's patients in the placebo group; $P > 0.15$). In addition, specific tests including bowel permeability (by measurement of urinary excretion of polyethylene glycol), and assessment of condition of the ulcerative colitis patients by flexible sigmoidoscopy, did not show any significant differences between the

two groups. The investigators concluded that olestra did not affect the activity of the ulcerative colitis or Crohn's disease in the patients.

A study in an animal model of inflammatory bowel disease showed that mucosal damage did not result in absorption of olestra (52). Guinea pigs with gastrointestinal tract lesions from consumption of poligeenan did not absorb ^{14}C-labeled olestra as shown by analyses of tissue lipids and sucrose in urine.

(iv) Olestra and microflora in the colon. The interaction of olestra with microbes in the colon has been studied. Early studies showed no evidence that olestra was metabolized by microflora in the human colon (10), since quantitative recovery of excreted sucrose polyester was consistent with the absence of degradation. This observation was important since the entry of triacylglycerols into the colon results in the symptoms of steatorrhea including severe diarrhea due to hydrolysis of triacylglycerols by bacterial lipases to fatty acids and soaps. *In vitro* studies confirmed microflora from human feces did not metabolize olestra (53). Fecal samples from subjects who had consumed olestra for 3–4 wk were used to inoculate anaerobic media containing ^{14}C-labeled olestra, and there was no evidence of change to the olestra molecule or production of metabolites from it.

It was shown that olestra in the diet reduced the chemical modification of sterols in the colon, and this observation was attributed to isolation of the sterols from microflora by sequestration in the olestra phase rather than an alteration in the microflora (54). Siigur *et al.* (55) confirmed this result. In addition to measuring the conversion of cholesterol to coprostanol, Siigur *et al*. found that olestra did not consistently change three other markers of intestinal microecology. Fecal tryptic activity degradation, mucin degradation, and β-aspartyl glycine (a product of microorganisms in the colon) were altered by dietary olestra. In that study, dietary olestra did reduce the conversion of bilirubin to urobilin. This effect was attributed to partitioning of bilirubin, a lipophilic substance, into the olestra phase with a reduction in its availability for bacterial modification.

There is other evidence that microecology of the large intestine is normal when olestra is part of the diet. A study of olestra's effect on breath hydrogen and methane production and fecal microbe counts confirmed this conclusion (56). Among 94 subjects who received olestra for 36 d, there were no significant effects on the numbers or distribution of colonic bacteria. Breath hydrogen and methane were unaltered by dietary olestra. A trend was seen for lower hydrogen in the breath of subjects who ate both olestra and a high level of fiber, but the authors concluded that olestra does not interfere with normal intestinal fermentation of dietary fiber and does not alter gut microflora population.

(v) Intestinal morphology and histology. Studies of chronic ingestion of olestra, including lifetime studies of rodents, showed that olestra does not cause changes in the histology or morphology of any intestinal tissues (57). These observations are consistent with a lack of cell injury from olestra itself as well as the absence of any reactive chemical species produced from olestra during gastrointestinal transit. Neither pancreatic nor bacterial enzymes alter olestra into products with activities that are known to alter mucosal structure or function.

CONCLUDING COMMENTS

Olestra is currently approved for use only in the preparation of snack foods, as described in the olestra food additive regulation (4). Given the understanding of the ingestion patterns of snack food items, it has been possible to estimate the expected use of olestra (50). The estimates of mean chronic intake of olestra in the United States ranged from 1.8 (females above 64 yr) to 4.7 g/d (males of ages 13–17 and 18–44). The 90th percentile range of intake was 4.1 (females above 64) to 11.0 g/d (males 18–44). Estimated mean acute intakes ranged from 5.5 (females above 64) to 16.5 g/d (males 13–17). The 90th percentile acute intakes ranged from 10.2 (females above 64) to 24.0 g/d (females 13–17). All of these estimates are based on the assumption that 100% of the lipid in the snacks will be olestra. Initial postmarketing surveillance data show consumption to be less than these estimates. Based on interviews with 1525 adults who consumed olestra snacks, the median consumption was 8.1 g of olestra per month, with 90th percentile consumption of 64.4 g per month (58).

Studies which have shown that gastrointestinal effects do not differ between olestra-based snacks and regular triacylglycerol-based chips included realistic consumption levels that reflect these snack intake patterns. In one study, the olestra-chip group ate approximately 18 g/d of olestra (2 ounces of chips) and reported no more gastrointestinal symptoms than the regular triacylglycerol chip group (2). Another study rechallenged subjects who had called a toll-free number with reports of gastrointestinal problems after the use of purchased snacks that contained olestra (1). In this double-blind, placebo-controlled test, there were no differences in gastrointestinal symptoms between subjects who received olestra-based chips and those who received regular, digestible fat-based chips. In this study, subjects ate 2 ounces of chips, which contained approximately 18 g of olestra. These studies were carried out with snacks fried in a formulation of olestra (Olean®) that resulted from a basic understanding of the phase behavior and rheology of olestra. Through optimization of the solid sucrose polyester fatty acid composition, Olean® was produced to address the gastrointestinal issues associated with a nonabsorbable fat substitute while delivering the desired taste and texture benefits of fat in the mouth. Olean® is a direct consequence of a fundamental understanding of the properties and chemistry of edible lipids and lipid substitutes.

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Effect of Dietary Docosahexaenoic Acid on Desaturation and Uptake *in vivo* of Isotope-Labeled Oleic, Linoleic, and Linolenic Acids by Male Subjects

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ABSTRACT: The effect of dietary docosahexaenoic acid (22:6n-3, DHA) on the metabolism of oleic, linoleic, and linolenic acids was investigated in male subjects (n = 6) confined to a metabolic unit and fed diets containing 6.5 or <0.1 g/d of DHA for 90 d. At the end of the diet period, the subjects were fed a mixture of deuterated triglycerides containing 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4]. Blood samples were drawn at 0, 2, 4, 6, 8, 12, 24, 48, and 72 h. Methyl esters of plasma total lipids, triglycerides, phospholipids, and cholesterol esters were analyzed by gas chromatography–mass spectrometry. Chylomicron triglyceride results show that the deuterated fatty acids were equally well absorbed and diet did not influence absorption. Compared to the low-DHA diet (LO-DHA), clearance of the labeled fatty acids from chylomicron triglycerides was modestly higher for subjects fed the high DHA diet (HI-DHA). DHA supplementation significantly reduced the concentrations of most n-6[d2] and n-3[d4] long-chain fatty acid (LCFA) metabolites in plasma lipids. Accumulation of 20:5n-3[d4] and 22:6n-3[d4] was depressed by 76 and 88%, respectively. Accumulations of 20:3n-6[d2] and 20:4n-6[d2] were both decreased by 72%. No effect of diet was observed on acyltransferase selectivity or on uptake and clearance of 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4]. The results indicate that accumulation of n-3 LCFA metabolites synthesized from 18:3n-3 in typical U.S. diets would be reduced from about 120 to 30 mg/d by supplementation with 6.5 g/d of DHA. Accumulation of n-6 LCFA metabolites synthesized from18:2n-6 in U.S. diets is estimated to be reduced from about 800 to 180 mg/d. This decrease is two to three times the amount of n-6 LCFA in a typical U.S. diet. These results support the hypothesis that health benefits associated with DHA supplementation are the combined result of reduced accretion of n-6 LCFA metabolites and an increase in n-3 LCFA levels in tissue lipids.

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Various animal, human, and epidemiology studies have suggested that dietary docosahexaenoic acid (22:6n-3, DHA) has a beneficial effect on a variety of chronic diseases. These and other studies related to the physiological and health effects of n-3 fatty acids were recently reviewed (1,2).

Results from the clinical part of this dietary DHA study showed that supplementation of normal diets with 6.5 g/d of DHA increased the DHA content of platelet lipids by threefold and the eicosapentaenoic acid (EPA, 20:5n-3) content by more than sevenfold. The DHA and EPA content of plasma total lipid increased by 4.4- and 8.9-fold, respectively. Triglyceride concentrations were decreased by 16.7% and apolipoprotein E levels increased by 71% (3,4). Dietary DHA supplementation did not produce measurable changes in blood coagulation, *in vivo* bleeding times, platelet function, or thrombotic tendencies. Inhibition of immune response was not observed, but white blood cell count was significantly reduced due to a decrease in granulocytes (5). Synthesis of thromboxane was reduced by 35% ($P = 0.013$), and prostacyclin was not significantly reduced. (6).

Several studies investigated the inhibition by dietary n-6 fatty acids on conversion of 18:3n-3 to n-3 long-chain fatty acid (LCFA) metabolites (1,2). Conversion of 18:3n-3 to n-3 LCFA metabolites is reduced by 18:2n-6, and the DHA content of tissue lipids is not greatly increased by diets containing large amounts of 18:3n-3 (2). EPA and linoleic acid are reported to inhibit delta-6 and delta-5 desaturase activities in human CaCo-2 enterocyte cell lines (7). Recently we showed that dietary arachidonic acid significantly inhibited the accumulation of n-6 LCFA metabolites synthesized from isotope-labeled 18:2n-6 (8) and, paradoxically, stimulated the conversion of isotope-labeled 20:3n-6 to 20:4n-6 (9). Incorporation and acyltransferase selectivity of deuterated 20:4n-6 were not altered in subjects fed arachidonic acid-supplemented diets but turnover was reduced (10). In general, desaturase activity is inhibited by most dietary n-6 and n-3 fatty acids, but acyltransferase selectivity is generally not influenced.

The results from studies with a variety of fatty acid structures suggest that the physiological effects of dietary DHA may be related to its effect on desaturation and incorporation of 18:1n-9, 18:2n-6, and 18:3n-3. Thus, the objective of this

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Abbreviations: C18, 18-carbon chain length; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; GC, gas chromatography; HI-DHA, high docosahexaenoic acid diet; LCFA, long-chain fatty acid; LO-DHA, low docosahexaenoic acid diet; MS, mass spectroscopy; TLC, thin-layer chromatography.

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stable isotope study was to determine if DHA supplementation inhibits desaturation and elongation of 18:2n-6 and 18:3n-3 and to investigate the effect of DHA on absorption, uptake, and turnover of 18:1n-9, 18:2n-6, and 18:3n-3 in human subjects.

EXPERIMENTAL PROCEDURES

Study design. Six Caucasian male subjects between the ages of 28 and 39 yr were housed in a metabolic ward. Physical examinations and clinical blood profile data indicated that the subjects were in good health. Medical histories indicated no evidence of congenital ailments. The subjects' height/weight ratios, blood pressures, fasting serum cholesterol, and triglyceride concentrations were within normal ranges. Physical characteristics and plasma lipid profiles for the subjects are summarized in Table 1. Plasma lipid data were obtained at the end of the 90-d diet period. Institutional ethical approval for the study protocol was obtained from the Agricultural Research Service's Human Studies Institutional Review Committee and the University of California at Davis, Human Subjects Committee. Informed consent was obtained from each subject before initiation of the study.

Diets. The subjects were provided a high DHA (HI-DHA) diet that contained 6.5 g/d of 22:6n-3 or a low DHA (LO-DHA) diet that contained <0.1 g/d of 22:6n-3. DHASCO™ oil (15 g/d) was added as the source of 22:6n-3 in the HI-DHA diet. The DHASCO™ oil was provided by Martek Bioscience Corp. (Columbia, MD), and is the Trademark name for a microalgal single-cell oil that contains a high level of DHA. An equal amount of safflower oil (15 g/d) was added to the LO-DHA diet. The subjects were fed these diets for 90 d prior to the deuterium isotope experiment. The fatty acid compositions of the diets were similar except for 22:6n-3. The fatty acid and macronutrient compositions of the diets are summarized in Table 2. The fatty acid composition of the DHASCO™ oil (wt%) was 3.5% 12:0; 13.1% 14:0; 11.4% 16:0; 1.4% 16:1; 1.2% 18:0; 30.0% 9*c*-18:1; 1.1% 18:2n-6; <0.01% 18:3n-3; 0.4% 22:0; 38.0% 22:6n-3. All meals were prepared from weighed food portions and their consumption was monitored. Duplicate meals were collected and analyzed for fatty acid composition. Values for total fat, protein, and carbohydrate were obtained by analysis of diet composites and were similar to calculated values based on food composition data in USDA Handbook 8 (11). No significant changes in the subjects' weights were observed during the controlled diet period, indicating a stable energy balance. Detailed information on the study design and diet compositions was described previously (3).

Deuterated fatty acids. Deuterium-labeled methyl *cis*-9-octadecenoate-14,14,15,15,17,18-d6 (18:1n-9[d6]) and methyl *cis*-9,*cis*-12-octadecadienoate-12,13-d2 (18:2n-6[d2]) were synthesized, purified, and converted to their triglycerides by previously described methods (12,13). Methyl *cis*-9,*cis*-12,*cis*-15-octadecatrienoate-12,13,15,16-d4 (18:3n-3[d4]) was prepared by a Lindlar-catalyzed deuteration of the diacetylene precursor (9c,12a,15a-18:3). The preparation of 9c,12a,15a-18:3 was achieved by modification of a previously described procedure (14). The isotopic purity of the deuterated fatty acids was: 88.6% for 18:1n-9[d6], 78.6% for 18:2n-6[d2], and 83.6% for 18:3n-3[d4]. Chemical purity was 99% for 18:1n-9[d6], 88% for 18:2n-6[d2], and 95% for 18:3n-3[d4]. The impurities in these samples were *trans* fatty acid isomers. The 18:2n-6[d2] sample also contained small amounts of nonlabeled 16:0, 18:0, and 18:1n-9.

Stable isotope study design. A mixture of deuterated triglycerides containing 52.6 g of deuterated 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4] was emulsified with 763.5 g of no-fat yogurt. After a 12-h overnight fast, each subject was fed an equal portion of the total mixture between 7:00 and 7:50 A.M. The amount of deuterated triglycerides fed to each subject was 3.1 g 18:1n-9[d6], 2.4 g 18:2n-6[d2], and 2.3 g 18:3n-3[d4] after adjusting the weights for chemical and isotopic purity. Subjects were provided a no-fat breakfast at 8:00 A.M. to avoid dilution of the labeled fatty acid with exogenous fatty acids. A low-fat (*ca.*15% fat calories) lunch containing no added safflower or DHASCO™ oil was provided at 12:00, and the usual diets were fed at 5:00 P.M. and on subsequent days.

Sample collection. Blood samples (6 to 10 mL each) were collected by venipuncture at 0, 4, 6, 8, 12, 24, 48, and 72 h and used to isolate plasma lipid classes. Additional blood samples (*ca.*12 mL) were collected at 2, 4, 6, 8, and 12 h and used to isolate chylomicron total lipid. Chylomicron fractions were isolated by standard preparative ultracentrifuge meth-

TABLE 1
Physical Characteristics and Plasma Lipid Profiles of Subjects[a]

Subject	Diet	Body weight (kg)	BMI[b] (kg/m^2)	Plasma lipids[c] (mg/dL) Triglyceride	Cholesterol	Total Lipid[d]
1	LO-DHA	74.5	25.2	66	201	319
2	LO-DHA	67.7	23.1	210	243	588
3	LO-DHA	66.8	24.3	70	138	297
4	HI-DHA	61.8	26.3	65	162	245
5	HI-DHA	73.6	25.2	68	202	329
6	HI-DHA	85.0	21.8	74	170	313

[a]Males, nonsmokers, ages 28–39 yr, normal hypertensive.
[b]BMI, body mass index.
[c]Fasting plasma lipid concentrations at end of the 90-d diet period.
[d]mg of total lipid as methyl ester/100 mL of plasma. LO-DHA, low docosahexaenoic acid diet; HI-DHA, high DHA diet.

TABLE 2
Composition of Diets Fed to HI-DHA and LO-DHA Subject Groups[a]

Fatty Acid	HI-DHA (%)	LO-DHA (%)	Macronutrient	HI-DHA	LO-DHA
Saturates	29.5	26.9	Protein, en%	15.2	14.6
t-18:1	6.2	7.0	Fat, en%	30.9	29.0
c-18:1	29.8	30.1	Carbohydrate, en%	53.9	56.4
18:2n-6	21.6	28.3			
18:3n-3	2.6	3.2	Cholesterol, mg/d	360	360
20:4n-6	0.1	0.1	Polyunsaturated, n-6, en%	6.5	8.5
20:5n-3	0.4	0.3	Polyunsaturated, n-3, en%	2.8	1.1
22:6n-3	6.5	< 0.1			
Other	1.2	1.1	Total Kcal/d	2800	2800
Unknown	2.0	2.8			
Total	99.9	99.9			

[a]See Table 1 for abbreviations.

ods (15). Several representative chylomicron samples were analyzed by electrophoresis to confirm the purity of the chylomicron fractions (16).

Analysis of plasma lipid fatty acids. Plasma lipids were extracted with 2:1 chloroform/methanol (17). Preparative thin-layer chromatography (TLC) was used to isolate triglyceride, cholesterol ester, and phospholipid fractions from plasma total lipid (18). Known weights of triheptadecanoin, cholesterol heptadecanoate, and diheptadecanyl-*sn*-phosphatidylcholine (Applied Science, State College, PA) were added as internal standards to the total lipid extract. Methyl esters of the isolated lipid classes were prepared by heating the samples with a 5% HCl–methanol solution (19).

The percentages of labeled and unlabeled fatty acids in the plasma lipid classes were obtained by gas chromatography–mass spectroscopy (GC–MS) analysis of their methyl esters. Data for the added 17:0 internal standards were used to determine the concentrations (μg/mL) of each deuterated and nondeuterated fatty acid. A Hewlett-Packard model 5988A quadrupole mass spectrometer (Palo Alto, CA) operated in a positive chemical ionization mode with isobutane as the ionization reagent was used to analyze the methyl ester samples. The GC–MS methodology utilized selected ion monitoring of the appropriate ion masses for the fatty acid in each GC peak. The areas for each of the ion masses monitored were obtained by integration of the peaks. The GC–mass spectrometer was equipped with a Supelcowax 10 fused-silica column (30 m × 0.25 mm; Supelco Inc., Bellefonte, PA). The column was temperature programmed from 165 to 265°C at 5°C/min with a 20-min final hold. The specific operating conditions and computer-assisted storage and processing of the MS data were described previously (20,21). Response factors were determined by analysis of standard mixtures containing weighed amounts of pure fatty methyl esters purchased from Nu-Chek-Prep Inc. (Elysian, MN) and Applied Science. The accuracy of the GC–MS data was determined by adding known weights of 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4] to samples of plasma triglyceride, phospholipid, and cholesterol ester from subjects that were not fed the deuterated fatty acid mixture. The weight for each of the deuterated fatty esters added was equal to about 0.3% of the total unlabeled fatty acids in the samples. Standard deviations were based on three replicate analyses. The standard deviations for the 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4] in the triglyceride, phospholipid, cholesterol ester spiked samples ranged from ± 0.002 to 0.004%.

The accuracy of the unlabeled methyl ester data obtained by GC–MS was checked by analysis of a subset of samples using a Varian model 3400 gas chromatograph (Varian, Walnut Creek, CA), equipped with a 100 m × 0.25 mm SP2560 fused-silica capillary column (Supelco) and a flame-ionization detector. Operating conditions were: split ratio, 1:100; linear velocity of helium, 21 cm/s; detector and injection temperature, 235°C. The identification and accuracy of the GC peaks areas were confirmed by comparison to data for authentic standards and mixtures of known composition.

Statistical analysis and calculations. Data were analyzed with the SAS-PC statistical software package from Statistical Analysis System Institute (Cary, NC). A two-tailed, unpaired *t*-test was used to test for significant differences between data from subjects fed the LO-DHA and HI-DHA diets (22). The concentration data (μg/mL) for the deuterated fatty acids and their metabolites were normalized to compensate for differences in subject plasma lipid concentrations. Normalization was achieved by dividing the μg of deuterated fatty acid/mL plasma data by the total plasma lipid methyl ester concentrations (mg/mL). The μg of deuterated fatty acid per mg of total plasma lipid data were then divided by the weight of deuterated fatty acids in the fed mixtures to normalize the data to a per gram of deuterated fatty acid fed basis. Time-course curves were produced by plotting the normalized μg deuterated fatty acid/mg of total lipid data for the eight samples collected over the 72-h study period. The n-6 and n-3 total area data shown in the bar graphs are weighted averages for the samples collected and were obtained by calculating the total area under the time-course curve, as described previously (8,9,23). The values obtained represent the weighted averages of the eight plasma lipid samples collected over the 72-h period.

RESULTS

Absorption. Time-course curves for the μg of deuterium-labeled fatty acids per mg of total chylomicron lipid are shown in Figure 1. The results show that the labeled fats were equally well absorbed. The shapes of all of the curves for the

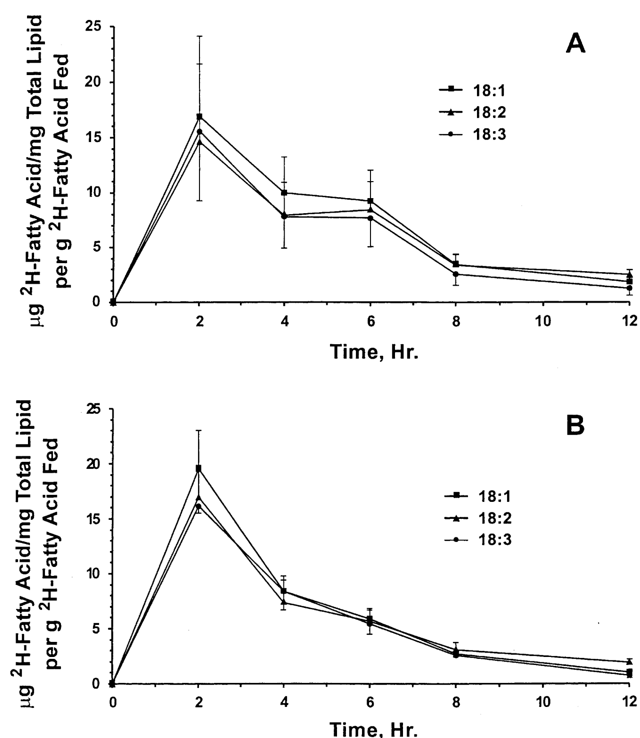

FIG. 1. Time-course plots for uptake and clearance of deuterium-labeled oleic, linoleic, and linolenic acids in chylomicron total lipids of subjects fed the low docosahexaenoic acid (LO-DHA) (A) and high docosahexaenoic acid (HI-DHA) (B) diets. Each data point is the average for data from three subjects. Error bars equal standard deviation. Error bars are not shown when less than the size of the symbol.

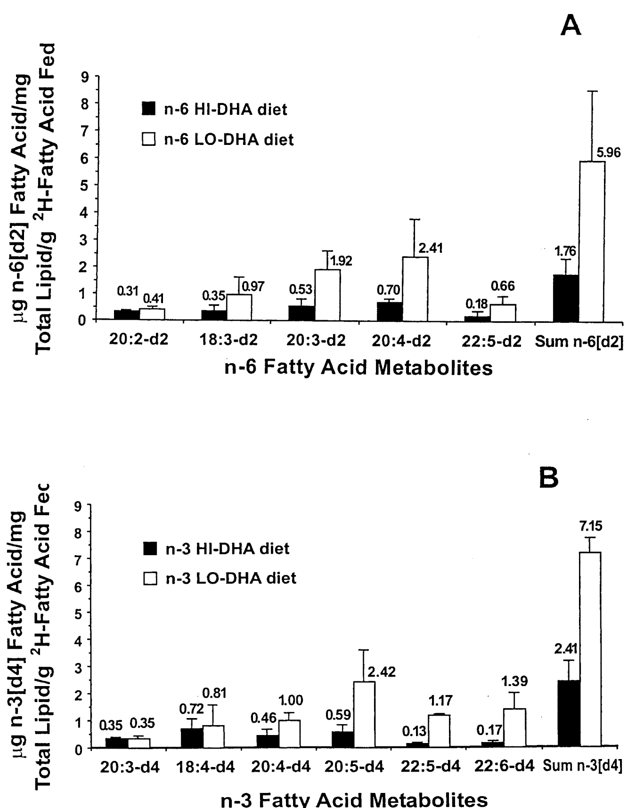

FIG. 2. Weights for n-6[d2] (A) and n-3[d4] (B) long-chain fatty acid metabolites in plasma total lipid from male subjects fed diets containing <0.1 g/d (LO-DHA) and 6.5 g/d (HI-DHA) of 22:6n-3. The value for each bar is the average of the area-under-curve data from three subjects. Error bars equal standard deviation. See Figure 1 for abbreviations.

LO-DHA and HI-DHA subject groups are similar except the amount of each labeled fatty acid was consistently about 30% lower ($P < 0.01$) in the 6-h samples from the HI-DHA group. These results suggest that dietary DHA supplementation produced a transient increase in chylomicron triglyceride clearance. The results are consistent with the decrease in triglyceride levels observed for the HI-DHA diet group in the clinical part of this study (4).

Conversion to LCFA metabolites. The incorporation of n-6 [d2] and n-3[d4] long-chain fatty acid products into plasma total lipid are compared in Figure 2 for subjects fed the LO-DHA and HI-DHA diets. Total weight data for the n-6[d2] and n-3[d4] LCFA metabolites were calculated from the areas under the time-course curves. Each bar represents the average for three subjects. The total amount of deuterated n-6 LCFA in the entire plasma pool was 9.3 ± 0.58 mg for the HI-DHA diet group and 40.25 ± 2.6 mg per g of 18:2n-6[d2] fed for the LO-DHA diet group. The total amount of deuterated n-3 LCFA was 12.7 ± 0.77 mg for the HI-DHA diet group and 48.25 ± 0.55 mg per g of 18:3n-3[d4] fed for the LO-DHA diet group.

The plasma total lipid data show that supplementation with dietary DHA significantly reduced the total amounts of n-6 [d2] and n-3[d4] LCFA metabolites synthesized by desaturation–elongation of 18:2n-6[d2] and 18:3n-3[d4]. Weights for the n-6[d2] LCFA metabolites were reduced by 64 to 73% with an overall decrease of 70% ($P < 0.05$), and weights for the n-3[d4] LCFA metabolites were reduced by 54 to 89% with an overall decrease of 66% ($P < 0.001$). The amount of elongated-only metabolites, 20:2n-6[d2] and 20:3n-3[d4], was not significantly different.

Uptake and turnover. Plasma triglyceride, total phospholipid, and cholesterol ester data for incorporation and disappearance of 18:1n-9[d6], 18:2n-6[d2], and 18:3n-3[d4] are compared in Figures 3, 4, and 5. Each time-course curve represents the average for three subjects from the LO-DHA and HI-DHA diet groups. The LO-DHA and HI-DHA diet time-course curves for each plasma lipid class are similar. Weights for the deuterated fatty acids were consistently lower in the plasma triglyceride 6-h samples for the subjects fed the HI-DHA diet ($P < 0.04$), but data for other time points were not significantly different. The average weight for 18:2n-6[d2] in the plasma total phospholipid and cholesterol ester samples from both diet groups was four to five times higher than for 18:1n-9[d6] and 18:3n-3[d4]. These results show that supplementation with dietary DHA does not affect uptake and turnover of dietary 18:1n-9, 18:2n-6, and 18:3n-3 in plasma triglyceride, total phospholipid, or cholesterol ester.

The total plasma lipid data for isotope-labeled oleic,

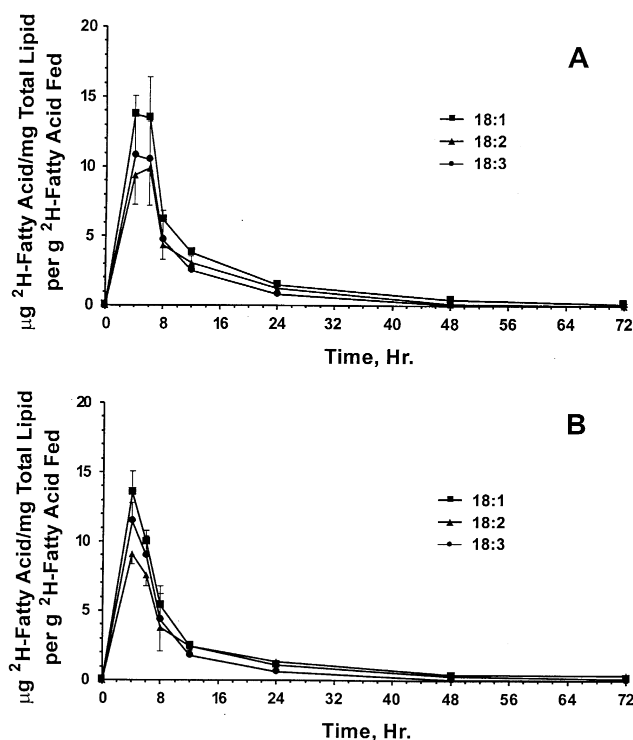

FIG. 3. Time-course plots for uptake and clearance of deuterium-labeled oleic, linoleic, and linolenic acids in plasma triglycerides of subjects fed the LO-DHA (A) and HI-DHA (B) diets. Each data point is the average for data from three subjects. Error bars equal standard deviation. Error bars are not shown when less than the size of the symbol. See Figure 1 for abbreviations.

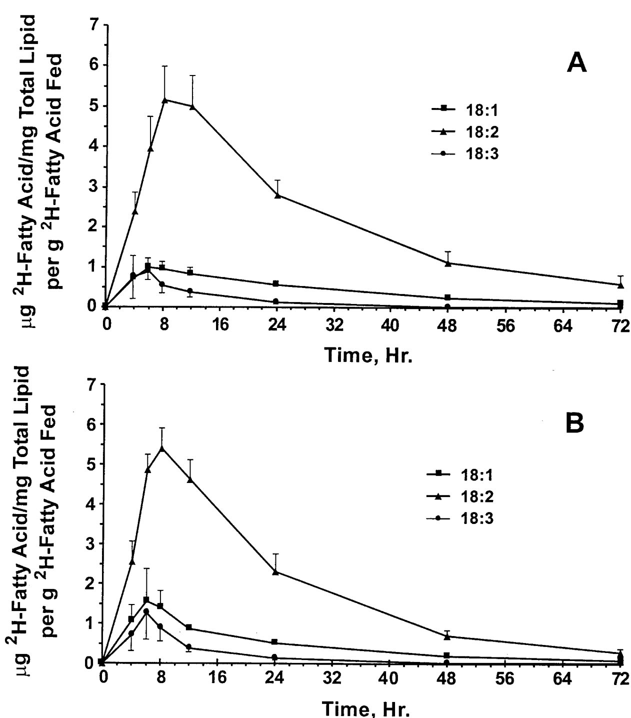

FIG. 4. Time-course plots for uptake and clearance of deuterium-labeled oleic, linoleic, and linolenic acids in plasma total phospholipid of subjects fed the LO-DHA (A) and HI-DHA (B) diets. Each data point is the average for data from three subjects. Error bars equal standard deviation. Error bars are not shown when less than the size of the symbol. See Figure 1 for abbreviations.

linoleic, and linolenic acids are plotted as a percentage of the total deuterated fatty acids in Figure 6. The percentage data are calculated from under-the-curve total weight data. The percentage of 18:2n-6[d2] was three to four times higher than for 18:1n-9[d6], and the percentage of 18:1n-9[d6] was 3 to 3.5 times higher than for 18:3n-3[d4]. Inspection of Figures 3, 4, and 5 indicates that plasma phospholipid and cholesterol ester lipid classes are responsible for most of the difference. These results illustrate the previously reported high selectivity for incorporation of 18:2n-6 and the strong discrimination against 18:3n-3 relative to 18:1n-9 (23). The combined results displayed in Figures 3 to 6 show that dietary DHA does not influence acyltransferase selectivity for the C18 unsaturated fatty acids.

DISCUSSION

In clinical studies long-chain n-3 fatty acids are observed to decrease fasting chylomicron and plasma triglyceride levels and to improve postprandial clearance (4,24,25). It was suggested that both effects reduce coronary heart disease risk (26–28). The enhanced clearance of the deuterium-labeled fatty acids in the 6-h chylomicron total lipid and plasma triglyceride samples from the HI-DHA diet group are consistent with results from clinical studies. These results provide additional evidence that supplementation with DHA containing no EPA may enhance postprandial triglyceride clearance.

Most clinical results, including this study, show that DHA supplementation does not affect serum cholesterol levels (4,24). These clinical results suggest that dietary DHA does not alter the mechanisms responsible for regulation of cholesterol synthesis and removal. This metabolic study shows that DHA supplementation did not alter phospholipid and lecithin/cholesterol acyltransferase selectivity or the relative proportions of deuterated 18:1n-9, 18:2n-6, and 18:3n-3 incorporated into plasma phospholipid and cholesterol ester. The absence of an effect of DHA supplementation on 18:1n-9, 18:2n-6, and 18:3n-6 metabolism is consistent with the lack of an effect of DHA on serum cholesterol levels. The plasma cholesterol ester results also suggest that dietary DHA may not alter liver cholesterol/cholesterol ester ratios and cholesterol oleate/cholesterol linoleate ratios that influence low density lipoprotein receptor activity. The metabolic results from this study suggest that the reason DHA does not influence serum cholesterol is that DHA does not affect dietary C18 unsaturated fatty acid metabolism and the cholesterol regulation mechanisms that are influenced by C18 unsaturated fatty acids.

DHA supplementation resulted in a substantial decrease in accumulation of most n-6[d2] and n-3[d4] LCFA metabolites. The results show that dietary 18:2n-6 and DHA do not

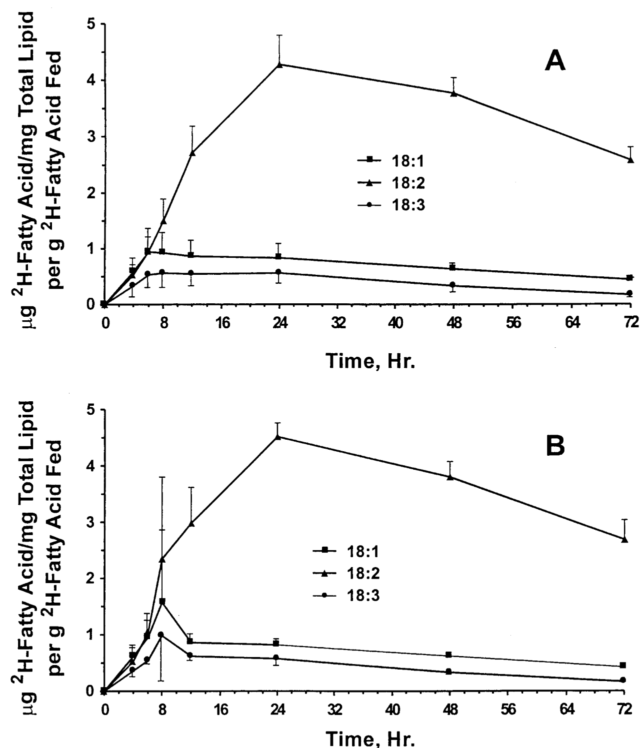

FIG. 5. Time-course plots for uptake and clearance of deuterium-labeled 18:1n-9, 18:2n-6, and 18:3n-3 in plasma cholesterol ester of subjects fed the LO-DHA (A) and HI-DHA (B) diets. Each data point is the average for data from three subjects. Error bars equal standard deviation. Error bars are not shown when less than the size of the symbol. See Figure 1 for abbreviations.

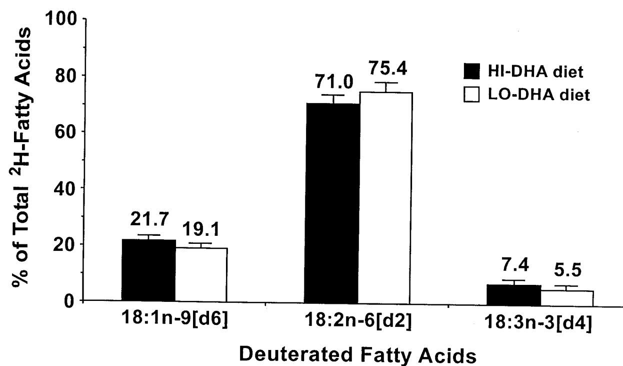

FIG. 6. Percentage of deuterated oleic, linoleic, and linolenic acids in human plasma total lipid. Each bar is the average for data from three subjects. Error bars equal standard deviation. See Figure 1 for abbreviations.

equally inhibit accretion of n-6 and n-3 LCFA metabolites. Dietary intake of 18:2n-6 was 7 g/d higher, and DHA intake was 6.5 g/d lower for the LO-DHA diet group compared with the HI-DHA group. The total plasma lipid data shown in Figure 2 for the HI-DHA and LO-DHA groups would have been similar if dietary 18:2n-6 and DHA equally inhibited accumulation of deuterated n-6 and n-3 LCFA metabolites.

Summary and implications. DHA supplementation slightly enhanced triglyceride clearance and reduced the concentrations of most desaturated-elongated n-6[d2] and n-3[d4] LCFA metabolites by more than 50%. Dietary 18:2n-6 and DHA did not equally inhibit accumulation of LCFA metabolites. DHA supplementation did not influence C18 unsaturated fatty acid absorption, incorporation or turnover in human plasma lipids. There was no effect on elongation or the relative selectivity of the acyltransferases for oleic, linoleic, and linolenic acids.

Feedback inhibition by 6.5 g/d of dietary DHA is estimated to reduce accumulation of n-3 LCFA metabolites synthesized from the 2.5 g of 18:3n-3 (29,30) in typical U.S. diets from 121 to 32 mg/d or by *ca.* 66%. Accumulation of n-6 LCFA metabolites synthesized from the 20 g of 18:2n-6 present in U.S. diets (29,30) is estimated to be reduced from 805 to 186 mg/d, or by *ca.* 70%. For the HI-DHA diet, inhibition by DHA supplementation on n-3 LCFA synthesis is relatively unimportant because it represents only about 1.5% of the *ca.* 6.5 g/d n-3 LCFA in the HI-DHA diet. Inhibition of n-6 LCFA metabolite accumulation is important. For the HI-DHA diet group, available n-6 LCFA metabolites were reduced from about 900 mg/d (100 mg from diet and 800 mg from 18:2n-6 conversion) to about 380 mg/d. This large difference suggests that an important effect of dietary DHA is the reduction of n-6 LCFA synthesized from 18:2n-6. The results of this study provide additional evidence to support the hypothesis that reduction of n-6 LCFA metabolite accretion and the increase in n-3 LCFA levels in tissue lipids both contribute to the health benefits attributed to fish oil and DHA supplementation.

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Fatty Acid Analysis of Blood Serum, Seminal Plasma, and Spermatozoa of Normozoospermic vs. Asthenozoospermic Males

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ABSTRACT: Docosahexaenoic acid (DHA; 22:6n-3) is found in extremely high levels in human ejaculate with the majority occurring in the spermatozoa. However, the relative concentration of DHA and other fatty acids, in blood serum, seminal plasma, and spermatozoa of asthenozoospermic vs. normozoospermic individuals is not known. We analyzed the phospholipid fatty acid composition of blood serum, seminal plasma, and spermatozoa of normozoospermic men and asthenozoospermic men in order to determine if DHA levels, as well as the levels of other fatty acids, differed. The serum phospholipid DHA levels were similar in the two groups, suggesting similar intakes of dietary DHA. On the other hand, seminal plasma levels of DHA (3.0 vs. 3.7%) and total polyunsaturated fatty acids (PUFA) (11.8 vs. 13.5%) were significantly lower in asthenozoospermic vs. normozoospermic men, respectively, while 18:1 (19.0 vs. 16.8%) and monounsaturated fatty acids (MUFA) (24.2 vs. 21.7%) were significantly higher in the asthenozoospermic vs. the normozoospermic men. Spermatozoa from asthenozoospermic men had higher levels of 18:1, 20:0, 22:0, 22:1, and 24:0 than sperm from normozoospermic men, and lower levels of 18:0 and DHA (8.2 vs. 13.8%). Furthermore, total MUFA (19.3 vs. 16.5%) was higher and total PUFA (19.0 vs. 24.0%), n-3 fatty acids (9.3 vs. 14.6%), and the ratio of n-3 to n-6 fatty acids (1.0 vs. 1.6) were lower in the asthenozoospermic men. Therefore, in asthenozoospermic individuals, lower levels of DHA in the seminal plasma, but not in the blood serum, mimic the decreased concentrations of DHA in the spermatozoa. This suggests that the lower concentrations of spermatozoon DHA in these individuals are due not to dietary differences but to some type of metabolic difference in the asthenozoospermic men.

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In recent years, the prognosis for infertile couples has improved dramatically. Male-factor infertility, however, plays a role in the inability of approximately 45% of childless couples to achieve pregnancy, and still remains difficult to treat (1,2). Poor sperm-forward motility (asthenozoospermia) is considered to contribute to the infertility of a significant number of males, and many cases of decreased sperm motility are not completely understood (3).

Docosahexaenoic acid (DHA; 22:6n-3) is found in extremely high levels in human ejaculate (4,5), and the concentration of DHA in both ejaculate and spermatozoa has been suggested to be positively associated with sperm motility in various species including chickens (6) and humans (4,5,7,8). Phospholipids (PL) in normal human spermatozoa are approximately 14.3% DHA (7,9). In rhesus monkeys, the majority of DHA appears to be in phosphatidylethanolamine and phosphatidylcholine fractions (10). Interestingly, DHA levels have been shown to be lower in complete ejaculates from asthenozoospermic individuals vs. normozoospermic individuals (4,5), as well as in certain sperm fractions from asthenozoospermic vs. normozoospermic individuals (7). DHA may contribute to the membrane fluidity necessary for the motility of sperm tails (11), and as such it makes up almost 20% of PL fatty acids in the tails of monkey sperm and approximately 14% of PL fatty acids in the tails of human sperm (7). In humans (7), the majority of PL DHA appears to be in the head (33.7 vs. 14.0% in the tail), whereas in monkeys (11), the majority of DHA appears to be in the tail (20.0 vs. 4.5% in heads). The role of DHA in the motility of the spermatozoa is not completely clear although it has been suggested that DHA is involved in the regulation of free fatty acid utilization by sperm (12,13). DHA can also be slowly metabolized to 19,20-dihydroxy-4,7,10,13,16-docosapentaenoic acid by monkey seminal vesicles (14).

Measures of eicosapentaenoic acid (EPA) and DHA in serum and plasma PL have been considered useful biological indicators for EPA/DHA intake and nutritional status (15–17). It is not clear whether DHA status in other biological fluids (i.e., seminal plasma, sperm) also reflects dietary intake or whether the levels of DHA in the sperm or seminal plasma reflect the DHA status of blood serum.

The purpose of the present study was to determine whether DHA status of blood-serum PL, seminal-plasma PL, and sperm PL differs between normozoospermic men and astheno-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MUFA, monounsaturated fatty acid; PL, phospholipid; PUFA, polyunsaturated fatty acid.

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zoospermic men, and if so, to determine whether DHA levels in the sperm or seminal plasma reflect serum DHA levels. The fatty acid analysis of total sperm from the ejaculate of asthenozoospermic vs. normozoospermic individuals has never been investigated, nor has the comparison been made in terms of the fatty acid analysis of seminal plasma in these two groups. The inclusion of blood serum was of interest in order to rule out possible dietary differences influencing the sperm/seminal plasma fatty acid status.

MATERIALS AND METHODS

Subjects and experimental design. The subjects were 73 healthy males (43 control and 30 asthenozoospermic individuals) who were patients of the Reproductive Endocrinology and Infertility Program at the London Health Sciences Centre seeking diagnostic semen analysis. Approval for this study was granted by the Human Ethics Committee of the London Health Sciences Centre and the University of Western Ontario, and written informed consent was obtained from each subject. Subjects were determined to be either normozoospermic (control) or asthenozoospermic after analysis of sperm motility. The sperm motility criteria applied in our study to the normozoospermic and asthenozoospermic individuals were ≥60% and ≤50%, respectively, based on the recommendation of Mortimer (18).

Semen analysis. Semen samples were produced on-site by masturbation into a sterile container and allowed to liquefy for 30 min before analysis. Conventional manual assessments were performed by an experienced technician, in accordance with the recommendations of the World Health Organization (19), for ejaculate volume and sperm motility, as well as sperm concentration using hemacytometer count. Following the assessment, 0.5 or 1.0 mL aliquots (volume depending on the availability of the individual) of each sample were placed into an Eppendorf tube (1.5 mL capacity) and centrifuged at high speed (15,000 rpm) for 15 min to pellet the sperm. The supernatant seminal plasma was then carefully removed and transferred to a separate container. Both sperm and seminal plasma fractions were stored at −70°C until needed for fatty acid analysis of total PL.

Blood collection. Blood was collected from each subject (asthenozoospermics and normozoospermics) by antecubital venipuncture into siliconized tubes and centrifuged at 1200 × *g* for 10 min to obtain serum. Serum was stored at −70°C until analysis of serum total PL fatty acid composition.

Fatty acid analysis of PL of serum, seminal plasma, and sperm. The fatty acid composition of total PL from serum and seminal plasma was determined following lipid extraction as described by Folch *et al.* (20). Sperm total PL fatty acid composition was determined following lipid extraction as described by Bligh and Dyer (21). Thin-layer chromatography, transmethylation, and gas–liquid chromatography were used in procedures similar to those previously described (22–24). Gas–liquid chromatography of the fatty acid methyl esters was performed using a Varian 3800 gas chromatograph (Palo Alto, CA) with a 30-m DB-23 capillary column (0.32 mm internal diameter). The sperm PL fatty acid composition is presented for only 30 normozoospermic subjects (as opposed to 43 subjects for seminal plasma and serum PL analyses) due to insufficient sample volumes from 13 of the individuals.

Statistical analysis. All data are reported as SEM. Data analyzed by unpaired *t*-test (Tables 1–4) were normally distributed before analysis using SAS (SAS Institute, Cary, NC). Data that could not be normally distributed were analyzed using nonparametric analysis (Wilcoxon). Data in Figures 1–3 were analyzed by regression analysis using SAS (SAS Institute). Significance is reported if $P \leq 0.05$.

RESULTS AND DISCUSSION

Table 1 shows the subject and sperm characteristics for both groups. There were no significant differences in height, weight, body mass index, or age between the two groups (asthenozoospermic vs. normozoospermic; $P > 0.05$). Sperm forward motility (% of total) was significantly lower in the asthenozoospermic group [31.5 (2.3)%, where value in parentheses is the SEM; range 1–50%] vs. the normozoospermic group [73.3 (1.2)%; range 60–95%] ($P < 0.0001$). Spermatozoa concentration was also significantly lower in the asthenozoospermic [42.4 (7.0) M/mL] vs. normozoospermic group [119.5 (13.5) M/mL]. The normal range of sperm concentration is 20–250 M/mL (18), and thus the mean sperm concentration for the asthenozoospermic group was within the normal range. Thirty-three percent of the asthenozoospermic individuals were also oligospermic (sperm concentration < 20 M/mL); however, this did not confound the results obtained in this study since the outcome parameters were expressed on a percentage basis.

The fatty acid composition of the serum total PL of asthenozoospermic and normozoospermic individuals is given in Table 2 (SEM wt%). The levels of the various fatty acids of serum PL were similar between the two groups. Interestingly, however, the amount of 18:1 and total monounsaturated fatty acids (MUFA) were increased in the asthenozoospermic group. The levels of total saturated fatty acids, MUFA, and polyunsaturated fatty acids (PUFA), as well as total PL, n-3, and n-6 levels and the ratio of n-3 to n-6 fatty acids also were similar between the two groups.

Table 3 shows the levels of fatty acids in the total PL of

TABLE 1
Subject and Sperm Characteristics of Asthenozoospermic and Normozoospermic Men[a]

Characteristics	Normozoospermic	Asthenozoospermic
Height (m)	1.8 (0.0)	1.8 (0.0)
Weight (kg)	83.0 (2.4)	85.5 (2.6)
BMI (kg/m^2)	25.6 (0.7)	26.4 (0.5)
Age (yr)	34.8 (1.0)	36.2 (0.7)
Sperm motility (%)	73.3 (1.2)	31.5 (2.3)*
Sperm concentration (M/mL)	119.5 (13.5)	42.4 (7.0)*

[a]Values in parentheses are SEM where $n = 43$ (normozoospermic men) and 30 (asthenozoospermic men), *$P < 0.05$. BMI, body mass index.

TABLE 2
Fatty Acid Analysis of Serum PL of Asthenozoospermic and Normozoospermic Men[a]

Fatty acid	Normozoospermic[b] (wt% of total)	Asthenozoospermic (wt% of total)
16:0	27.6 (0.2)	27.4 (0.3)
16:1	0.43 (0.03)	0.46 (0.04)
18:0	13.7 (0.2)	14.2 (0.2)
18:1	11.4 (0.2)	12.4 (0.3)*
18:2n-6	21.0 (0.3)	20.4 (0.5)
18:3n-3	0.22 (0.01)	0.21 (0.01)
20:3n-6	3.1 (0.1)	2.9 (0.1)
20:4n-6	11.5 (0.2)	11.5 (0.3)
20:5n-3	0.75 (0.04)	0.83 (0.06)
22:4n-6	0.40 (0.01)	0.36 (0.03)
22:5n-3	1.0 (0.0)	1.1 (0.0)
22:6n-3 (DHA)	2.5 (0.1)	2.5 (0.1)
Total SFA	45.0 (0.2)	44.9 (0.2)
Total MUFA	13.9 (0.2)	14.9 (0.3)*
Total PUFA	41.0 (0.2)	40.2 (0.3)
Total n-3	4.7 (0.1)	4.7 (0.2)
Total n-6	36.4 (0.2)	35.6 (0.4)
n-3/n-6	0.13 (0.00)	0.13 (0.01)
Total PL (mg/dL)	204.8 (4.9)	213.5 (10.0)

[a]Values in parentheses are SEM where $n = 43$ (normozoospermic) and 30 (asthenozoospermic) individuals. Values with an asterisk are significantly different as measured by *t*-test ($P < 0.05$); DHA, docosahexaenoic acid; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; PL, phospholipid.

human seminal plasma. Seminal plasma total PL fatty acid differed between the two groups. The level of 18:1 was higher (by 13%) in the seminal plasma from asthenozoospermic men. Concentrations of 22:0 and DHA were lower in the seminal plasma from the asthenozoospermic men (7.2 vs. 8.2% and 3.0 vs. 3.7%, respectively; asthenozoospermic vs. control). Total MUFA were significantly higher [24.2 (0.4) vs. 21.7 (0.3) wt%] and total PUFA were significantly lower [11.8 (0.4) vs. 13.5 (0.8) wt%] in total seminal plasma PL from the asthenozoospermic vs. the control men. Interestingly, total PL (mg/dL) was also significantly lower in the seminal plasma from the asthenozoospermic men [31.5 (2.0) vs. 57.3 (5.1) mg/dL]. There were no differences in the total n-3 fatty acid levels or the total n-6 fatty acid levels or in the ratio of n-3 to n-6 fatty acids.

The fatty acid composition of total sperm PL (asthenozoospermic and normozoospermic) is given in Table 4. Levels of 18:1 (13.6 vs. 11.6 wt%), 20:0 (4.2 vs. 2.6 wt%), 22:0 (5.2 vs. 3.2 wt%), 22:1 (0.60 vs. 0.39 wt%), and 24:0 (4.4 vs. 2.6 wt%) were significantly higher in the sperm PL of asthenozoospermic vs. control men. On the other hand, levels of DHA (8.2 vs. 13.8 wt%) were significantly lower in the sperm PL of asthenozoospermic individuals. As observed with seminal plasma PL, the total MUFA levels were higher [19.3 (0.5) vs. 16.5 (0.5) wt%] and total PUFA significantly lower [19.1 (1.0) vs. 24.0 (1.2) wt%] in the sperm PL of asthenozoospermic vs. the normozoospermic men. Total n-3 fatty acid levels and the ratio of n-3 to n-6 fatty acids were also significantly lower in sperm PL from the asthenozoospermic subjects [9.3 (0.8) vs. 14.6 (0.9) wt% and 1.0 (0.1) vs. 1.6

TABLE 3
Fatty Acid Analysis of Seminal Plasma PL of Asthenozoospermic and Normozoospermic Men[a]

Fatty acid	Normozoospermic (wt% of total)	Asthenozoospermic (wt% of total)
16:0	27.2 (0.4)	28.1 (0.2)
18:0	15.4 (0.1)	14.9 (0.1)
18:1	16.8 (0.4)	19.0 (0.3)*
18:2n-6	2.5 (0.3)	2.3 (0.1)
18:3n-3	0.19 (0.04)	0.19 (0.01)
20:0	6.4 (0.1)	6.0 (0.2)
20:1	1.1 (0.1)	1.1 (0.0)
20:3n-6	2.2 (0.1)	2.0 (0.1)
20:4n-6	3.3 (0.1)	3.1 (0.1)
22:0	8.2 (0.2)	7.2 (0.3)*
22:1	0.33 (0.10)	0.34 (0.01)
22:4n-6	0.36 (0.02)	0.44 (0.02)
22:5n-3	0.41 (0.03)	0.44 (0.03)
22:6n-3 (DHA)	3.7 (0.3)	3.0 (0.2)*
24:0	6.3 (0.1)	6.3 (0.2)
24:1	3.1 (0.1)	3.0 (0.1)
Total SFA	64.8 (0.6)	64.1 (0.4)
Total MUFA	21.7 (0.3)	24.2 (0.4)*
Total PUFA	13.5 (0.7)	11.8 (0.4)*
Total n-3	4.8 (0.6)	3.6 (0.2)
Total n-6	8.7 (0.3)	8.2 (0.3)
n-3/n-6	0.54 (0.05)	0.45 (0.02)
Total PL (mg/dL)	57.3 (4.7)	31.5 (2.0)*

[a]Values in parentheses are SEM where $n = 43$ (normozoospermic) and 30 (asthenozoospermic) individuals. Values marked with an asterisk are significantly different as measured by *t*-test ($P < 0.05$). For abbreviations see Table 1.

TABLE 4
Fatty Acid Analysis of Sperm PL of Asthenozoospermic and Normozoospermic Men[a]

Fatty acid	Normozoospermic (wt% of total)	Asthenozoospermic (wt% of total)
16:0	33.5 (0.7)	31.9 (0.4)
16:1	0.44 (0.05)	0.41 (0.05)
18:0	15.7 (0.4)	14.1 (0.2)
18:1	11.6 (0.5)	13.6 (0.4)*
18:2n-6	3.2 (0.2)	2.9 (0.1)
20:0	2.6 (0.2)	4.2 (0.2)*
20:1	0.65 (0.18)	0.85 (0.04)
20:2n-6	0.48 (0.05)	0.49 (0.03)
20:3n-6	2.5 (0.2)	2.7 (0.2)
20:4n-6	2.5 (0.2)	2.6 (0.1)
22:0	3.2 (0.2)	5.2 (0.3)*
22:1	0.39 (0.05)	0.60 (0.06)*
22:4n-6	0.38 (0.04)	0.42 (0.03)
22:5n-3	0.77 (0.05)	0.73 (0.06)
22:6n-3 (DHA)	13.8 (0.9)	8.2 (0.7)*
24:0	2.6 (0.3)	4.4 (0.2)*
24:1	2.3 (0.1)	2.5 (0.1)
Total SFA	59.5 (1.0)	61.5 (0.7)
Total MUFA	16.5 (0.5)	19.3 (0.5)*
Total PUFA	24.0 (1.2)	19.1 (1.0)*
Total n-3	14.6 (0.9)	9.3 (0.8)*
Total n-6	9.4 (0.4)	9.8 (0.4)
n-3/n-6	1.6 (0.1)	1.0 (0.1)*
Total PL (mg/dL)	38.1 (3.0)	21.8 (1.6)*

[a]Values in parentheses are as SEM where $n = 30$ (normozoospermic) and 30 (asthenozoospermic) individuals. Values marked with an asterisk are significantly different as measured by *t*-test ($P < 0.05$). For abbreviations see Table 2.

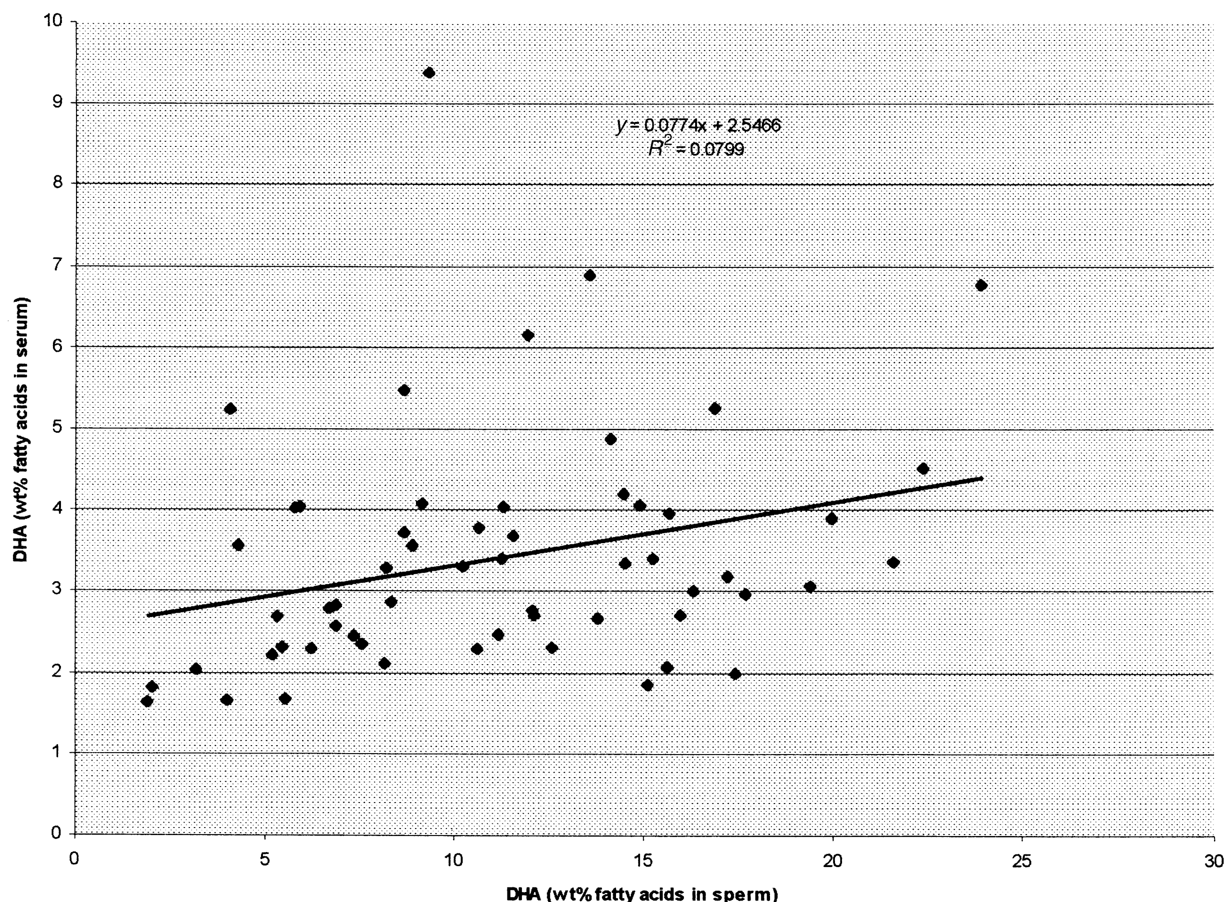

FIG. 1. Relationship of docosahexaenoic acid (DHA) concentration in serum to DHA concentration in sperm. Regression analysis is for a total of 58 men (both asthenozoospermic and normozoospermic). Statistical analysis was done using SAS (SAS Institute, Cary, NC). $P = 0.03$.

(0.1), respectively]. As would be expected due to the differences in total sperm count, the levels of total PL were also significantly lower in the sperm from the asthenozoospermic individuals [21.8 (1.6) vs. 38.1 (3.0) mg/dL].

Figure 1 shows the relationship between serum PL DHA concentration and sperm PL DHA concentration (% fatty acids). Serum PL DHA concentration was found to correlate with sperm PL DHA concentration ($r^2 = 0.08$, $P < 0.05$).

Figure 2 shows the relationship between sperm PL DHA concentration and sperm motility (%). Sperm motility was found to be highly correlated with PL DHA concentration ($r^2 = 0.26$, $P < 0.001$).

Figure 3 shows the relationship between sperm PL DHA concentration and sperm concentration (M/mL). Sperm concentration was found to be highly correlated with PL DHA concentration ($r^2 = 0.42$, $P < 0.0001$).

This is the first study to examine the differences and similarities in PL compositions of serum, seminal plasma, and total sperm of normozoospermic vs. asthenozoospermic individuals. In order to determine if dietary intakes of n-3 fatty acids were similar between the two groups of men, comparisons were made in the PL DHA status of serum. Dietary n-3 fatty acids consist mainly of α-linolenic acid (18:3n-3), EPA (20:5n-3), and DHA. Serum n-3 fatty acid status (including DHA status) represents n-3 dietary intake (15–17). Although metabolism of α-linolenic acid to EPA and DHA occurs in humans, it is limited (25). The similarities in fatty acid profile between the two subject groups as presented herein suggest that intakes of long-chain n-3 fatty acids (EPA and DHA) were similar between the normozoospermic and asthenozoospermic men. The serum PL levels of EPA (0.8%) and DHA (2.5%), as observed in this study, can be compared with previous results in our laboratory that suggest that omnivorous subjects with average North American n-3 intakes have PL EPA levels of 0.7% and PL DHA levels of 2.2% (23). A DHA level of greater than 6% can be observed in subjects consuming large amounts of this fatty acid (22–24). Although there is no published information suggesting that infertile men have different levels of n-3 fatty acids in blood serum, Lenzi *et al.* (26) have suggested that infertile men have a lower arachidonic acid/linoleic acid ratio (used as an indicator of PUFA metabolism) in red blood cell membranes.

The similarities in serum fatty acid status between the asthenozoospermic and normozoospermic men are of interest due to the differences both in the compositions of seminal plasma PL and sperm PL. Very little has been done on the

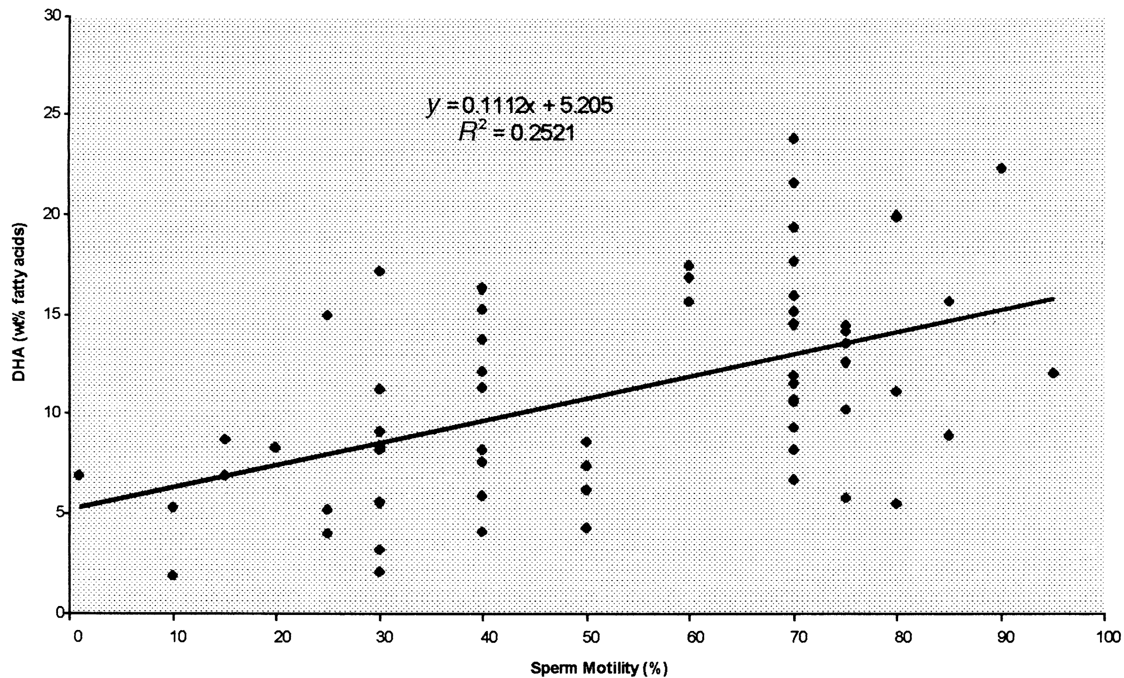

FIG. 2. Relationship of DHA concentration in sperm to sperm motility. Regression analysis is for a total of 60 men (both asthenozoospermic and normozoospermic). Statistical analysis was done using SAS (SAS Institute, Cary, NC). $P = 0.0001$. See Figure 1 for abbreviation.

lipid composition of the seminal plasma (27). Vignon *et al.* (27) determined the breakdown of the major lipid groups and published the nonesterified fatty acid composition of seminal plasma. They found minor levels of DHA (1.5%) in the seminal plasma nonesterified fatty acid. There was no indication of PL DHA levels in the seminal plasma. In addition, there was no mention of possible differences between the seminal plasma composition of asthenozoospermic vs. normozoosper-

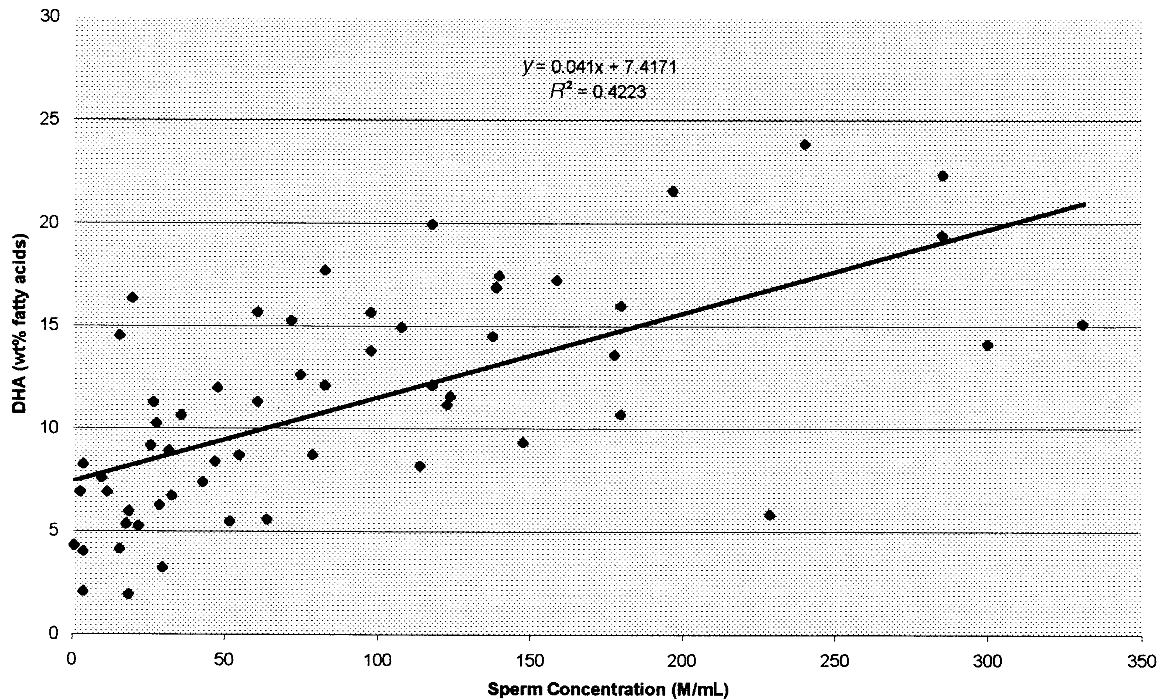

FIG. 3. Relationship of DHA concentration in sperm to sperm concentration. Regression analysis is for a total of 58 men (both asthenozoospermic and normozoospermic). Statistical analysis was done using SAS (SAS Institute, Cary, NC). $P = 0.0001$. For abbreviation see Figure 1.

mic men. In our study, we observed that seminal plasma from asthenozoospermic males was higher in total MUFA (mainly 18:1) and lower in total PUFA (mainly DHA) than seminal plasma from normozoospermic males. Interestingly, although DHA has been shown to retroconvert to EPA in humans, as evidenced by an increase in serum EPA with DHA supplementation (22–24), seminal plasma is essentially devoid of EPA and has extremely low levels of docosapentaenoic acid (22:5n-3). It is not clear why seminal plasma PL is lower in EPA and docosapentaenoic acid, and higher in DHA, than blood serum PL. Furthermore, it is not clear what effect, if any, an altered fatty acid composition of seminal plasma PL, in the asthenozoospermic men, would have on sperm motility. Decreased levels of DHA may be indicative of an altered seminal plasma oxidant status (28–31), but may not have an actual effect on progressive sperm motility.

The extremely high levels of DHA in spermatozoa, as suggested in the literature, are supported by our results, since the DHA levels in both groups were highest in sperm (13.8%) and lowest in blood serum (2.5%). The higher levels of MUFA (18:1, 22:1) and lower levels of PUFA, including DHA, in the sperm of the asthenozoospermic vs. normozoospermic individuals support the hypothesis of an increased breakdown and/or decreased accumulation of DHA in the sperm PL of asthenozoospermic males. We also observed higher levels of certain saturated fatty acids, including 20:0, 22:0, and 24:0, in the sperm PL of the asthenozoospermic group. As with seminal plasma PL, sperm PL is essentially devoid of EPA and has very low levels of docosapentaenoic acid. The fatty acid composition of sperm PL, as reported here, can be compared with those of past studies. Two studies comparing the PL fatty composition of total ejaculate suggest decreased levels of DHA in asthenozoospermic vs. normozoospermic men (4,5). In a third study (7), sperm PL fatty acid of asthenozoospermic individuals differed from that of normozoospermic individuals. In this case, sperm were separated on a Percoll (Pharmacia Biotech AB, Uppsala, Sweden) gradient (in order to separate the more active sperm from the least active sperm in a given individual) and recovered from the 90% Percoll and 47% Percoll fractions. In the 90% Percoll fraction, levels of 18:0, 18:2n-6, and total saturated fatty acids were higher in the asthenozoospermic group, and levels of DHA and total PUFA were lower. Interestingly, in the sperm recovered from the 47% Percoll gradient, DHA was similar between the normo- and asthenozoospermic individuals. Finally, in a study (8) involving sperm from patients with retinitis pigmentosa, (a hereditary disease causing blindness, which is accompanied by extremely low serum DHA levels), higher levels of 20:0, 22:0, 16:1, and 18:1 and lower levels of total PUFA, 20:3n-6, 20:4n-6, and 22:6n-3 were observed, in combination with reduced sperm motility. The exact mechanism by which this may occur is not completely clear although a lower antioxidant status and/or higher oxidant status may be at least partially responsible (9,28–31). It would also be of interest to determine possible differences in DHA concentration of the tails of sperm from asthenozoospermic vs. normozoospermic men.

This study suggests that the fatty acid composition of blood serum is similar between asthenozoospermic and normozoospermic men. However, the concentration of various fatty acids, including DHA, differs between the two groups in seminal plasma and sperm. The effect on sperm function of sperm/seminal plasma fatty acid composition, which is lower in polyunsaturates/DHA and higher in MUFA, is not clear, although previous publications in the literature suggest that DHA may play a role in sperm motility (4,5,7,8). Our results, showing a good correlation between sperm motility, as well as sperm concentration, and sperm PL-DHA concentration are in agreement with results of Nissen and Kreysel (4), in which they demonstrated an excellent correlation between sperm motility and concentration with PL DHA in total ejaculate. With respect to their findings, our group has recently undertaken a study in order to determine the effect of DHA supplementation on both sperm and seminal plasma DHA status, as well as sperm motility. Previous reports suggest that the synthesis of DHA is reduced in infertile men and that increased intake of preformed DHA (in the presence of EPA) is associated with poorer sperm parameters (32). Our results suggest a slight correlation between serum PL DHA and sperm PL DHA. Thus, we will examine next whether increasing dietary DHA, in the absence of EPA, will increase sperm DHA status as well as sperm motility in asthenozoospermic men.

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Increased Diaphragm Expression of GLUT4 in Control and Streptozotocin-Diabetic Rats by Fish Oil-Supplemented Diets

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ABSTRACT: Dietary fat intake influences plasma glucose concentration through modifying glucose uptake and utilization by adipose and skeletal muscle tissues. In this paper, we studied the effects of a low-fat diet on diaphragm GLUT4 expression and fatty acid composition in control and streptozotocin-induced diabetic rats. Control as well as diabetic rats were divided into three different dietary groups each. Either 5% olive oil, 5% sunflower oil, or 5% fish oil was the only fat supplied by the diet. Feeding these low-fat diets for 5 wk induced major changes in fatty acid composition, both in control and in diabetic rats. Arachidonic acid was higher in diabetic olive and sunflower oil-fed rats with respect to fish oil-fed, opposite to docosahexaenoic acid which was higher in diabetic fish oil-fed rats with respect to the other two groups. Animals receiving a fish oil diet had the lowest plasma glucose concentration. GLUT4 expression in diaphragm, as indicated by GLUT4 protein and mRNA, is modulated both by diabetes and by diet fatty acid composition. Diabetes induced a decrease in expression in all dietary groups. Plasma glucose levels correlated well with the increased amount of GLUT4 protein and mRNA found in fish oil-fed groups. Results are discussed in terms of the influence that arachidonic and n-3 polyunsaturated fatty acids may exert on the transcriptional and translational control of the GLUT4 gene.

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Skeletal muscle is the major site of insulin-mediated glucose utilization *in vivo* (1–3). In muscle, glucose is taken up through facilitated diffusion by two transporters, GLUT1 and, mainly, GLUT4 (2–4). Most of the GLUT4 protein is found in an intracellular organelle in the basal state from where it can be rapidly translocated to the plasma membrane (2–5). This translocation is typically elicited by insulin (2,3) or exercise (5,6) whereas GLUT1 transporter is generally considered to be insulin independent (2).

Both insulin- and noninsulin-dependent diabetes mellitus decrease muscle glucose utilization due to a defective expression and/or regulation of GLUT4 translocation (1–3). In fact, it has been reported that GLUT4 protein and mRNA levels are decreased in skeletal muscle of several rodent models of both genetically and experimentally induced diabetes (1,2).

Fatty acid composition of membranes is also a factor that may influence the action of insulin within skeletal muscle. It has been shown that incorporation of unsaturated fatty acids into muscle membrane phospholipids is associated with improved insulin action. Correspondingly, a higher proportion of saturated fats is linked to an impairment of insulin action (7–10).

Low-fat diet feeding is associated with changes in fatty acid cell membrane composition in normal rats (10,11) but the information is more limited in the diabetic state (12). Moreover, whether a low-fat diet also affects gene expression of GLUT4 glucose transporter in skeletal muscle of nondiabetic animals and of an animal model of diabetes is not known. In the present work, we studied the relationship between dietary fat and fatty acid composition of diaphragm membranes in nondiabetic and streptozotocin-induced diabetic rats and the ways in which differences in polyunsaturated fatty acid percentages in muscle membranes can influence gene expression of GLUT4 transporter in skeletal muscle.

MATERIALS AND METHODS

Animals and treatments. Female Wistar rats (Granada University breeding colony) (150–160 g) were housed at 22°C in a light-controlled (12-h cycle) animal facility. They were randomly divided into three groups of animals; each one had free access to water and food. They were fed for 1 wk with the same purified diet supplemented differentially with 50 g/kg of olive oil, sunflower oil, and fish oil, respectively. The composition of the diet per kg was 650 g corn starch, 50 g cellulose, 200 g vitamin-free casein, 35 g mineral mix, 10 g vitamin mix, 3 g DL-methionine, and 2 g choline bitartrate. The energy content of the three diets was 1.03 kJ/g distributed as (% energy) 68.0 carbohydrate, 21.5 protein, and 10.5 lipids. The fatty acid composition of the three diets is shown in Table 1. All three diets had equal levels of antioxidants per kg of oil: 1.3 g (1300 IU) α-tocopherol, 1.2 g (13.2 IU) γ-to-

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Abbreviations: SSC, sodium chloride/sodium citrate buffer; STZ, streptozotocin.

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TABLE 1
Fatty Acid Composition of Diets[a]

Fatty acid	Olive oil[b]	Sunflower oil[c]	Fish oil[d]
16:0	15.22	7.02	23.50
16:1n-7	1.18	0.18	12.83
18:0	3.80	3.90	4.18
18:1n-9	72.55	34.28	18.77
18:2n-6	4.87	54.19	1.31
18:3n-6			0.84
18:3n-3	0.57	0.29	0.84
18:4n-3			3.20
20:5n-3			21.07
22:5n-3			2.29
22:6n-3			6.8
Other PUFA > 20 carbons			4.62

[a]Results are expressed as the percentages of total fatty acid methyl esters. The fatty acid composition of the diet was determined by gas-liquid chromatography. PUFA, polyunsaturated fatty acids.
[b]Carbonell, Carbonell y Cia, Cordoba, Spain.
[c]Koipesol, Koipe S.A., San Sebastian, Spain.
[d]Fish oil from AFAMSA, Vigo, Spain.

copherol, and 1 g *tert*-butylhydroquinone to prevent peroxidation during 1 wk storage at 4°C. The final concentration of vitamin E (DL-α-tocopheryl acetate, γ-tocopherol, and α-tocopherol was 215 IU/kg of diet.

Within each group, experimental diabetes was induced in half of the rats by a single streptozotocin (STZ) injection (60 mg/kg i.p. in 50 mM sodium citrate buffer, pH 4.5) whereas control animals received citrate buffer alone. Only fed animals with blood glucose levels greater than 20 mM were considered diabetic.

Diabetic rats and the corresponding controls were maintained for five additional weeks on the corresponding dietary regime. At the end of the treatments, following one night of food deprivation, rats were killed by decapitation between 0900 and 0930. After blood collection, diaphragm was removed, rapidly frozen in liquid nitrogen and kept at −70°C until analysis. Animals were studied in compliance with our institution's guidelines for animal research. Ethical approval of the protocol was obtained in conformity with regulations for the care and use of laboratory animals.

Fatty acid composition of diaphragms. Muscle tissue was homogenized 1:10 (wt/vol) in 10 mM NaH_2PO_4 buffer, pH 7.4, and diaphragm lipids were extracted by a direct transesterification method according to Lepage and Roy (13). Basically, 100 µL of diaphragm homogenate were precisely pipetted into glass tubes and 3 mL of methanol/benzene (4:1) were added to the biological samples. While stirring, 200 µL of acetyl chloride was slowly added to each tube, over a period of 1 min. Tubes were tightly capped and subjected to methanolysis at 100°C for 1 h. After cooling the tubes, 5 mL of 6% K_2CO_3 solution was added to stop the reaction and neutralize the mixture. The tubes were then shaken and centrifuged, and an aliquot of the benzene upper phase was injected into a gas chromatograph (Hewlett-Packard, model 5880) as described (11) and expressed as the percent distribution of fatty acid methyl esters.

Preparation of total membrane fractions from diaphragm. Total membrane fractions from diaphragm muscle were prepared according to Kahn *et al.* (14). Briefly, 200 mg of tissue was minced in liquid nitrogen and homogenized by 10 strokes in a motor-driven Potter-Elvehjem homogenizer equipped with a Teflon pestle in a buffer containing 20 mM $NaHCO_3$, 0.25 M sucrose, 5 mM Na_3N, 1 mM leupeptin, 1 mM apoprotinin A, 1 mM pepstatin, pH 7.0 at 4°C. This homogenate was centrifuged at 1200 × *g* for 10 min and the pellet was resuspended, homogenized and recentrifuged to remove debris. The combined supernatants were centrifuged at 9000 × *g* for 10 min at 4°C to sediment mitochondria and nuclei. The resulting supernatant was then centrifuged at 200,000 × *g* for 60 min at 4°C to obtain the total membrane fraction. The membrane pellets were resuspended in the homogenization buffer, and total membrane protein content was assayed using the Bradford method (15).

GLUT4 and GLUT1 protein analyses. Muscle membrane fractions (10 µg protein for GLUT4 and 30 µg protein for GLUT1 from a pool of six different membrane fraction samples per condition) were subjected to electrophoresis in 12% SDS-polyacrylamide gels, and the resolved proteins were transferred to nitrocellulose filter membranes (Millipore, Bedford, MA). Samples were also analyzed individually by blotting 10 µg (GLUT4) or 20 µg protein (GLUT1) of each membrane fraction sample onto nitrocellulose membranes using a slot blot apparatus (Bio-Rad, Hercules, CA). The blots were incubated with a 1:500 dilution of a polyclonal anti-GLUT4 antibody or a 1:1500 dilution of a polyclonal anti-GLUT1 antibody (East Acres Biologicals, Southbridge, MA). The antigen-antibody complexes were detected with horseradish peroxidase-conjugated antibodies and an enhanced chemiluminescence detection system (Boehringer Mannheim, Mannheim, Germany). The Western blot autoradiograms revealed uniques bands of 45 kDa, compatible with the molecular weight of the transporters. Quantification was performed by densitometry after scanning of the autoradiographs using the NIH Image Software (16).

RNA isolation and analysis. Total RNA was extracted from diaphragm of control and diabetic rats fed different diets using a guanidinium thiocyanate method (17). All samples had a 260:280 absorbance ratio above 1.7. No differences in the yield per gram of tissue in control and diabetic rats were found. For Northern blot analysis, RNA pooled samples from six different animals per condition (20 µg) were denatured, electrophoresed in 1.0% agarose/formaldehyde gel and alkaline-transferred using a 5× sodium chloride/sodium citrate buffer (5× SSC; a 20× SSC stock solution is 3M NaCl/0.3 M Na citrate, pH7)/10 mM NaOH solution on BrightStar-Plus positively charged nylon membranes (Ambion Inc., Austin, TX) according to the manufacturer's instructions. After the transfer, the RNA in filters was visualized with ethidium bromide and photographed by ultraviolet transillumination to ensure the integrity of RNA, to check the loading of equivalent amounts of total RNA, and to confirm proper transfer. For slot blot analysis, 10 µg of the individual RNA samples were spotted onto BrightStar-Plus membranes by using a slot blot

apparatus. Blots were initially prehybridized in 10 mL preheated 50% formamide, 5× Denhardt's solution (1× Denhart's solution is 0.02% polyvinylpyrolidone, 0.02% Ficoll, 0.02% bovine serum albumin), 0.5% SDS, 5× SSC, and 100 mg/L of denatured salmon sperm DNA solution for 3 h at 42°C and then were hybridized with the random primer-labeled cDNA probes (5×10^4 Bq/mL) for 18 h at 42°C in the same solution with an added 10% dextran sulfate. Finally, the blots were washed in 0.1× SSC and 0.1% SDS at 65°C. Blots were exposed to Kodak Biomax film at −70°C using intensifying screens (Cronex Lightening Plus, Du Pont, Wilmington, DE). The relative amounts of mRNA in each sample were quantitated by densitometric analysis using NIH Image Software (16), and the data were normalized to that of β-actin mRNA.

cDNA probes. GLUT4 glucose transporter mRNA was quantitated by using a 2400 bp *Eco*RI fragment inserted in the pSM-1-1-1 plasmid (18) as probe, and β-actin mRNA was assayed by using a 1900 bp *Hind*III fragment of the gene (19) inserted in a pGEM 7Z plasmid. Glycogen synthase mRNA was measured by using two *Hind*III fragments coding for the human muscle glycogen synthase inserted in the pHMGS plasmid (20). A mouse glyceraldehyde-3-phosphate dehydrogenase probe was obtained from Ambion Inc. cDNA probes were labeled with α-[^{32}P]-deoxycytidine triphosphate (Amersham, Buckinghamshire, United Kingdom) by random oligonucleotide-priming (21).

Statistical methods. Significant differences among the groups of results were considered when $P \leq 0.05$ using two-way analysis of variance and *posthoc* Tukey tests. Statistical analyses were performed using the BMDP Computer Software program (Berkeley, CA) (22).

RESULTS

The three diets differ in the origin of lipid supplementation, olive oil, sunflower oil and fish oil, enriched, respectively, in monounsaturated, n-6 polyunsaturated and n-3 long-chain polyunsaturated fatty acids (Table 1). The amount of fat supplied in the diets was always 5% oil, so they were basically low-fat diets, fat contribution to energy being only 10.5% of the total intake.

After induction of diabetes by a single-dose STZ injection, animals were fed the supplemented diets for 5 wk. Plasma glucose levels significantly increased in diabetic compared to control animals ($P < 0.001$) (Table 2). Control fish oil-fed rats had significantly lower glycemia than those fed olive or sunflower oil ($P < 0.05$). No differences in body weight among groups were observed as an effect of diet. In diabetic rats body weight was moderately decreased ($P < 0.05$).

Table 3 shows the percentages of major fatty acids in diaphragms of control and diabetic animals fed diets varying in fat type. Dietary fatty acids clearly affected the relative abundance of the different fatty acids. Thus, the highest levels of oleic, linoleic, or n-3 polyunsaturated fatty acids were found in diaphragms of rats fed olive oil, sunflower oil, or fish oil diets, respectively. Arachidonic acid was similar in control and diabetic animals fed either olive or sunflower oil diets. Diabetes produced a significant increase ($P < 0.001$) in arachidonic acid when the diet was supplemented with either olive oil or sunflower oil. Arachidonic acid levels in control and diabetic fish oil-fed rats were similar and they were lower than in the other two diabetic groups ($P < 0.001$). Eicosapentaenoic acid was only detected in fish oil-fed animals. Docosahexaenoic acid increased by diabetes ($P < 0.001$) in the fish oil group compared to controls.

Total diaphragm membrane fractions were purified from control and diabetic rats after 5 wk of dietary treatment, and there were no differences in the yield of membrane protein obtained per gram of tissue. The content of GLUT4 protein was assayed by Western blot and by slot blot. The Western blot of six pooled membrane samples per condition showed a single band of 45 kDa (Fig. 1). Quantification of the signal detected for individual samples by densitometric scans gave the results shown in Table 4. Diabetes caused a decrease in protein content 40% higher than in any of the other groups ($P < 0.001$). The amount of GLUT4 protein was similar in the groups receiving olive or sunflower oil diets while muscle membranes isolated from animals having fish oil diets had significant higher concentration ($P \leq 0.05$) of the glucose transporter in both control and diabetic rats. The amount of GLUT1 protein was assayed by Western and slot blot. No differences in GLUT1 content were found between control and diabetic animals fed the different diets (data not shown).

We next determined the levels of diaphragm glucose transporter GLUT4 mRNA by Northern and slot blot. The Northern blot of six pooled RNA samples per treatment (Fig. 2) shows a single band of 2.8 kb corresponding to that described for the glucose transporter (18). The relative intensity (in arbitrary units) of the signal detected for six individual samples measured by slot blot are presented in Table 4. There is an agreement between data obtained by quantitation of the

TABLE 2
Plasma Glucose and Body Weight of Control and Streptozotocin-Diabetic Rats Fed 5% Olive Oil, Sunflower Oil, or Fish Oil Diets for 5 wk[a]

	Olive oil-fed		Sunflower oil-fed		Fish oil-fed	
Parameter	Control	Diabetic	Control	Diabetic	Control	Diabetic
Glucose (mM)	8.2 ± 0.7	42.3 ± 3.5[c]	9.2 ± 1.0	39.5 ± 2.3[c]	6.4 ± 0.2[a,b]	34.8 ± 0.8[c]
Body weight (g)	201.3 ± 5.2	169.7 ± 9.8[c]	195.3 ± 10.3	174.3 ± 6.1	203.2 ± 7.5	171.6 ± 4.4[c]

[a]Values are means ± SEM, n = 6–8. Statistically significant differences ($P \leq 0.05$) are as follow: [a]Olive oil- vs. sunflower or fish oil-fed rats; [b]sunflower vs. fish oil-fed rats; [c]control vs. diabetic rats.

TABLE 3
Diaphragm Fatty Acid Composition of Control and Streptozotocin-Diabetic Rats Fed Olive Oil, Sunflower Oil, or Fish Oil for 5 wk[a]

	Olive oil-fed		Sunflower oil-fed		Fish oil-fed	
Fatty acid	Control	Diabetic	Control	Diabetic	Control	Diabetic
Saturated fatty acids	32.0 ± 0.5	29.6 ± 1.0	33.3 ± 0.6	32.7 ± 0.9	35.9 ± 0.4[a]	35.5 ± 0.9[a]
18:1n-9	38.0 ± 0.7	36.4 ± 3.3	28.4 ± 1.2[a]	15.0 ± 2.2[a,c]	26.8 ± 1.8[a]	24.4 ± 0.9[a,b]
18:2n-6	11.5 ± 0.3	11.8 ± 0.2	20.4 ± 0.7[a]	24.2 ± 0.3[a]	10.1 ± 0.4[b]	12.0 ± 0.5[b]
20:3n-6	0.3 ± 0.01	0.7 ± 0.03[c]	0.3 ± 0.02	0.7 ± 0.05[c]	0.3 ± 0.02	0.6 ± 0.04[c]
20:4n-6	6.9 ± 0.1	12.2 ± 0.9[c]	6.7 ± 0.7	15.6 ± 1.5[c]	5.0 ± 0.7[a]	6.9 ± 0.7[a,b]
20:5n-3	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	2.6 ± 0.4[a,b]	2.2 ± 0.2[a,b]
22:6n-3	2.8 ± 0.1	3.5 ± 0.9	1.5 ± 0.2[a]	2.5 ± 0.3	6.9 ± 1.1[a,b]	10.0 ± 0.8[a,b,c]

[a]Results are expressed as means ± SEM (n = 6–8) of the percentages of total fatty acids methyl esters. Statistically significant differences ($P \le 0.05$) are as in Table 2.

Northern and slot blots. In both cases, data have been normalized to the values obtained for the expression of the β-actin gene. GLUT4 mRNA expression was significantly depressed ($P < 0.001$) in diabetes, as expected. Control animals fed the fish oil diet presented the higher content of GLUT4 mRNA ($P < 0.001$) when compared to olive or sunflower oil groups. No differences were found in GLUT4 mRNA levels among diabetic animals receiving the different diets.

As a control, the expression levels of two genes known to be down-regulated in diabetes (5,23,24), glyceraldehyde-3-phosphate dehydrogenase, implicated in glucose utilization, and glycogen synthase, implicated in glucose storage in the muscle, have been studied. Quantitation of the mRNA content for these proteins, after β-actin normalization, was carried out by Northern blot, detecting two single bands of 1.4 and 3.5 kb corresponding to the glyceraldehyde-3-phosphate dehydrogenase and muscle glycogen synthase, respectively. We measured a decrease in the mRNA levels of both genes (60% decrease in the glyceraldehyde-3-phosphate dehydrogenase mRNA levels and 39% decrease in the glycogen synthase mRNA levels) due to STZ-induced diabetes without significant differences among the dietary groups, confirming the purity of our RNA preparations (data not shown).

DISCUSSION

Glucose transporter GLUT4 is believed to be responsible for the insulin-mediated glucose uptake in muscle, and therefore, the reduction of its concentration in plasma membranes will account for development of insulin resistance. As dietary manipulations may improve the response to insulin, we have administered three experimental diets to assess the importance of different dietary fatty acids on GLUT4 expression. These diets have been fed to control and STZ-diabetic rats in order to study their effect in basal expression (control rats) or low-level expression (diabetic rats) of the GLUT4 glucose transporter (1–3,25).

The amount of fat supplied in the diets was always 5%, so they were basically low-fat diets. Low-fat diets prevent insulin resistance, whereas high-fat diets impair insulin-dependent glucose transport by reducing translocation of the transporter to the plasma membrane (26). Furthermore, low-fat diets allow rapid modifications in fatty acid content of membrane phospholipids (10,27).

Induction of diabetes by a single-dose STZ injection was effective, and after 5 wk plasma glucose was significantly increased in all diabetic animals compared to the controls.

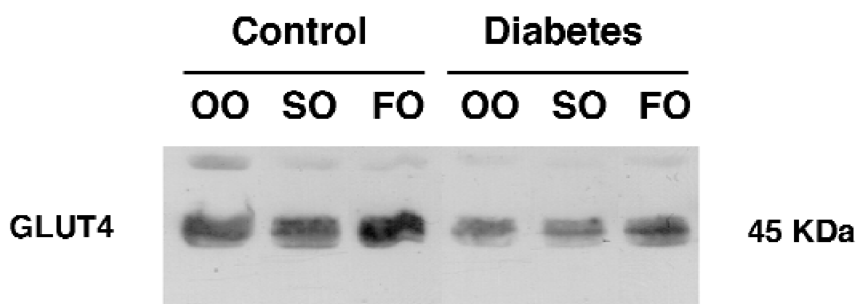

FIG. 1. Western blot analysis of GLUT4 protein levels in diaphragm of control and streptozotocin-diabetic rats fed 5% olive oil, sunflower oil, or fish oil diets for 5 wk. Aliquots of six different membrane fractions per condition were pooled and applied (10 μg) to a 12% SDS-polyacrylamide gel. Gels were blotted, and GLUT4 protein was detected using a polyclonal antibody. Bands were detected using chemiluminescence as indicated in the Materials and Methods section. OO, Olive oil-fed rats; SO, sunflower oil-fed rats; FO, fish oil-fed rats.

TABLE 4
Diaphragm GLUT4 mRNA and GLUT4 Protein Levels of Control and Streptozotocin-Diabetic Rats Fed Olive Oil, Sunflower Oil, or Fish Oil for 5 wk[a,b,c]

	Olive oil-fed		Sunflower oil-fed		Fish oil-fed	
Parameter	Control	Diabetic	Control	Diabetic	Control	Diabetic
GLUT4 mRNA	1517 ± 42	793 ± 48[c]	1224 ± 32	795 ± 68[c]	1890 ± 32[a,b]	782 ± 63[c]
GLUT4 protein	2611 ± 38	1474 ± 95[c]	2570 ± 61	1522 ± 83[c]	3515 ± 146[a,b]	2048 ± 76[a,b,c]

[a]Results are expressed as relative amounts of GLUT4 protein or mRNA. Quantification was performed by densitometry after scanning the autoradiographs corresponding to the slot blot determinations as described in the Material and Methods section.
[b]Values are means ± SEM, $n = 6–8$. Statistically significant differences ($P \leq 0.05$) are as in Table 2.
[c]Aliquots of individual mRNA (20 μg) and membrane fraction (10 μg) samples were analyzed by slot blot as described in the Material and Methods section.

Control fish oil-fed animals presented significantly lower glycemia than fed those olive or sunflower oil as an indication that glucose uptake by peripheral tissues may be favored by n-3 long-chain polyunsaturated fatty acids.

Low levels of fat intake, as those in our experimental protocol, induce changes in fatty acid composition of diaphragm membranes, and the type of oil supplement in the diet greatly influences the relative abundance of different fatty acids. However, a great deal of phospholipid remodeling occurs prior to deposition in muscle plasma membranes. As an example, oleic acid content in olive oil-supplemented diet is more than 70% while in the corresponding muscle membranes it only reaches 38%. Arachidonic acid is similar in membranes of animals fed either olive or sunflower oil supplements despite the big difference in the concentration of linoleic acid between these diets. Animals fed fish oil had the lowest levels of arachidonic acid, probably owing to a diminished Δ^5-desaturase activity as evidenced by the differences in 20:4n-6/20:3n-6 index. Significant changes in fatty acids in diabetes affect arachidonic acid, which is increased when the diet is supplemented with either olive or sunflower oil, while a rise in docosahexaenoic acid (22:6n-3) has been detected in diabetic fish oil-fed rats. Similar results have been shown in liver microsomal phospholipids (28).

Fatty acid composition within membranes may modulate the action of membrane proteins involved in glucose transport by several mechanisms. Borkman *et al.* (7) showed that long-chain polyunsaturated fatty acid content in muscle-membrane phospholipids may influence the action of insulin through the effects of their physical properties on proteins such as insulin receptors and glucose transporters. It has previously been shown that diets high in polyunsaturated fatty acids facilitate glucose transport into cells and that insulin action is partly affected by changes in the physicochemical micro environments of membrane lipid domains associated with glucose transporters. These changes can include an increased membrane fluidity of muscle tissue, which consequently increases or facilitates the penetration of glucose transporters into the membrane. Fatty acids have been demonstrated as physiological regulators of the glucose transport system (29). Oleic and arachidonic acids particularly seem to destabilize the mRNA for GLUT4. Arachidonic acid suppresses the transcription rate of GLUT4 gene in adipocytes (29,30). Our results clearly agree with this mechanism, as olive and sunflower oils in the diet occasion the highest concentration of arachidonic acid in muscle membranes and minimal levels of GLUT4 mRNA and protein.

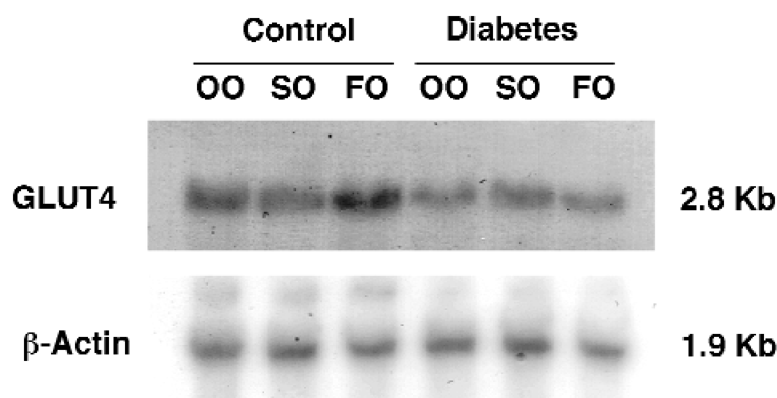

FIG. 2. Northern blot analysis of GLUT4 mRNA levels in diaphragm of control and streptozotocin-diabetic rats fed 5% olive oil, sunflower oil, or fish oil diets for 5 wk. Aliquots of six different RNA samples per condition were pooled and 20 μg (GLUT4 mRNA) or 10 μg (β-actin mRNA) were electrophoresed on 1% agarose/formaldehyde gel. Samples were transferred to positively charged nylon membranes and probed as described under the Material and Methods section either with a GLUT4 or a β-actin probe. For abbreviations see Figure 1.

mRNA and protein GLUT4 levels may be modulated both by the quality of fat in the diet and by diabetes when animals are subjected to a low-fat intake. In our experimental groups, dietary supplementation with fish oil enhances the amount of GLUT4 mRNA and protein in controls. Diabetes causes a decrease in GLUT4 mRNA levels in all groups that is not modulated by dietary fat. Thus, GLUT4 mRNA levels are similar, independent of the type of fat intake, although GLUT4 protein is increased by fish oil feeding both in control and diabetic rats. Fish oil-fed rats have the highest levels of eicosapentenoic acid (20:5n-3) and other n-3 polyunsaturated fatty acids. Fish oil, apart from its lowest content in n-6 fatty acids, could well provide high levels of substrates for prostaglandins and leukotrienes, particularly prostaglandin E_3, which would also contribute to increase the number of copies of GLUT4 transporter in muscle after fish oil feeding (8). Similar results have been shown by Ezaki *et al.* (31) in adipocytes from rats fed a high-fish oil diet or a high-safflower oil diet.

The increase found in GLUT4 protein levels after fish oil feeding, which does not correlate with GLUT4 mRNA levels in diabetes, has to be explained in terms of the stability of the protein. Olson and Pessin (32) reported that GLUT4 is apparently a very stable protein, whereas the GLUT4 message has a relatively rapid turnover rate. Thus, the depletion of GLUT4 mRNA occurs before any decrease in GLUT4 protein levels. Furthermore, there appears to be a compensatory mechanism that reduces the rate of GLUT4 protein degradation, for example in STZ-diabetic rats, which spares GLUT4 at a time when there is a pretranslational synthetic defect.

In summary, data presented in this paper indicate that fish oil supplementation to the diet seems to correlate well with a diminution of glycemia through changes in fatty acid composition of muscle membranes that directly or indirectly may influence GLUT4 protein levels through a transcriptional or translational mechanism control. Thus, the fatty acid composition of the diet may be an important regulator of the glucose transporter, GLUT4, in rat muscle.

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Apolipoprotein B mRNA Editing and Apolipoprotein Gene Expression in the Liver of Hyperinsulinemic Fatty Zucker Rats: Relationship to Very Low Density Lipoprotein Composition

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ABSTRACT: We previously demonstrated increased apolipoprotein B (apoB) mRNA editing, elevated levels of mRNA for the catalytic component of the apoB mRNA editing complex, apobec-1, and increased secretion of the product of the edited mRNA, apoB48, in very low density lipoproteins (VLDL) in primary cultures of Sprague-Dawley rat hepatocytes following insulin treatment. In order to determine the effect of *in vivo* hyperinsulinemia on these processes, we determined apoB mRNA editing, apobec-1 expression, hepatic expression of mRNA for apoB and other VLDL apoproteins, and the quantity and composition of plasma VLDL in the hyperinsulinemic fatty Zucker rat. Total apoB mRNA content of the livers of the fatty rats and lean littermates did not differ; however, edited apoB message coding for hepatic apo B48, and abundance of mRNA for the catalytic subunit of the apoB mRNA editing complex, apobec-1, was increased by 1.7- and 3.3-fold, respectively, in fatty rats. ApoCIII mRNA abundance was increased in livers of fatty rats as well, but the abundance of hepatic apoE mRNA in the fatty animal was not different from that of the lean rat. Hepatic apoAI mRNA abundance was also increased in the fatty rats. Associated with increased apoB mRNA editing, was the 1.7-fold increase in the fraction of apoB in plasma as apoB48 in fatty rats. VLDL-triglyceride and -apoB in plasma were 15- and 3-fold higher, respectively, in fatty Zucker rats compared to lean littermates, indicating both enrichment of VLDL with triglycerides and increased accumulation of VLDL particles. Increased hepatic expression of mRNA for apoCIII and apoAI was associated with increased content of apoC (and relative depletion of apoE) in VLDL of fatty rats, and plasma apoAI was increased in fatty Zucker rats, primarily in the HDL fraction. The current study provides further evidence that chronic exposure to high levels of insulin influences both the quantity of and lipid/apoprotein composition of VLDL in plasma. The increased apoC and decreased apoE (as well as increased triglyceride) content of VLDL in the fatty Zucker rat observed in the current study may affect VLDL clearance and therefore may be a factor in the observed accumulation of VLDL in the plasma of the fatty hyperinsulinemic Zucker rats.

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Apolipoprotein B (apoB) is an essential component of chylomicrons, very low density lipoprotein (VLDL), intermediate density lipoprotein (IDL), and low density lipoprotein (LDL) (1,2). It is expressed in a full-length form, apoB100, and in a truncated form, apoB48, which is the amino terminal 48% of apoB100. A single gene generates both apoB100 and apoB48 through apoB mRNA editing, in which the CAA codon for glutamine 2153 in apoB is changed to UAA, an in-frame translation stop signal (3). Although human liver secretes only full-length apoB100, the livers of other species including rat, mouse, horse, and dog produce both apoB100 and apoB48 (4). The ratio of hepatic apoB48 to apoB100 produced in the rat is regulated nutritionally (5) and by several hormones, including insulin (6,7), estradiol (8), and thyroid hormone (9).

We previously demonstrated that insulin treatment in primary cultures of hepatocytes from Sprague-Dawley rats increases editing of apoB mRNA and increases hepatic expression of mRNA for apobec1, the catalytic subunit of the editing complex (6,7). Increased editing of apoB mRNA was associated with an increase in the secretion of apoB48 in VLDL (6). These findings support the hypothesis that insulin regulates both the quantity and composition of VLDL secreted by the liver. It is important to determine whether these effects of insulin are also observed *in vivo* and to determine the effect of hyperinsulinemia on hepatic apolipoprotein gene expression. The fatty Zucker rat (fa/fa), a strain with inherited obesity and hyperinsulinemia, provides a useful model to examine the effects of hyperinsulinemia (10). Our prior *in vitro* experiments predicted that livers of fatty Zucker rats would demonstrate increased expression of mRNA for apobec-1 and increased apoB mRNA editing, and that this would be reflected by increased apoB48 content in plasma lipoproteins in comparison with lean littermates (Fa/–). A recent preliminary communication by Phung *et al.* indicates that this indeed occurs (11). The present study confirms and extends these findings by demonstrating that hyperinsulin-

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Abbreviations: ApoB, apolipoprotein B; HDL, high density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; PCR, polymerase chain reaction; VLDL, very low density lipoprotein.

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emia in the fatty Zucker rat not only results in increased apoB mRNA editing but is also accompanied by increased quantity and altered apolipoprotein composition of plasma VLDL, and that the expression of other apolipoprotein genes (apoCIII and apoAI) is also altered in the fatty Zucker rat.

MATERIALS AND METHODS

All chemicals and reagents used in these studies were analytical or molecular biology grade. Sources of special reagents are described below.

Animals and sample collection. Male Zucker rats were obtained from Harlan Industries (Indianapolis, IN) and fed standard Purina rodent chow diet *ad libitum*. Fatty Zucker rats (fa/fa) were received at an average weight of 390 g, and their lean littermates (Fa/–) were received at 260 g (about 10 wk of age). After 3 wk, obese animals had gained an average of 128 ± 11 g, and lean rats 63 ± 4 g. Livers were surgically removed from three fatty and three lean Zucker rats. Livers were quickly frozen in liquid nitrogen and stored at −70°C until processed. Blood was removed from the abdominal aorta and placed in tubes containing EDTA. Plasma samples were obtained after brief centrifugation to sediment the erythrocytes and analyzed immediately as described below. Lipids were extracted from aliquots of frozen liver and were analyzed as described below.

Preparation of RNA and cDNA. Total RNA was isolated from Zucker lean and fatty rat liver samples using RNA Stat-60 (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions and quantitated by absorbance at 260 nm. Following digestion with RNase-free DNase I (Ambion, Austin, TX) and heat denaturation of the enzyme, 5.0 µg of RNA from each liver sample was used to prepare cDNA with a cDNA Cycle Kit (Invitrogen, San Diego, CA), employing avian myeloblastosis reverse transcriptase. A 304 bp fragment of the rat apoB cDNA containing the edited site was amplified by reverse transcriptase-polymerase chain reaction (RT-PCR) using the cDNA samples generated from the Zucker rat liver RNA as templates as described (6).

Primer extension analysis. Editing of apoB mRNA was quantitated by primer extension analysis using a 27-base oligonucleotide primer complementary to the region starting eight bases downstream from the edited site in rat apoB mRNA, as described by Wu *et al.* (12). The primer (10 pmol) was 5′ end-labeled using a kit from Promega (Madison, WI), employing 150 µCi of (gamma-^{32}P)ATP (6000 Ci/mmol; DuPont NEN, Boston, MA). About 25 ng of the product from each PCR was assayed by denaturing the DNA at 95°C for 5 min, then annealing with a fivefold molar excess of labeled primer in 34 mM Tris-HCl (pH 8.3), 50 mM NaCl, and 5 mM dithiothreitol at 58°C for 30 min. The extension was carried out at 42°C for 30 min after the addition of dATP, dTTP, and dCTP to 0.5 mM and 2′,3′-dideoxy-GTP to 3 mM, plus 5 units of avian myeloblastosis virus reverse transcriptase (Invitrogen) according to Driscoll *et al.* (13). The reaction products were resolved on a 12% polyacrylamide sequencing gel and analyzed by autoradiography with Kodak X-Omat AR film (Kodak, Rochester, NY). The bands corresponding to edited or nonedited apoB mRNA were cut out of the gel and quantitated by liquid scintillation spectrometry.

DNA probes. A rat apoB cDNA probe (+126 to +643) was generated by PCR from rat liver cDNA using the following oligonucleotide primers: 5′-AGCAGTTATCTTCCCAAAGAAACG and 5′-CATGAAGATGTAGTGGAGAAACAAG. PCR reagents came from a GeneAmp kit (Perkin Elmer, Norwalk, CT), and the starting parameters used were those recommended by the manufacturer. After an initial denaturation for 3 min at 94°C, 35 cycles of amplification were carried out, with each cycle consisting of 30 s at 94°C, 1 min at 55°C, and 1 min at 72°C. A rat apobec-1 cDNA probe (+86 to +479) was also generated by PCR, following the above protocol, from rat liver cDNA using the primers 5′-GAGAAGAATTGAGCCCCACGAG and 5′-CTCCGTCATGATCTGGATAGTA. After PCR, all probes were purified through silica-binding using a Gene-Clean kit (Bio-101; Vista, CA). Probes were prepared for apoAI, apoCIII, and apoE using primers based on published sequences of rat liver cDNA for the corresponding proteins. Primers for apoAI were 5′-AAAGCTGCAGTGTTGGCTGTGGCC (upstream) and 5′ GTTCAGCTTCTTTTTGGCCTCATC (downstream), corresponding to sequences at the translation start and stop sites (14). Primers for apoCIII were 5′-CGGCTCAAGAGTTGGTGTTGTTAG (upstream) and 5′-CAGCCCCGAATGCTGCTCATCGTG (downstream). Primers for apoE were 5′-AAGGCTCTGTGGGCCCTGCTGTTG (upstream) and 5′-TTGATTTCTCCAGGGCACTGTGGT (downstream) (15).

Slot blot analysis of apoB mRNA. Five µg of total RNA from each sample was transferred to nylon membranes (Schleicher & Schuell, Keane, NH) in triplicate using a Schleicher & Schuell slot blot apparatus and ultraviolet cross-linked. Blots were prehybridized for 6 h at 42°C in 50% formamide, 5× SSPE (1× = 0.18 M sodium chloride, 0.01 M sodium phosphate, 1 mM EDTA, pH 7.4), 5× Denhardt's solution (5 Prime-3 Prime, Boulder, CO) (16), 7.5% dextran sulfate, 1.5% sodium dodecyl sulfate (SDS), and 100 µg/mL of sheared salmon sperm DNA (5 Prime-3 Prime). ApoB mRNA on the blots was detected by hybridization overnight with the apoB cDNA probe which was ^{32}P-labeled using the random primer method (17). The unbound probe was removed by washing membranes twice with 2× SSC, 0.1% SDS (1× = 0.015 M sodium chloride, 0.0015 M sodium citrate, pH 7.0) for 30 min at room temperature, and twice with 0.1× SSC, 0.1% SDS (1× = 0.015 M sodium chloride, 0.0015 M sodium citrate, pH 7.0) for 30 min at 65°C. Membranes were exposed to X-Omat AR film (Kodak) at −70°C for 1–2 d. The resulting films were digitized using an Alpha Innotech image analysis workstation (Alpha Innotech Corp., San Leandro, CA), and relative apoB mRNA was determined from the images using NIH Image (version 1.61) for Macintosh (National Institutes of Health USA shareware). Blots were corrected for mRNA loading by reprobing for poly A RNA using a biotinylated oligo (dT) probe from R&D Systems (Abingdon, United Kingdom) as described by Johnson *et al.* (18).

Northern blot analysis of apobec-1, apoCIII, apoE, and apoA1 mRNA. About 25 µg of total RNA, adjusted to contain the same amount of poly(A) RNA based on slot blot analysis, was loaded per lane onto a 1% agarose gel containing 2.2 M formaldehyde (16), electrophoresed, and transferred to a nylon membrane by capillary blotting with a Turbo Blotter (Schleicher & Schuell) using 20× SSC, according to manufacturer's instructions. RNA on the blot was UV cross-linked, and the blot was probed with the apobec-1 cDNA probe ^{32}P-labeled by the random primer method (17) using the same conditions employed for slot blot analysis.

These blots were subsequently reprobed for apoC III, apoE, and apoA1 using the probes described above.

Analyses of plasma, plasma lipoproteins, and liver tissue. Plasma lipoproteins were isolated by sequential ultracentrifugation at d = 1.006 (VLDL), d = 1.006–1.063 (IDL/LDL), and d = 1.063–1.21 high-density lipoprotein (HDL) after an initial centrifugation at 12,000 g (30 min) of plasma layered under a "mock plasma" (d = 1.006) to remove the "chylomicron" fraction. The "chylomicron" fraction was not further analyzed. The lipoprotein fraction isolated in the d 1.006–1.063 range contain some unmetabolized IDL from the liver and chylomicron remnants from the intestine, and is therefore referred to as IDL/LDL. Chemical analyses of all major classes of lipids (triglyceride, phospholipid, cholesterol, free fatty acid, and cholesteryl ester) from extracts of whole plasma, liver, and plasma lipoproteins were carried out after separation by thin-layer chromatography on silica gel G (19). Protein analyses on the isolated lipoproteins were carried out by the Lowry method as modified by Markwell *et al.* (20). Apolipoproteins of the various lipoprotein fractions of plasma were separated on Laemmli gradient gels (4 to 22% polyacrylamide) and stained with Coomassie Brilliant Blue R-250 (21). The mass of each apoprotein was estimated from densitometric scanning of the stained gels to determine the relative percentages in each lipoprotein. Separation of apoC species was not sufficient using this methodology to allow separate quantitation of apoCIII and apoCII. Therefore, total apoC content of lipoproteins is presented. Plasma apoB was also determined by radioimmunoassay as described previously (19). Plasma insulin levels were determined using a Micromedic Insulin RIA kit obtained from ICN (Costa Mesa, CA). DNA levels in liver samples were determined with a Hoefer DyNA Quant 200 (Pharmacia Biotech, Piscataway, NJ).

Statistics. All data are expressed as the mean ± SE (from values derived from three rats in each of the fatty and lean groups). Statistical comparisons were made with Student's t test (unpaired). Significance of difference was set at $P < 0.05$.

RESULTS

Characteristics of this group of fatty rats and their lean littermates are shown in Table 1. Fatty rats had plasma levels of insulin five times greater than those of the lean controls (273 vs. 58 µIU/dL). Although of similar age, the fatty rats were 1.6 times larger and their livers were twice as heavy as the lean controls. While there was a moderate increase in the total DNA content (54%) in livers from obese rats, total RNA and total protein contents were increased to a much greater extent (103 and 267%, respectively). Although total RNA content in the livers of fatty rats was increased, there was a reduction (28%) in mRNA as a fraction of total RNA (data not shown), indicating disproportionate increases in the ribosomal and/or transfer RNA content of the fatty livers.

TABLE 1
Characteristics of Lean and Fatty Zucker Rats[a]

	Lean (n = 3)	Fatty (n = 3)
Animal weight (g)	328.7 ± 7.5	515.7 ± 2.9*
Liver weight (g)	10.7 ± 0.7	22.0 ± 1.2*
Plasma insulin (µIU/mL)	58 ± 2	273 ± 62*
Protein (mg/g liver)	160.8 ± 5.7	210.0 ± 10*
Protein (g/liver)	1.72 ± 0.17	4.60 ± 0.12*
DNA (mg/g liver)	2.91 ± 0.03	2.19 ± 0.12*
DNA (g/liver)	31.1 ± 2.2	48.0 ± 1.7*
RNA (mg/g liver)	7.27 ± 0.70	7.14 ± 0.40
RNA (g/liver)	76.9 ± 6.5	156.4 ± 8.5*

[a]Results are means ± SE of 3 animals.
*$P < 0.05$ compared to lean rats.

Although total DNA per liver was greater in fatty Zucker rats, the content of DNA per gram of liver was significantly reduced. These relationships are consistent with a combination of hypertrophy and hyperplasia in the fatty rat livers. Because of significant differences in RNA and mRNA content in livers of fatty vs. lean Zucker rats, specific mRNA determinations are corrected for differences in total mRNA between livers of lean vs. fatty rats, and the abundance of the mRNA of interest in fatty livers is expressed relative to corresponding mRNA in lean controls. This provides an accurate representation of the abundance of specific mRNA species; however, relative to mRNA, since there is a twofold increase in total RNA per liver (28% less for mRNA) in the fatty Zucker rat (Table 1), this actually underestimates the amount of mRNA available for translation per liver by a factor of 1.7.

The fraction of hepatic apoB mRNA in the edited form was determined by primer extension assay (Fig. 1). Quantitative analysis of the bands revealed significantly higher levels of edited apoB mRNA in livers of fatty vs. lean Zucker rats [82.0

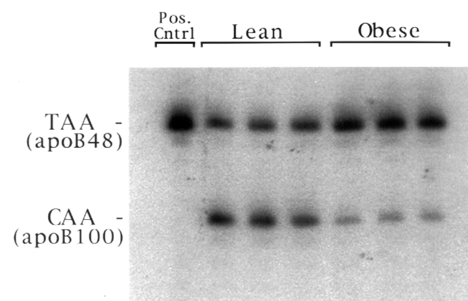


FIG. 1. The extent to which hepatic apolipoprotein B (apoB) mRNA had undergone editing was determined by primer extension as described in the Materials and Methods section. TAA and CAA refer to the sequence at codon 2153 of the apoB mRNA. CAA is the original, unedited sequence, while UAA is the edited sequence (TAA after polymerase chain reaction amplification).

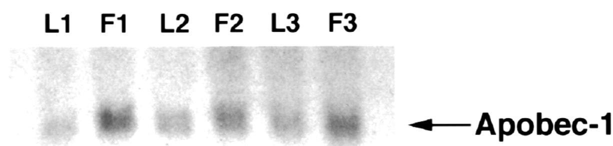


FIG. 2. Northern blot analysis of total RNA samples from lean (L) and fatty (F) Zucker rat livers, adjusted to contain equal amounts of poly (A) RNA, were analyzed for apobec-1 mRNA abundance as described in the Materials and Methods section.

± 0.4% (n = 3) vs. 47.6 ± 4.7% (n = 3)]. A direct correlation of the extent of hepatic apoB mRNA editing and of abundance of mRNA for the catalytic subunit of the editing complex, apobec-1, has been reported during induced hyperinsulinemia (high carbohydrate feeding) (22) and with insulin and estrogen treatment in rat hepatocytes (6,7). We therefore determined the abundance of apobec-1 mRNA in livers of lean and fatty Zucker rats by Northern blot analysis (Fig. 2). Apobec-1 mRNA was 3.3-fold more abundant in livers from obese rats than lean rats (16.4 ± 3.1 vs. 5.0 ± 3.4 arbitrary density units) when expressed relative to total mRNA (determined separately by slot blot analysis as described in the Materials and Methods section).

In addition to increased apoB mRNA editing, insulin may also regulate VLDL by altering overall expression of apoB and other VLDL apolipoproteins. We determined the abundance of mRNA for apoB, apoCIII, apoE, and apoA1 in the livers of fatty and lean Zucker rats by slot blot and Northern analysis. Apolipoprotein mRNA abundance was corrected for mRNA content measured with an oligo(dT) probe. Slot blot analysis of apoB mRNA revealed an average density of 0.85 ± 0.12 for fatty rats, and 1.00 ± 0.14 for lean rats (P < 0.10) (data not shown). The abundance of both apoCIII and apoAI mRNA, corrected for poly(A) mRNA content of fat vs. lean livers, was significantly greater in livers of fatty Zucker rats (1.77 ± 0.16 fold higher and 2.70 ± 0.31 fold higher than lean livers for apoCIII and apoAI, respectively, P < 0.05, n = 3) (Fig. 3), whereas that of mRNA for apoE was not significantly different (1.0 ± 0.13 vs. 0.87 ± 0.04 for lean and fatty livers, respectively).

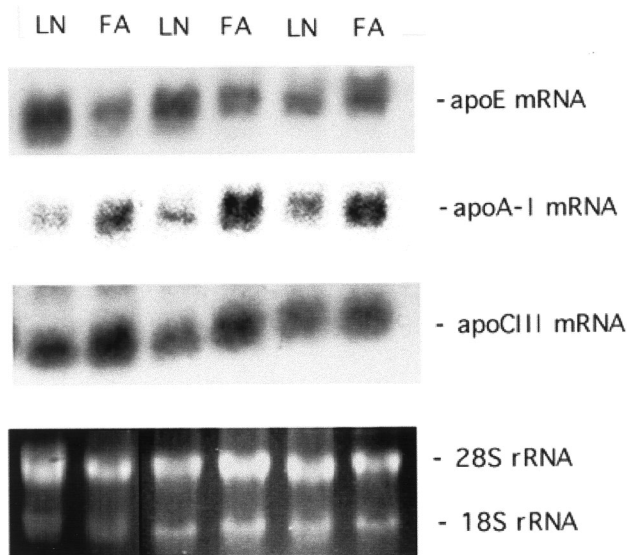


FIG. 3. After probing for apobec-1 mRNA, the blots were reprobed for apoCIII, apoE and apoAI mRNA. (LN) refers to individual liver samples lean and (FA) to liver samples from fatty rats.

To determine the effect of increased hepatic apoB mRNA editing and increased apoCIII and apoA1 gene expression on plasma lipoproteins in the fatty Zucker rat, we assessed plasma lipid and apolipoprotein levels in VLDL, LDL/IDL, and HDL fractions in lean vs. fatty Zucker rats. There was a marked increase in the amount of VLDL protein and lipid in plasma of fatty Zucker rats. In addition, there was a marked and disproportionate (32-fold) enrichment of VLDL of fatty Zucker rats with triglyceride (Table 2). Calculated molar ratios of surface/core lipids, i.e., cholesterol and phospholipid/triglyceride and cholesterol ester, for the VLDL of the lean and fatty rats were 0.79 ± 0.07 and 0.19 ± 0.02, respectively, indicating significantly larger particle size for circulating VLDL in the fatty rats. Similarly, VLDL of fatty Zucker rats also demonstrated a markedly higher ratio of triglyceride to apoB (17.25 ± 3.00 μmol/nmol vs. 1.70 ± 0.41 μmol/nmol for fatty and lean Zucker rats, respectively). Although the amount of IDL/LDL-protein, -phospholipid, and -cholesterol was not different in the plasma of the two groups, there was a significant triglyceride enrichment of this fraction in the plasma of the fatty animals as well (Table 2). In the fatty rats there was also a 1.6-fold elevation in the amount of plasma HDL protein accompanied by an increase in the lipid content (Table 2).

We determined plasma fatty acid and hepatic lipid content in lean vs. fatty Zucker rats. Total plasma-free fatty acid levels were not significantly higher in fatty Zucker rats than in lean rats (64.9 ± 6.1 vs. 51.7 ± 3.9 μmol/dL plasma, respectively). Livers of fatty Zucker rats had significantly higher levels of hepatic triglyceride compared to lean littermates [9.9 ± 0.7 and 4.3 ± 0.4 μmol/g liver, respectively (P < 0.05)]. The hepatic con-

TABLE 2
Plasma Lipoprotein-Lipid and -Protein Levels of Lean and Fatty Zucker Rats[a]

	μmol/dL plasma		
	VLDL[b]	IDL/LDL[b]	HDL[b]
Lean			
Triglyceride	7.8 ± 0.9	16.3 ± 2.8	<1.0
Phospholipid	4.8 ± 0.5	13.3 ± 2.3	40.1 ± 2.8
Cholesterol	3.8 ± 2.0	7.3 ± 1.8	13.4 ± 1.8
Cholesteryl ester	3.0 ± 1.0	25.1 ± 3.6	77.2 ± 18.5
Protein (mg/dL)	2.5 ± 0.4	9.6 ± 0.8	52.3 ± 3.9
Fatty			
Triglyceride	246.4 ± 44.5*	57.7 ± 10.9*	<1.0
Phospholipid	34.0 ± 9.1*	15.5 ± 0.7	64.1 ± 3.7*
Cholesterol	16.7 ± 4.8*	6.3 ± 0.4	23.4 ± 2.5*
Cholesteryl ester	7.2 ± 1.7*	23.2 ± 2.3	90.3 ± 18.3
Protein (mg/dL)	17.0 ± 4.7*	9.2 ± 0.3	83.6 ± 3.6*

[a]Results are means ± SE of 3 animals.
[b]Lipoproteins were isolated at d < 1.006 (very low density lipoprotein, VLDL), d =1.006–1.063 (intermediate density lipoprotein, IDL/low density lipoprotein, LDL), and d = 1.063–1.21 (high density lipoprotein, HDL). *P < 0.05.

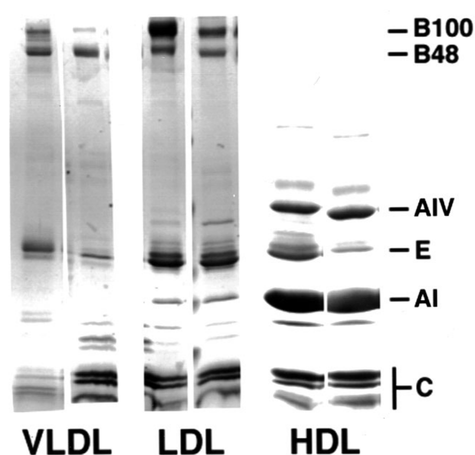
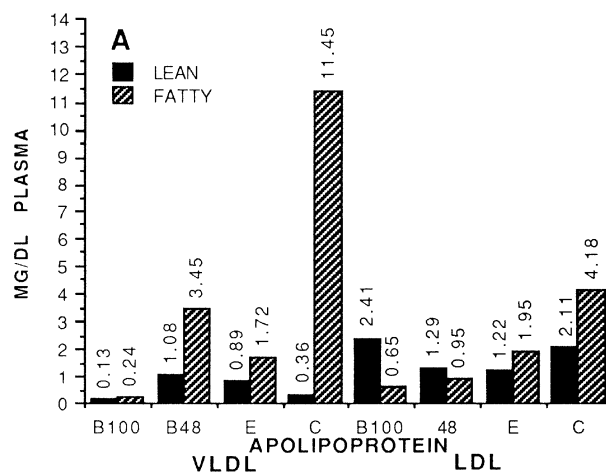


FIG. 4. A representative apoprotein pattern is presented for the very low density lipoprotein (VLDL), intermediate density lipoprotein/low density lipoprotein (IDL/LDL) and high density lipoprotein (HDL) isolated from the plasma of a lean and fatty Zucker rat. Within each lipoprotein fraction the left lane is from the lean Zucker rat and the right lane is from the fatty Zucker rat. An equal amount of protein was loaded in each lane, therefore this figure depicts the relative distribution of apoproteins in lipoprotein fractions. The increased relative content of apoB48, apoC, and decreased (relative to apoB) apoE is apparent in VLDL and LDL/IDL (apoB48 only). Quantitative data derived from densitometric scanning and calculated using total protein determination (Table 2) are presented in Figures 5A and 5B.



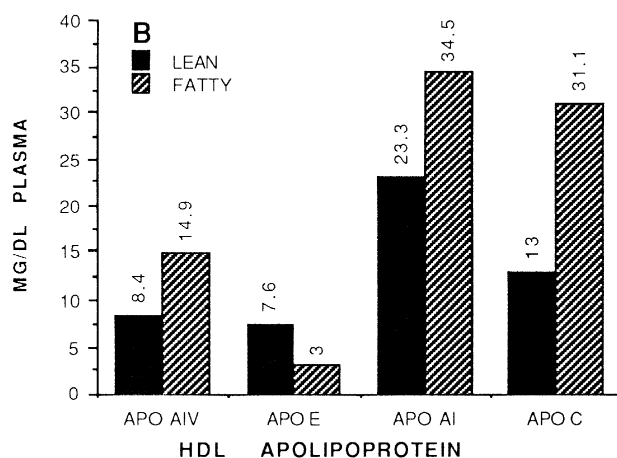


FIG. 5. (A) Apoproteins of VLDL and IDL/LDL, (B) apoproteins of HDL. Coomassie blue-stained polyacrylamide gels were digitized using Alpha Innotech image analysis workstation software (Alpha Innotech Corp., San Leandro, CA) and optical density of the protein bands quantitated using NIH Image (version 1.61) software for Macintosh (National Institutes of Health USA Shareware). Areas within a sample lane were totaled and the percentage of each individual apoprotein calculated. Corrections for potential differences in chromogenicity of each individual band were not made. The mass of each apoprotein was then calculated based on the measured mass of protein in the lipoprotein and the percentage of each apoprotein in each lipoprotein. Data are the mean of values derived from three experiments. See Figure 4 for abbreviations.

centrations of phospholipid and cholesterol in the fatty rat were significantly decreased (23 and 31% lower, respectively) but a 14% decrease in cholesteryl ester content was not statistically significant (data not shown).

Plasma lipoprotein–apoprotein patterns were examined by gradient sodium dodecyl sulfate polyacrylamide gel electrophoresis (Fig. 4). The mean calculated levels ($n = 3$ experiments) for apoprotein species in VLDL and IDL/LDL were derived from the densitometric scan of the stained gel. The amount of the lipoprotein-protein of each lipoprotein isolated from the plasma of each rat are presented in Figure 5A, while the data for the HDL fractions are given in Figure 5B. Although VLDL from fatty Zucker rats had higher contents of both apoB100 (twofold) and apoB48 (threefold), apoB48 content was disproportionately increased (Figs. 5A, Table 3), which is consistent with an increase in apoB mRNA editing in the liver of these animals. The isolated IDL/LDL fraction of the plasma of the fatty animals also had a much higher proportion of apoB48-containing particles than the lean rats (Fig. 5A, Table 3). The apoC content of both the IDL/LDL and HDL fractions were also increased both absolutely and relative to apoB (LDL/IDL) and apoE (Figs. 5A, and 5B). A higher plasma concentration of apoAI and apoAIV was also observed in the fatty Zucker rats, the majority of which was associated with HDL (Fig. 5B). The amount of apoAI associated with the VLDL and LDL was also increased, but this amount was small compared to that in the HDL.

The overall molar ratio of apoB48 to total apoB in the total lipoproteins of the plasma was 0.58 ± 0.06 and 0.85 ± 0.05 for the lean and fatty rats, respectively, which correlates with the proportion of edited apoB mRNA observed in the two groups. Altered levels of apoB48, apoC, and apoE in VLDL may reflect the presence of an increased number of particles, but also may reflect enrichment or relative depletion of apoproteins in individual VLDL particles. Therefore we calculated the relative content of apoC, apoE, and apoB48 relative to apoB100 or total apoB, using apoB as an index of VLDL particle number (each VLDL particle has one apoB molecule) to calculate molar ratios (Table 3). Molar ratios of apoB48 to apoB100 were increased in the VLDL and IDL/LDL of fatty vs. lean Zucker rats (Table 3). Although the total content of apoE in VLDL (and IDL/LDL) was higher in fatty rats (Fig. 5A), the molar ratio of apoE to apoB in the VLDL of fatty Zucker rats was reduced, in-

TABLE 3
Molar Ratios of ApoB48, ApoB100, ApoC, and ApoE in Selected Plasma Lipoproteins of Fatty vs. Lean Zucker Rats[a]

Apolipoprotein ratio (lipoprotein)	Fatty (n = 3)	Lean (n = 3)
ApoB48/apoB100		
VLDL	28.75 ± 5.75	16.60 ± 2.50
IDL/LDL	1.47 ± 0.27	0.54 ± 0.10
ApoE/apoB (VLDL)	3.60 ± 0.33	5.80 ± 1.93
ApoC/apoB (VLDL)	100.66 ± 43.4	9.83 ± 6.30

[a]Data are the relative content of apoproteins in VLDL (and IDL/LDL) expressed as molar ratios to apoB as an assessment of the difference in per particle content of apoE, apoC, and apoB48 between lean and fatty Zucker rats. See Table 2 for abbreviations.

dicating a relative reduction in the content of apoE per particle (Table 3). On the other hand the VLDL particles of the fatty rats were markedly enriched in apoC, both in absolute terms and on a per particle basis, compared to lean rats (Fig. 5A, Table 3).

Figure 6 depicts the total level of each apoprotein in plasma (the sum of the calculated values for VLDL, IDL/LDL, and HDL). The increase in apoB associated with VLDL in fatty Zucker rats was accompanied by an equivalent reduction in apoB in IDL/LDL, so that total plasma apoB (Fig. 6) was not different (4.9 ± 1.5 vs. 5.3 ± 1.1 mg/dL plasma for lean and fatty rats, respectively). The altered distribution of apoB in plasma of fatty Zucker rats (increased apoB48) might result in the underestimation of the number of apoB molecules using protein staining methodology (sodium dodecyl sulfate polyacrylamide gel electrophoresis) since the protein content of apoB48 is half that of apoB100. The similarity of the total apoB plasma levels, however, was confirmed using a method that assesses particle number (radioimmunoassay) rather than protein reaction (Coomassie blue). Total plasma apoB by radioimmunoassay was 4.3 ± 0.6 mg/dL in plasma of lean Zucker rats, compared to 3.0 ± 0.4 mg/dL in fatty Zucker rats (n = 3, P > 0.05).

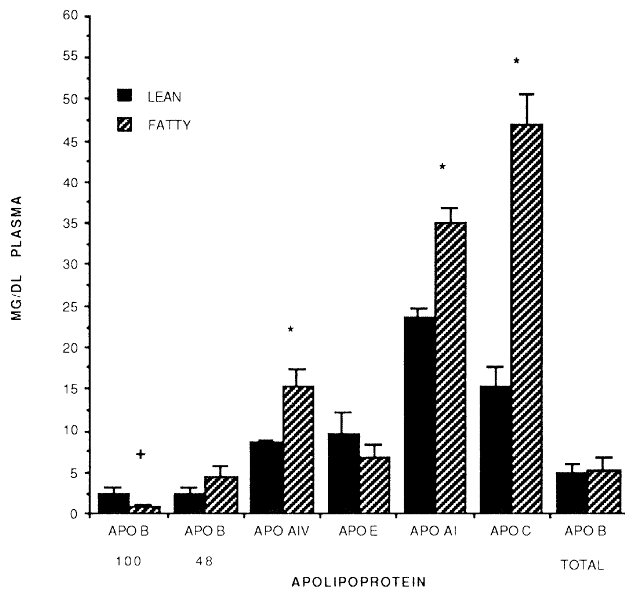


FIG. 6. Data represent the sum of the individual apoproteins in VLDL, IDL/LDL, and HDL. Also represented is the total apoB level in the plasma derived from the sums of apoB 48 and apoB 100 in the three lipoprotein fractions. The majority of the apoAI, apoAIV, and apoC was present in HDL (99, 98, and 84% for the lean rats and 99, 97, and 66% for that of the fatty rats, respectively). See Figure 5 for abbreviations. *(P < 0.05) and + (P < 0.10) compared to the lean controls; n = 3.

DISCUSSION

Our current findings on increased apobec-1 mRNA expression and increased apoB mRNA editing along with the resultant enrichment of plasma lipoproteins with apoB48 in the hyperinsulinemic fatty Zucker rat are consistent with our own previous studies of *in vitro* insulin treatment in primary cultures of Sprague-Dawley hepatocytes (6,7) and with those of Phung *et al.* (11). We also report here associated alterations in quantity and composition of plasma VLDL in fatty Zucker rats, specifically increased number of plasma VLDL particles (apoB) as well as enrichment of the VLDL with apoC and relative reduction in VLDL apoE content. In addition, we report that in the fatty Zucker rat, hyperinsulinemia is accompanied by increased hepatic expression of apoCIII and apoAI , but not total apoB or apoE mRNA. These alterations have significant implications for the pathogenesis of hyperlipidemia in the fatty Zucker rat and indicate that prolonged hyperinsulinemia, as found in the fatty Zucker rat, results in altered lipid and apoprotein composition of the VLDL, and in altered hepatic mRNA expression of key apoproteins involved in VLDL synthesis and clearance.

The increased content of apoB48 relative to apoB100 in plasma VLDL and IDL/LDL observed in the Zucker fatty rat in this study and in other reports (11,23) suggests that increased editing of the apoB message results in increased secretion of apoB48 relative to apoB100. Our previous *in vitro* studies and the current study indicate that this is a specific effect of insulin which is mediated, at least in part, by increased expression of apobec-1 (6,7). Other *in vivo* studies also support the hypothesis that insulin regulates apoB mRNA editing in the hyperinsulinemic fatty Zucker rat (11). During fasting, apoB mRNA editing is decreased concomitant with decreased levels of insulin (24). Conversely, the apoB48/apo-100 ratio is elevated in plasma (25) and the hepatic perfusate VLDL (26) of fed vs. fasted rats. Similarly, in the Goto-Kakizaki rat, a slightly obese, hyperinsulinemic, hyperglycemic model of noninsulin-dependent diabetes, editing of apoB mRNA has been shown to be increased (27) as well as the plasma levels of apoB48, but not apoB100.

The current study also demonstrates that hypertriglyceridemia in the fatty Zucker rat is the result both of marked enrichment of plasma VLDL with triglyceride and increased accumulation of VLDL particles (as indicated by increased plasma VLDL apoB content). Enrichment of VLDL with triglyceride in the fatty Zucker rat results from increased hepatic triglyceride synthesis and secretion (23,28,29). The reason for the presence of an increased number of VLDL particles in the plasma of fatty Zucker rats is less certain. Although

we have previously demonstrated that secretion of apoB by hepatocytes of the Sprague-Dawley rat is increased by insulin (6), studies of apoB secretion by perfused livers and hepatocytes of fatty Zucker rats by other workers have, with the exception of one report (23), failed to demonstrate increased secretion of apoB (29–32). Our findings that accumulation of apoB in plasma VLDL was accompanied by a concomitant reduction of apoB in IDL/LDL fractions also suggests that decreased clearance of VLDL may be, in part, responsible for net accumulation of VLDL in plasma of fatty Zucker rats.

Clearance of chylomicron remnant particles has been reported to be reduced in the fatty Zucker rat (33). Although, to our knowledge, VLDL clearance has not been specifically studied in the fatty Zucker rat, chylomicron remnants and VLDL share a common removal mechanism (34). Although the mechanisms which alter clearance of triglyceride-rich lipoproteins in the fatty Zucker rat have not been elucidated, our observation of increased apoC and reduced apoE content in VLDL of fatty Zucker rats, and of increased hepatic apoCIII expression, may provide insight into this process. ApoE (and apoCII) promote clearance of triglyceride-rich lipoproteins by the liver; whereas apoCIII reduces VLDL clearance by inhibiting lipoprotein lipase activity (35–39). Therefore, the VLDL composition which we have observed in the fatty Zucker rats, specifically enrichment with apoC and relative depletion of apoE, might be expected to result in delayed clearance of chylomicron remnants (and VLDL), as observed in the studies of Redgrave (33). Although the methodology used in the present study did not allow us to assess the distribution of apoC subspecies (apoCI, CII, CIII), Witztum and Schonfeld (29) observed previously that both apoCII and apoCIII are increased in VLDL of fatty Zucker rats. The interaction of apoCII and apoCIII on VLDL clearance when both are elevated deserves further study. Furthermore, our novel observation that steady-state levels of apoCIII mRNA are increased in livers of fatty Zucker rats indicates a possible role of altered apoCIII expression in the pathogenesis of hyperlipidemia in the fatty Zucker. This latter finding is unexpected in that the apoCIII gene is transcriptionally downregulated following insulin treatment of diabetic mice and in HepG2 cells transfected with an apoCIII luciferase reporter construct (39). The presence of increased apoCIII mRNA levels in the face of markedly increased plasma insulin in the fatty Zucker rat has important implications and is currently the subject of further study.

We found that increased apoA1 expression in livers of hyperinsulinemic fatty Zucker rats was accompanied by increased plasma apoA1, predominantly in HDL. Murao *et al.* (40) have, in fact, shown that insulin will induce apoAI mRNA in human hepatoma cells (Hep G2). Increased levels of HDL and apoAI have also been reported in the fatty Zucker rat by Witztum and Schonfeld (29).

The DNA, RNA, and protein data summarized in Table 1 reveal some interesting characteristics of the fatty Zucker liver. Although livers from the fatty rats were twice as large as those of lean rats the DNA, RNA, and protein contents increased to widely differing extents. The DNA content of the livers of the fatty rats was 1.5-fold greater, while those of RNA and protein were elevated 2- and 2.7-fold, respectively, indicating that there are both more and larger cells in the fatty livers. Metabolic and/or hormonal changes, including insulin, specific to the fatty Zucker rat may alter liver cell ploidy in these animals (41,42). It is also noteworthy that the content of poly(A) RNA, a measure of mRNA, per μg of total liver RNA was decreased by 28% in the fatty rats. This indicates that ribosomal and/or transfer RNA is increased to a greater extent in the livers of the fatty rats. These differences should be taken into consideration in interpreting both mRNA levels and lipoprotein production rates, and may explain why increased VLDL apoB secretion has been observed in perfused livers of fatty Zucker rats (23), but not in cultured hepatocytes where output is expressed as a function of cell number or mass (30–32).

In summary, we have demonstrated that hyperinsulinemia in the fatty Zucker rat is accompanied by increased apoB mRNA editing, increased expression of mRNA for the catalytic subunit of the editing complex, apobec-l, and increased hepatic mRNA expression of apoCIII and apoAI. These changes are accompanied by increased content of apoB48 relative to apoB 100 in plasma triglyceride-rich lipoproteins, and net accumulation of VLDL lipid and apoB in the plasma of fatty Zucker rats compard to lean littermates. In addition, the observed changes in VLDL lipid and apoprotein composition specifically, enrichment in triglyceride and apoC and relative reduction in apoE content, may alter VLDL clearance in and thereby contribute to hypertriglyceridemia in the fatty Zucker rat.

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Effects of Cholestyramine on Hepatic Cholesterol 7α-Hydroxylase and Serum 7α-Hydroxycholesterol in the Hamster

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ABSTRACT: Cholestyramine increases activities of hepatic cholesterol 7α-hydroxylase and serum levels of 7α-hydroxycholesterol. To examine if serum 7α-hydroxycholesterol levels parallel with enzyme activity, 0, 0.5, 1, 2, 4, and 10% of cholestyramine was administered to female golden Syrian hamsters for 28 d in the dose-dependent study, and 2% cholestyramine for 0, 1, 3, 7, 14, 21, and 28 d in the time-dependent study. In the dose-dependent study, hepatic and serum cholesterol levels were significantly decreased dose-dependently when more than 0.5% of cholestyramine was fed for 28 d. Cholestyramine increased the cholesterol 7α-hydroxylase activity in a dose-dependent manner, while the serum 7α-hydroxycholesterol level was essentially unchanged. No correlation was found between the serum level and the hepatic enzyme activity. In the time-dependent study, hepatic and serum cholesterol levels markedly decreased when 2% cholestyramine was fed for longer than 3 d. The serum triglyceride level increased significantly for the first 7 d and then decreased. Cholesterol 7α-hydroxylase activity increased significantly as early as day 1, reached maximum activity level on day 7, and then kept the significantly high values until day 28. The serum 7α-hydroxycholesterol level significantly increased for the first 7 d and decreased to the pretreatment level thereafter. 7α-Hydroxycholesterol levels significantly correlated with serum cholesterol and triglyceride levels. We conclude that the serum 7α-hydroxycholesterol level does not always reflect the activity of hepatic cholesterol 7α-hydroxylase, when cholesterol metabolism is severely disturbed by cholestyramine.

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The initial and rate-limiting step in bile acid biosynthesis is 7α-hydroxylation of cholesterol to form 7α-hydroxycholesterol by hepatic microsomal cholesterol 7α-hydroxylase (cholesterol 7α-monooxygenase, EC 1.14.13.17) (1). The activity of the enzyme is regulated by bile acids and cholesterol levels (1–3). Interruption of the enterohepatic circulation of bile acids by biliary diversion (4) and cholestyramine feeding (5) increases the cholesterol 7α-hydroxylase activity as well as the enzyme mass and mRNA expression. Cholesterol feeding (6,7) and depletion of dietary cholesterol by furnishing a lymph fistula (8) also stimulate the enzyme activity.

Enzyme activity was determined by isotope-incorporation technique, which measures the conversion of labeled cholesterol to 7α-hydroxycholesterol (9). Recently, more sensitive, accurate, and reproducible methods have been developed, measuring the actual mass of 7α-hydroxycholesterol by gas chromatography–mass spectrometry (GC–MS) (10) and by high-performance liquid chromatography (11). However, all these methods have a crucial limitation in human studies, because of the requirement of liver biopsy specimens.

We have recently shown a positive correlation between the serum level of 7α-hydroxycholesterol and the hepatic cholesterol 7α-hydroxylase activity in humans (12–14) and in experimental animals (15–17). From these results, we proposed that the serum level of total 7α-hydroxycholesterol may be a good indicator in assessing the enzyme activity and the amount of bile acid synthesized in the liver.

Cholestyramine is a sequestrant of bile acids and accelerates hepatic bile acid synthesis. The activities of cholesterol 7α-hydroxylase and the serum levels of 7α-hydroxycholesterol have been reported to increase with the administration of cholestyramine in rats (15) and humans (18). However, it has not been known if serum 7α-hydroxycholesterol levels reflect hepatic enzyme activity when variable doses of cholestyramine are fed for variable periods. In the present study, we examined dose- and time-dependent effects of cholestyramine on hepatic enzyme activity and serum 7α-hydroxycholesterol levels in hamsters.

MATERIALS AND METHODS

Materials. Cholestyramine (Questran, Bristol-Myers Co., Evansville, IN) was purchased from a commercial source. This preparation contains 4 g of cholestyramine in 9 g of Questran. Cholesterol, EDTA, and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). 7α-Hydroxycholesterol and 5α-cholestane-3β,7β-diol were synthesized as described previously (10). Bond-Elut silica cartridge columns were purchased from Analytichem International (Harbor City, CA). TMSI-H (hexamethyldisilazane/trimethylchlorosilane/pyridine, 2:1:10, by vol) was purchased from Gasukuro Kogyo (Tokyo, Japan). NADPH was obtained

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Abbreviations: DTT, dithiothreitol; GC, gas chromatography; GC–MS, gas chromatography–mass spectrometry; LDL, low density lipoprotein.

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from Kojin (Tokyo, Japan). All solvents used in the present study were of analytical grade or distilled prior to use.

Equipment. A Shimadzu GC15A GC, equipped with a flame-ionization detector, a van den Berg's solventless injector, and a data-processing system (Chromatopack C-R3A; Shimadzu, Kyoto, Japan), and a Shimadzu Auto GC–MS 9020DF GC–MS system with a data-processing system (SCAP 1123) were employed. A fused-silica capillary column (15 m × 0.2 mm) coated with a 0.25-μm layer of cross-linked methylsilicon (Hicap CBP1; Shimadzu, Kyoto, Japan) was used. Conditions for GC were as follows: column oven temperature, 280°C; injection port temperature, 290°C; detector temperature, 290°C; flow rate of helium carrier gas, 2.7 mL/min. Conditions for determination of 7α-hydroxycholesterol by GC–MS were the same as described previously (10).

Animal experiment. Female golden Syrian hamsters (Kyudo, Fukuoka, Japan), weighing 90–110 g, were used. The animals were kept in groups of 2–3 animals per cage and had free access to water and standard powder rodent chow (Oriental Yeast Co. Ltd., Tokyo, Japan) under a controlled 12-h light–dark cycle (light period from 8:00 A.M. to 8:00 P.M.) for at least a 1-wk acclimation period. In the dose-dependent study, varying concentrations of cholestyramine (0, 0.5, 1, 2, 4, and 10% in chow) were fed for 28 d to four animals in each group. In the time-dependent study, 2% cholestyramine was fed for various periods (0, 1, 3, 7, 14, 21, and 28 d, 5 to 6 animals in each group). After an overnight fast, the animals were sacrificed under ether anesthesia between 10:00 and 12:00 A.M. Blood was obtained by cardiac puncture and serum was separated and stored at −20°C until analyzed. The liver was excised, rinsed with an ice-cold homogenizing solution, and weighed. This protocol was reviewed and preapproved by the Committee of the Ethics on Animal Experiments in the Faculty of Medicine, Kyushu University, and was carried out under the control of the Guidelines for Animal Experiments of the Institute.

Determination of hepatic microsomal cholesterol 7α-*hydroxylase activity*. Cholesterol 7α-hydroxylase activity was determined by the method previously described (10). Liver homogenate was prepared in 50 mM Tris-HCl buffer (pH 7.4) containing 0.3 M sucrose, 10 mM DTT, and 10 mM EDTA. The homogenate was centrifuged at 20,000 × *g* for 15 min. The microsomal fraction was obtained by centrifugation of the supernatant fluid at 100,000 × *g* for 1 h. The microsomal pellet was suspended in the homogenizing medium without DTT and recentrifuged at 100,000 × *g* for 1 h. The resulting microsomal pellet was resuspended in 0.1 M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA. An aliquot of microsomal suspension was used for protein determination using the method of Lowry *et al.* (19). The standard assay system consisted of 0.5 mL of the microsomal preparation corresponding to 0.5–1.0 mg of microsomal protein and 0.1M potassium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM NADPH in a total volume of 1.0 mL. The enzyme reaction was conducted for 15 min at 37°C. The reaction was terminated by addition of chloroform/methanol (2:1, vol/vol). 5α-Cholestane-3β,7β-diol was added as an internal standard. After extraction with chloroform/methanol (2:1, vol/vol), the organic phase was evaporated to dryness under a stream of nitrogen. Following purification with Bond-Elut silica cartridge column and TMS derivatization (10), the actual mass of 7α-hydroxycholesterol was analyzed by GC–MS. In the selected ion monitoring mode, the ion at *m/z* 456 (M − 90) was scanned for the trimethylsilyl ether derivative of 7α-hydroxycholesterol, and *m/z* 458 (M − 90) for that of the internal standard.

Assay of serum 7α-hydroxycholesterol. The serum concentration of 7α-hydroxycholesterol was determined as described previously (13). In brief, 200 pmol of 5α-cholestane-3β,7β-diol dissolved in 50 μL of ethanol was added to 0.5 mL of serum as the internal standard. After the addition of 0.7 mL of 0.9% NaCl solution and 1.8 mL of ethanol, sterols were extracted with *n*-hexane. The collected *n*-hexane layer was evaporated to dryness under a stream of nitrogen. Sample was then hydrolyzed in 2.0 mL of 2.5% KOH in 90% ethanol at 55°C for 45 min. After the addition of 1 mL of distilled water, the sterols were extracted with *n*-hexane and the solvent was evaporated under nitrogen. Following purification and derivatization, the serum total 7α-hydroxycholesterol level was quantitated as described above.

Determination of hepatic cholesterol and serum lipids. Approximately 100 mg of the liver was hydrolyzed with 10% KOH in 95% ethanol, containing 20.2 mg of 5α-cholestane as an internal standard, at 70°C for 2 h. Lipids were extracted and the cholesterol content was determined by GC (20). Serum cholesterol and triglyceride concentrations were determined by an automatic multichannel analyzer (Hitachi 736-40 Automatic Analyzer, Hitachi Co., Tokyo, Japan).

Statistical analysis. Values were expressed as means ± SEM. The statistical difference was evaluated using the Mann–Whitney U test. Probability values less than 0.05 were considered statistically significant. Correlations between the parameters were examined by Spearman's rank correlation test.

RESULTS

Dose-dependent study. The animals ingested approximately 10 g of chow per day, and there were no significant differences in food intake among the groups. Body weight and liver weight are summarized in Table 1. The growth of the animals was suppressed significantly in animals fed 10% cholestyramine. In 4 and 10% cholestyramine groups, one out of 4 and 2 out of 4 animals died during the experiment, respectively. The liver weight decreased significantly in animals fed more than 1% cholestyramine for 28 d. In the 10% cholestyramine group, the liver weight was approximately 50% of the controls. Serum triglyceride, serum cholesterol, and liver cholesterol concentrations are depicted in Figure 1. Serum total cholesterol concentration decreased significantly and was dose-dependent. The serum cholesterol level of animals in the 10% cholestyramine group was only 32% of the control value (186.0 ± 12.6 mg/dL in the control group). Serum triglyceride level became less than that of the control animals (322.3 ± 57.8 mg/dL). In cholestyramine-fed animals, however, a sta-

TABLE 1
Effects of Cholestyramine on the Body and Liver Weight of the Hamster, Dose-Dependent Study[a]

Dose (%)	Body weight (g)	Liver weight (g)
0	145.8 ± 3.2 (4)	6.4 ± 0.4 (4)
0.5	151.3 ± 2.7 (4)	6.3 ± 0.2 (4)
1	144.3 ± 5.3 (4)	5.2 ± 0.3 (4)
2	143.4 ± 2.6 (4)	4.4 ± 0.2[b] (4)
4	139.7 ± 7.2 (3)	4.3 ± 0.4[b] (3)
10	103.2 ± 8.3[b] (2)	3.0 ± 0.2[b] (2)

[a]A variable dose of cholestyramine (0, 0.5, 1, 2, 4, and 10% in chow) was fed for 4 wk to 4 hamsters in each group. Values are expressed as mean ± SEM; and figure in parentheses indicates number of animals.
[b]$P < 0.01$, significantly different from the value of the control group (0% cholestyramine).

tistically significant decrease was observed only in the 10% cholestyramine group. The total cholesterol concentration in the liver was significantly lower in groups fed more than 0.5% cholestyramine for 28 d; the level was nearly one-tenth that of the controls.

The cholesterol 7α-hydroxylase activities and the serum 7α-hydroxycholesterol levels after the feeding of different doses of cholestyramine for 28 d are shown in Figure 2. Cholestyramine caused a dose-related increase of the cholesterol 7α-hydroxylase activity and the activity was 6.2 times that of the control values (2.96 ± 0.59 pmol/min/mg protein) when 10% cholestyramine was fed. In contrast, the serum 7α-hydroxycholesterol levels did not increase in a dose-dependent manner and were practically unchanged compared with the control level (322 ± 58 pmol/mL), although a statistically significant decrease was observed in the 2% cholestyramine group. There was no correlation between the serum 7α-hydroxycholesterol level and the hepatic cholesterol 7α-hydroxylase activity (data not shown).

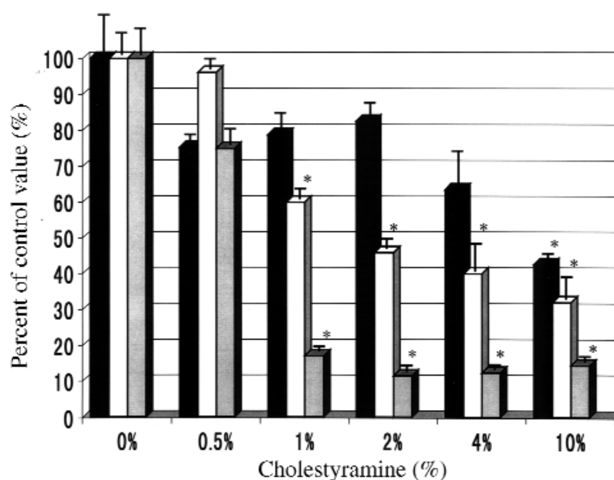

FIG. 1. Effect of cholestyramine on tissue lipid concentrations in the hamster, dose-dependent study. Variable dose of cholestyramine (0, 0.5, 1, 2, 4, and 10% in chow) was fed to 4 hamsters in each group for 4 wk. Values are expressed as percent of the control value. Closed column, light column, and gray column indicate serum triglyceride, serum total cholesterol, and hepatic total cholesterol, respectively. Vertical bar indicates SEM of 3 to 4 hamsters. *$P < 0.01$ vs. the controls.

Time-dependent study. The body and liver weights of the hamsters, which were administered 2% cholestyramine for 1 to 28 d are listed in Table 2. The animals gradually gained weight until day 14 and then the body weight was stable. The liver weight was essentially unchanged throughout the experiment. However the liver weight on the day 28 was significantly less than that of animals fed only chow (refer to Table

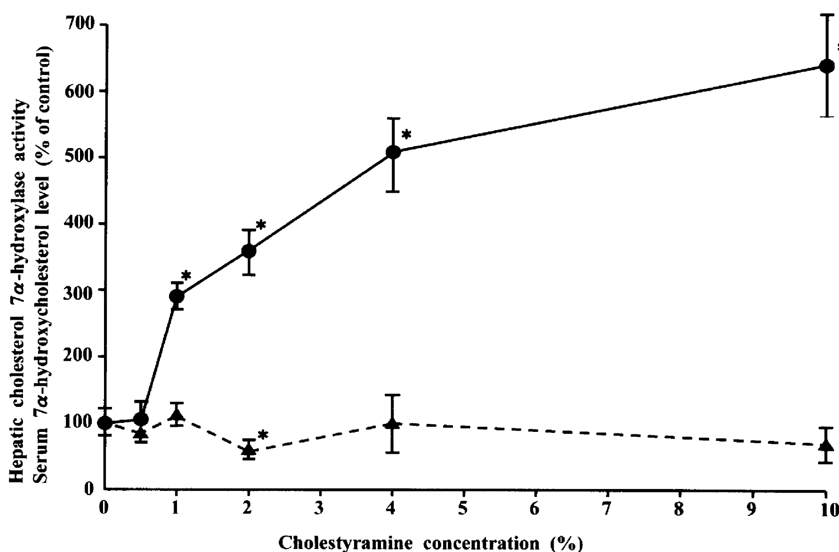

FIG. 2. Effect of cholestyramine on hepatic microsomal cholesterol 7α-hydroxylase activity and serum 7α-hydroxycholesterol level in the hamster, dose-dependent study. Variable dose of cholestyramine (0, 0.5, 1, 2, 4, and 10% in chow) was fed to hamsters for 4 wk and the hepatic enzyme activity (●) and the serum 7α-hydroxycholesterol level (▲) were determined. Values are expressed as percent of the control value. Vertical bar indicates SEM of three to four hamsters. *$P < 0.01$ vs. the controls.

TABLE 2
Effects of Cholestyramine on the Body and Liver Weight of the Hamster, Time-Dependent Study[a]

Days	Body weight (g)	Liver weight (g)
0	99.1 ± 3.1 (5)	4.7 ± 0.2 (5)
1	115.2 ± 3.4[c] (5)	5.2 ± 0.2 (5)
3	110.7 ± 1.3[b] (5)	4.5 ± 0.2 (5)
7	118.6 ± 2.5[c] (5)	5.2 ± 0.2 (5)
14	135.8 ± 4.3[c] (5)	4.6 ± 0.2 (5)
21	133.7 ± 4.2[c] (5)	4.5 ± 0.3 (5)
28	134.2 ± 1.7[c] (6)	4.5 ± 0.2 (6)

[a]Cholestyramine (2% in chow) was administered for 1, 3, 7, 14, 21, and 28 d to five to six hamsters in each group. Values are expressed as mean ± SEM and figure in parentheses indicates number of animals.
[b]$P < 0.05$ and [c]$P < 0.01$, significantly different from the value of the control group (day 0).

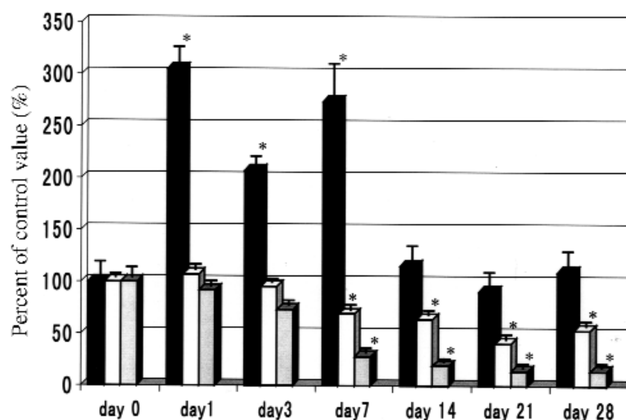

FIG. 3. Effect of cholestyramine on tissue lipid concentrations in the hamster, time-dependent study. Cholestyramine (2% in chow) was administered for 1, 3, 7, 14, 21, and 28 d to five to six hamsters in each group. Values are expressed as percent of the control value. Closed column, light column, and gray column indicate serum triglyceride, serum total cholesterol, and hepatic total cholesterol, respectively. Vertical bar indicates SEM of five to six hamsters. *$P < 0.01$ vs. the controls.

1). Figure 3 shows serum triglyceride, serum cholesterol, and liver cholesterol concentrations of the animals in the time-dependent study. Serum triglyceride increased significantly on day 1, had significantly higher levels until day 7, and then decreased. The level on day 28 was not statistically different from the pretreatment level. Serum cholesterol level was not significantly changed during the first 3 d and then significantly decreased. Serum cholesterol became about 50% of the pretreatment level on day 28. The liver cholesterol level significantly decreased from day 7 and kept the significantly low level until day 28. The liver cholesterol on day 28 was only 14% of the pretreatment value.

Hepatic cholesterol 7α-hydroxylase activities and serum 7α-hydroxycholesterol levels after feeding 2% cholestyramine for various periods are shown in Figure 4. The cholesterol 7α-hydroxylase activity increased significantly as early as day 1, and reached the maximal level on day 7, keeping significantly higher values than the basal value until day 28. The serum 7α-hydroxycholesterol level increased significantly on day 1, was significantly higher than the pretreatment value until day 7, and then decreased. The levels between day 14 and day 28 were not significantly different from the pretreatment value.

The correlation between the serum 7α-hydroxycholesterol levels and the serum cholesterol levels is shown in Figure 5. There was a significant positive correlation between the serum 7α-hydroxycholesterol level and the serum cholesterol levels ($n = 28$, $r = 0.58$, $P < 0.01$). There also was a significant positive correlation between the serum 7α-hydroxycholesterol levels and the serum triglyceride levels (Fig. 6, $n =$

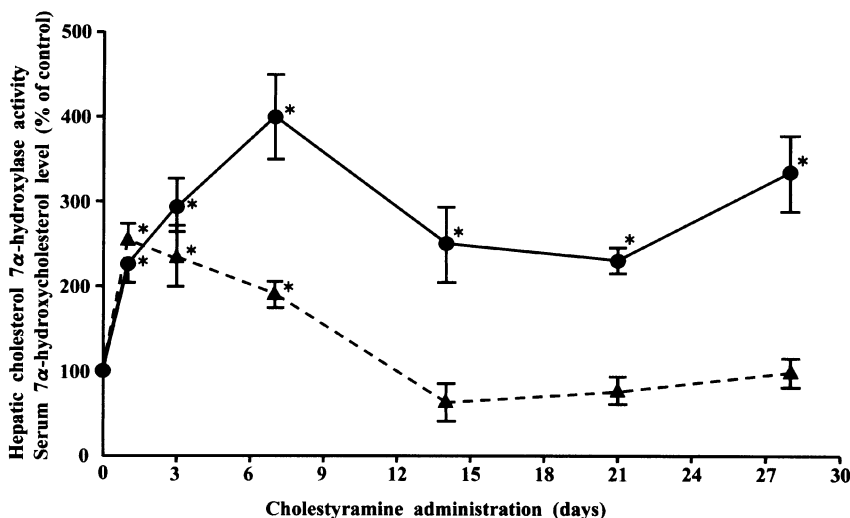

FIG. 4. Effect of cholestyramine on hepatic microsomal cholesterol 7α-hydroxylase activity and serum 7α-hydroxycholesterol level in the hamster, time-dependent study. Cholestyramine (2% in chow) was administered for 1, 3, 7, 14, 21, and 28 d and the hepatic enzyme activity (●) and the serum 7α-hydroxycholesterol level (▲) were determined. Values are expressed as percent of the control value. Vertical bar indicates SEM of five to six hamsters. *$P < 0.05$ vs. day 0.

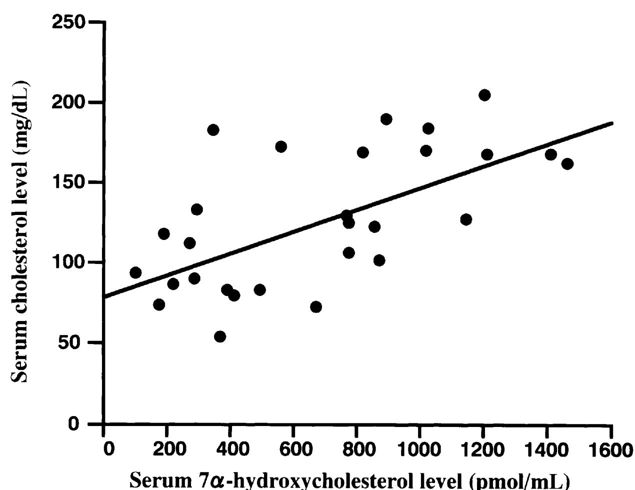

FIG. 5. Correlation between the serum 7α-hydroxycholesterol level and the serum cholesterol level, time-dependent study. A significant positive correlation was observed ($y = 0.07x + 80.8$, $n = 28$, $r = 0.58$, $P < 0.01$).

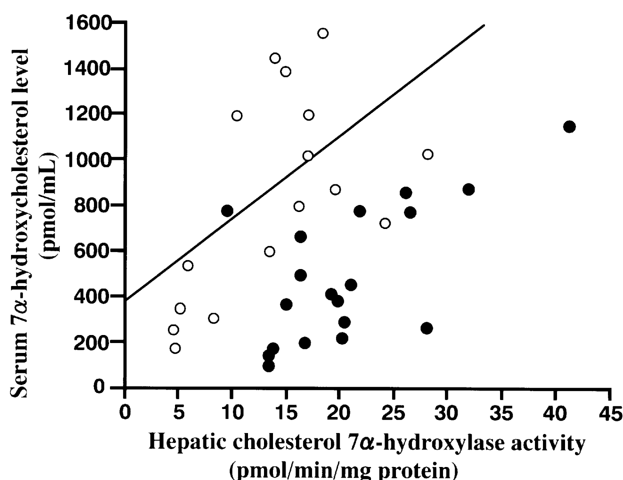

FIG. 7. Relationship between the hepatic cholesterol 7α-hydroxylase activity and the serum 7α-hydroxycholesterol level, time-dependent study. Open circles (○) indicate animals fed 2% cholestyramine for 0–3 d (16 animals). There was a significant positive correlation between the two parameters ($y = 36.4x + 379$, $n = 16$, $r = 0.58$, $P < 0.01$). Closed circles (●) indicate animals fed 2% cholestyramine for 7 to 28 d (19 animals). There was no significant correlation in all animals ($n = 35$, $r = 0.264$, $P = 0.126$).

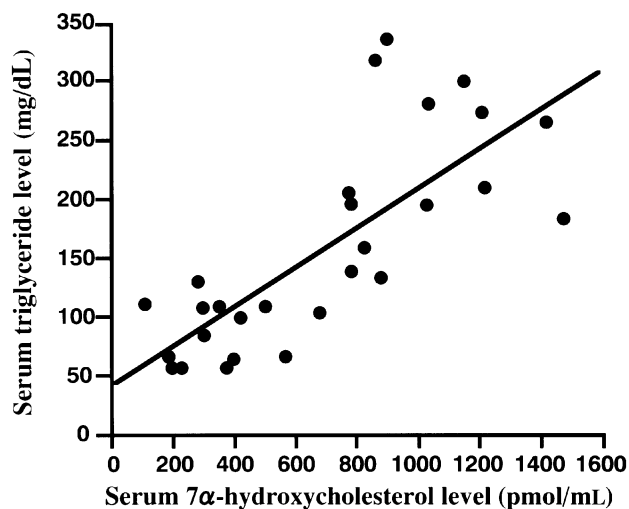

FIG. 6. Correlation between the serum 7α-hydroxycholesterol levels and the serum triglyceride levels, time-dependent study. A significant positive correlation was observed ($y = 0.17x + 43.4$, $n = 28$, $r = 0.78$, $P < 0.01$).

28, $r = 0.78$, $P < 0.01$). The relationship between the hepatic cholesterol 7α-hydroxylase activities and the serum 7α-hydroxycholesterol levels in the time-dependent study is shown in Figure 7. There was no significant correlation between the hepatic cholesterol 7α-hydroxylase activities and the serum 7α-hydroxycholesterol levels in all animals (open and closed circles). However, there was a significant positive correlation between the enzyme activities and the serum 7α-hydroxycholesterol levels during the first 3 d (Fig. 7, open circles, $n = 16$, $r = 0.58$, $P < 0.01$), when serum and hepatic cholesterol levels were not decreased significantly (Fig. 3).

DISCUSSION

Cholestyramine is an ion exchange resin that binds anions such as bile acids (9). Because bile acids play an important role in the absorption of cholesterol (21), the binding of bile acids by cholestyramine decreases intestinal cholesterol absorption (9). Interruption of the enterohepatic circulation of bile acids by cholestyramine decreases bile acid concentration in the portal blood and increases bile acid synthesis by a negative feedback mechanism (15,22). It is generally accepted that the hepatic cholesterol 7α-hydroxylase activity increases several fold by cholestyramine feeding (23–27). As a result, elimination of cholesterol from the body is greatly accelerated by cholestyramine. Although the liver may respond to compensate for the situation by increasing *de novo* cholesterol synthesis (28–30), the serum and liver cholesterol concentrations are decreased by the oral administration of cholestyramine in experimental animals (31) as well as in humans (32).

As cholestyramine is not absorbed by the intestine, it is considered to be a safe hypocholesterolemic agent. In the present study, however, animals fed extraordinarily high concentrations of cholestyramine (10%) did not gain weight, and some of them died. The liver was apparently atrophic, and cholesterol concentrations of the liver and serum were dramatically decreased. Serum transaminase levels were not measured in this study, however, it is not likely that cholestyramine has direct hepatotoxicity, as we previously showed unchanged serum transaminase levels when 2% cholestyramine was fed to hamsters for 5 wk (33). The cholesterol metabolism of the hamster is known to be very sensi-

tive to dietary cholesterol manipulation when compared to rats and humans (31). The administration of high concentrations of cholestyramine to hamsters is reported to decrease the body cholesterol pool by extensive losses of bile acids and cholesterol into the feces (31). Humans are reported to be less sensitive to cholesterol load (34) and cholestyramine (32,35) and the toxic effect of cholestyramine observed in the present study may not be directly applicable to humans. However, it should be pointed out that there might be a possible toxicity of cholestyramine treatment in patients with disturbed cholesterol synthesis, such as those with liver cirrhosis (35).

In the dose-dependent study, the activity of hepatic cholesterol 7α-hydroxylase significantly increased in hamsters when more than 0.5% of cholestyramine was fed. In contrast to the increase in the enzyme activity, the concentrations of serum 7α-hydroxycholesterol did not increase. No correlation between the cholesterol 7α-hydroxylase activity and the serum 7α-hydroxycholesterol level was found. The result was not expected from the previous report in which cholestyramine increased both the enzyme activity and the serum 7α-hydroxycholesterol level in rats (15). We also reported the increased serum 7α-hydroxycholesterol levels in cholestyramine-fed humans (35). We hypothesized that the serum 7α-hydroxycholesterol level may increase from a short-term cholestyramine feeding and then decrease to the pretreatment levels after a prolonged administration of cholestyramine. Thus, the time-dependent study was conducted.

For the time-dependent study, 2% cholestyramine was selected. At this dose, serum and liver cholesterol levels began to decrease significantly on day 7 after the administration of cholestyramine. The activity of cholesterol 7α-hydroxylase increased significantly as early as the first day, reached the maximum level on day 7, and remained high thereafter.

As hypothesized above, the serum 7α-hydroxycholesterol levels did not respond as hepatic cholesterol 7α-hydroxylase activity did after the 2% cholestyramine feeding. The time-dependent study has clearly shown that the serum 7α-hydroxycholesterol levels significantly increased for the first 7 d and then decreased to the pretreatment levels between the 14th and 28th days, when hepatic cholesterol 7α-hydroxylase activity remained significantly higher than the pretreatment value.

This is the first report to reveal the inconsistent behaviors of the cholesterol 7α-hydroxylase activity and the serum 7α-hydroxycholesterol level. In our previous study, we reported that the serum 7α-hydroxycholesterol level reflects the enzyme activity in humans and rats under various conditions (12–17). In the present study, however, there was no correlation between the cholesterol 7α-hydroxylase activities and the serum 7α-hydroxycholesterol levels. It should be noted, however, that there was a significant positive correlation between the two parameters during the first 3 d after 2% cholestyramine feeding, when serum and hepatic cholesterol concentrations were not altered significantly. These results suggest that the serum level of 7α-hydroxycholesterol could still be an indicator of hepatic cholesterol 7α-hydroxylase activity when tissue cholesterol levels are not significantly affected. However, these results do not reflect the hepatic enzyme activity under a severely disturbed cholesterol metabolism.

The key to solving the mystery of inconsistent serum 7α-hydroxycholesterol behavior may be the participation of low density lipoprotein (LDL) receptors. It has been well documented that LDL receptors on the surface of the hepatocytes increase in animals treated with cholestyramine (28,29,36). Because most of serum 7α-hydroxycholesterol is present in the LDL fraction (37), the increased uptake of serum LDL *via* LDL receptors should result in the elimination of serum 7α-hydroxycholesterol. The statistically significant positive correlation between the serum cholesterol or serum triglyceride levels and the serum 7α-hydroxycholesterol levels shown in the present study (Figs. 5 and 6) support the mechanism.

In summary, the activity of hepatic microsomal cholesterol 7α-hydroxylase increased in relation to time and dose in cholestyramine fed hamsters. Also, we found that the serum 7α-hydroxycholesterol level does not always reflect the activity of hepatic cholesterol 7α-hydroxylase, when cholesterol metabolism is severely affected

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Incorporation of [1-^{13}C]Oleate into Cellular Triglycerides in Differentiating 3T3L1 Cells

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ABSTRACT: Oleate is one of the most abundant dietary fatty acids, and much remains to be learned about its metabolism in fat cells. We studied the incorporation of exogenous [1-^{13}C]-oleate into triglycerides (TG) in differentiating 3T3L1 preadipocytes using ^{13}C NMR spectroscopy. The quantity of oleate incorporated into TG was found to increase as preadipocytes differentiated into fat cells. The ratio of unesterified [1-^{13}C]oleate to total stored fatty acids was higher in less differentiated cells, and declined at later stages of differentiation as cells accumulated fatty acids through *de novo* synthesis. When added as the only exogenous fatty acid, oleate was largely esterified at the *sn*-2 position. When equimolar unlabeled linoleate was co-provided at the same time, the ratio of [1-^{13}C]oleate esterified at the *sn*-1,3 position increased, implying competition between linoleate and oleate for esterification, especially at the *sn*-2 position. When cells pre-enriched with [1-^{13}C]oleate (esterified to TG) were treated with isoproterenol, a lipolytic agent, most of the [1-^{13}C]oleate was still found in TG, despite a high rate of lipolysis determined by measuring glycerol release. This implies extensive re-esterification of the oleate released by lipolysis.

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Oleic acid is present in a variety of acylated lipids, including phospholipids, cholesteryl esters, and triglycerides. The metabolism of oleate plays an important role in a number of diseases, particularly obesity and atherosclerosis (1–6). As adipose tissue is the major organ for fatty acid storage and release, elucidation of how oleate is metabolized in adipocytes and how this is affected by other fatty acids is of considerable importance.

^{13}C nuclear magnetic resonance (NMR) spectroscopy has proven very useful for investigating the metabolism of fatty acids in adipocytes, particularly for identifying esterification products (7–12). A ^{13}C NMR analysis of perfused cultured 3T3L1 adipocytes showed the utility of [1-^{13}C] labeled fatty acids for monitoring incorporation of exogenous fatty acids and their release through lipolysis (8). Cellular enzymes tended to esterify saturated fatty acids to the *sn*-1,3 positions and unsaturated fatty acids to the *sn*-2 position of glycerol. Under basal conditions, the rate of fatty acid release was the same from both carbonyl positions. When stimulated by a lipolytic agent, the rate of fatty acid release was greater from the *sn*-1,3 position than from the *sn*-2 position (8). Such studies employing perfused cells can detect cell metabolic products, providing valuable information about cellular events. However, perfusion experiments have to be completed in relatively short periods of time.

We recently used a different strategy to study the incorporation of exogenous fatty acids into cellular lipids (9). Cells maintained in monolayer culture were incubated with fatty acids at appropriate stages of differentiation for various periods of time. Cultures were terminated and the cellular lipids were extracted for NMR analysis. A known amount of [1-^{13}C]methyl myristate was added before extraction to serve as an internal integration reference. This method is advantageous for its optimal spectral resolution, sensitivity for a given mass of lipid, and flexible cell incubation conditions. We applied this method to study the incorporation of palmitate in rat preadipocytes, and found that palmitate could be esterified into both phospholipids (particularly at the *sn*-1 position) and triglycerides (with even distribution between *sn*-1,3 and *sn*-2 positions). As cells became more differentiated, the incorporation of palmitate into phospholipids decreased, whereas incorporation into triglycerides increased (9).

In this work, we used a similar strategy to study the incorporation of oleate into lipids in 3T3L1 cells at different stages of differentiation and to study the effects of lipolysis on the esterified oleate.

EXPERIMENTAL PROCEDURES

Cell culture and sample preparation. NIH-3T3L1 cells were cultured in basal medium [DMEM, fetal bovine serum (10%), penicillin (100 units/mL), and streptomycin (100 μg/mL)] until 2 d post confluency. To induce differentiation, cultures were exposed to basal medium supplemented with MDI [methylisobutylxanthine (120 μg/mL), dexamethasone (0.39 μg/mL), and insulin (100 μU/mL)]. Two days later, cells were

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Abbreviations: BSA, bovine serum albumin; DMEM, 10% fetal bovine serum, 100 units/mL penicillin, 100 μg/mL streptomycin; FFA, free fatty acid; ISO, isoproterenol; MDI, 120 μg/mL methylisobutylxanthine, 0.39 μg/mL dexamethasone, 100 μU/mL insulin; TG, triglyceride.

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incubated in basal medium supplemented with insulin (25 μU/mL). Medium was changed every 2–3 d. Multiple, small intracellular lipid droplets were visible by phase contrast microscopy 2 d after MDI treatment.

Sample preparation. A stock solution containing [1-^{13}C]-oleate complexed to bovine serum albumin (BSA) was added to the cell culture medium [concentration of [1-^{13}C]oleate = 1 mM, BSA = 0.2 mM, at the beginning of free fatty acid (FFA) incubation] during a regular medium change. Incubations were terminated at various time points by removing the medium. Cells were washed three times with phosphate buffer saline solution (pH = 7.4), scraped off into methanol, and Folch-extracted as described previously (9). Lipids were dissolved in deuterated chloroform and stored at 4°C under dry N_2 to prevent oxidation until the NMR experiments were performed. For each sample preparation, cells were harvested from one 100-mm culture dish. All cultures used were treated with MDI 2 d post confluency. As cells differentiated, confluency was maintained. Within the time frame of our experiment, we did not notice significant loss of cells (>5%). The total DNA value was 136 ± 10 μg/culture. Incubation with 1 mM of fatty acids complexed to BSA solution with a 5:1 molar ratio did not affect cell viability. We measured DNA instead of protein as the index for cell number because cellular protein levels change as cells differentiate, whereas the total DNA level generally remains constant after MDI treatment.

Chemicals. [1-^{13}C]oleate and deuterated solvent ($CDCl_3$) were purchased from CIL (Cambridge, MA), [1-^{14}C]oleate from NEN (Boston, MA), glycerol analysis reagent from Sigma Chemical Co. (St. Louis, MO), unlabeled linoleate and all other organic solvents were HPLC grade and purchased from Aldrich (Milwaukee, WI). [1-^{13}C]methyl myristate was prepared as described previously (9).

NMR spectroscopy. ^{13}C NMR spectra were obtained at 125 MHz on a Bruker DMX-500 spectrometer (Billerica, MA) with a 5-mm triple resonance probe. A 90° pulse width (12 μs), ^{1}H decoupling with the inverse-gated Waltz-16 sequence, and a pulse interval of 7.0 s were used for generating all spectra. The T_1 effects on integrated intensities were calibrated as described before (9). All NMR experiments were performed at 12°C to minimize solvent evaporation. The center peak of $CDCl_3$ in the spectrum was used as an internal chemical shift reference (77.0 ppm). All spectra were acquired with 4000 scans and processed without linebroadening to optimize resolution.

Determination of total triglycerides (TG) by densitometry. After the NMR experiments, thin-layer chromatography was performed to quantitate total TG (labeled plus unlabeled). About 10 μg of the total lipid mixture were spotted on a 10 × 10 cm HPTLC-GHL silica gel plate (Analtech, Newark, DE) and eluted in a solvent mixture for neutral lipids (hexane/ethyl ether/acetic acid, 70:30:1). The plate was sprayed with sulfuric acid and charred. The plate was then scanned and the volume of the TG component in each sample was quantified on a Diversity pdi 420oe™ scanner (Huntington Station, NY) with a reference to a known external thin-layer chromatograph reference standard (18-5A; Nu-Chek-Prep, Elysian, MN) eluted on the same plate. The total amount of TG so measured was converted to molar scale by assuming an average molecular weight of 887 (triolein).

Isotope tracing of [1-^{14}C]oleate in the culture medium. At appropriate stages of differentiation, cells were incubated with 1 mM unlabeled oleate/BSA complex containing a trace amount of [1-^{14}C]oleate (~3 μCi). Aliquots of the culture medium were taken at various time points and acidified to expel dissolved CO_2. Then the sample was mixed with 10 mL scintillation liquid and counted on a Wallace 1217 Rackbeta liquid scintillation counter (Gaithersburg, MD). Samples prepared in this way could contain minor amounts of soluble metabolites. However, other investigators have reported that exogenous long-chain fatty acids are exclusively (>98%) used for TG synthesis in differentiating preadipocytes (13). We also found that the amount of $^{14}CO_2$ produced from ^{14}C-labeled exogenous long-chain fatty acids in cells prepared under our conditions was negligible (unpublished results). Hence, we assumed that the amount of isotope remaining in the medium represented the amount of residual fatty acids.

Measurement of lipolysis. Lipolysis was measured by assaying glycerol released into the incubation medium. Briefly, cells at appropriate stages of differentiation were incubated with [1-^{13}C]oleate for 5 h to enrich the intracellular TG pool. Control cultures were terminated at this point to prepare samples for NMR and total TG measurement. Parallel cultures were washed three times with PBS and incubated with lipid-free, serum-free, and BSA-free DMEM containing ISO (10 μM) with and without insulin (100 μU/mL). At the end of the incubation, the medium was aspirated and assayed enzymatically for glycerol (Sigma Procedure No. 337) (14). Because fat cells do not have glycerol kinase, glycerol released from lipolysis cannot be metabolized (31), and is thus generally used as an index for lipolysis (24). Cellular lipids were extracted as described above.

RESULTS

^{13}C NMR spectral features. A complete ^{13}C NMR spectrum of the total lipid extract from cells incubated with [1-^{13}C]-oleate is illustrated in Figure 1. Signals in the upfield region (10–40 ppm) represent the background of the naturally abundant acyl chain carbons and are similar to those observed in pure triglycerides (8,10). Peaks in the region of 60–70 ppm are from glyceryl carbons, and those at ~130 ppm are from olefinic carbons. Compared to these naturally abundant signals, the carbonyl region (172–175 ppm) is dominated by two intense peaks with chemical shifts corresponding to the carbonyls at the *sn*-1,3[TG(1,3)] and *sn*-2 [TG(2)] positions in the TG molecules. Signals from *sn*-1 and *sn*-3 carbonyls are not discriminated in this spectrum. The farthest downfield signal (174.5 ppm) is from added [1-^{13}C]methyl myristate that serves as an internal integration reference.

Signals from phospholipids or other acylated lipids were not detected in this spectrum, indicating that TG was the main labeled component in the lipid mixture. In principle, [1-^{13}C]-

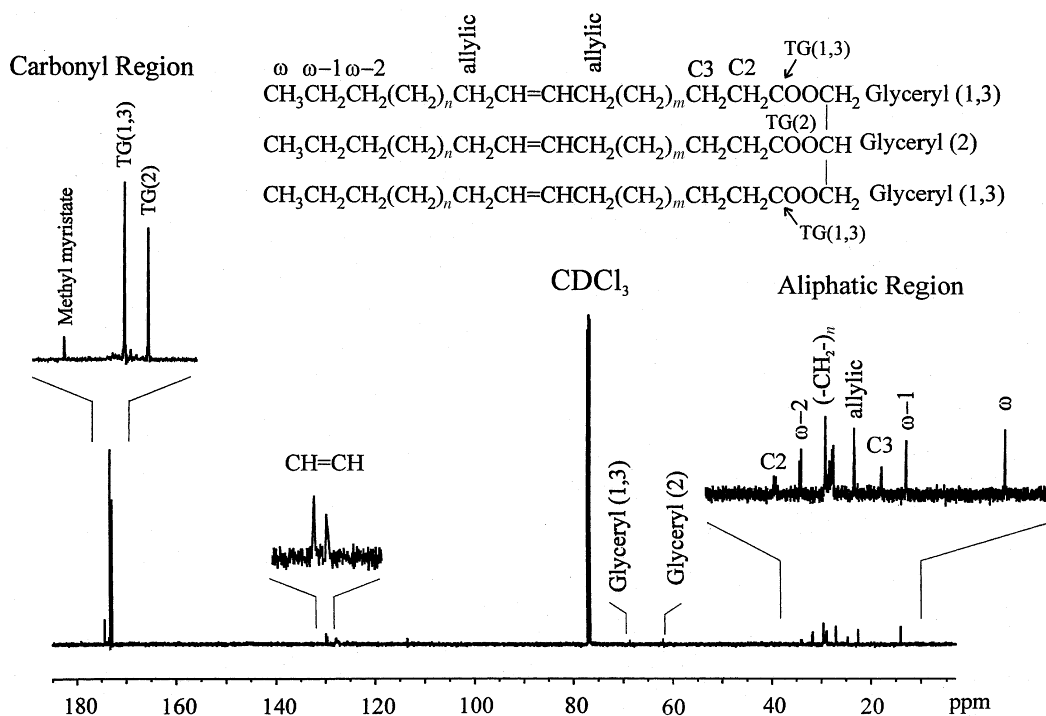

FIG. 1. The full ^{13}C nuclear magnetic resonance spectrum of a lipid extract from differentiated 3T3L1 cells incubated with [1-^{13}C]oleate for 1 h. After longer periods, signals in the aliphatic region became insignificant compared to the carbonyl signals as accumulation of esterified [1-^{13}C]oleate increased. In this spectrum, 50 μL (1 mg/mL) of internal standard solution was added. For all other samples studied, 50–200 μL of the same standard solution was added to the cells before the Folch extraction. The amount of standard added was based on the cellular TG content estimated by microscopy before cell harvesting, so as to minimize any integration error due to large differences between the intensities of the reference signal and the lipid signals.

oleate could be β-oxidized to produce [1-^{13}C]acetylCoA for *de novo* synthesis of long-chain fatty acids. If this occurred to a significant extent, ^{13}C enrichment would appear in the aliphatic carbons, and signal enhancement would have been detected in the aliphatic spectral region. The absence of such signals indicated that utilization of acetylCoA derived from the β-oxidation product of [1-^{13}C]oleate was not significant. Therefore, the incorporation of exogenous [1-^{13}C]oleate into TG can be determined by the integrated intensity of [TG(1,3) + TG(2)], and the distribution of [1-^{13}C]oleate between the *sn*-1,3 and *sn*-2 positions can be determined by the peak intensity ratio of TG(1,3)/TG(2).

Incorporation of [1-^{13}C]oleate increases as cells differentiate. Treatment with MDI stimulates differentiation of 3T3L1 cells (15). Using the NMR method described above, we measured the incorporation of exogenous [1-^{13}C]oleate into TG in cells at different stages of differentiation (Table 1). In essentially undifferentiated cells (day 0), total cellular TG content was very low (Table 1), and the exogenous [1-^{13}C]-oleate incorporated into TG (within 5 h incubation) accounted for ~30 mol% of the total stored fatty acids. This ratio decreased to ~10% when cells were highly differentiated (Table 1), probably because *de novo* synthesis from glucose also provides a significant quantity of fatty acids for storage

TABLE 1
Total Free Fatty Acid Content, [1-^{13}C]Oleate Incorporated Into TG, Esterification at TG(1,3)/(TG(2), and Ratio of [1-^{13}C]Oleate to Free Fatty Acid[a]

	Days					
	0	2	3	4	5	7
Total FFA (μmol)	0.54 ± 0.2	1.2 ± 0.3	5.1 ± 1.5	16.2 ± 2.4	23.5 ± 2.7	27 ± 1.1
[1-^{13}C]Oleate (μmol)	0.15 ± 0.03	0.2 ± 0.03	0.5 ± 0.07	1.5 ± 0.2	2.1 ± 0.2	2.4 ± 0.2
TG(1,3)/TG(2)	2.0 ± 0.1	1.8 ± 0.1	1.7 ± 0.1	1.3 ± 0.1	1.22 ± 0.1	1.2 ± 0.1
[1-^{13}C]Oleate/stored FFA (%)	28 ± 1	17 ± 2	10 ± 3	9 ± 2	8.9 ± 1	8.9 ± 1

[a]Results are presented as ±SE per culture (n = 3). Readings were taken from lipids extracted from cells after a 5-h incubation with [1-^{13}C]oleate on different days after MDI treatment. FFA, free fatty acid; MDI, methylisobutylxanthine (120 μg/mL), dexamethasone (0.39 μg/mL), and insulin (100 μU/mL); TG, triglyceride.

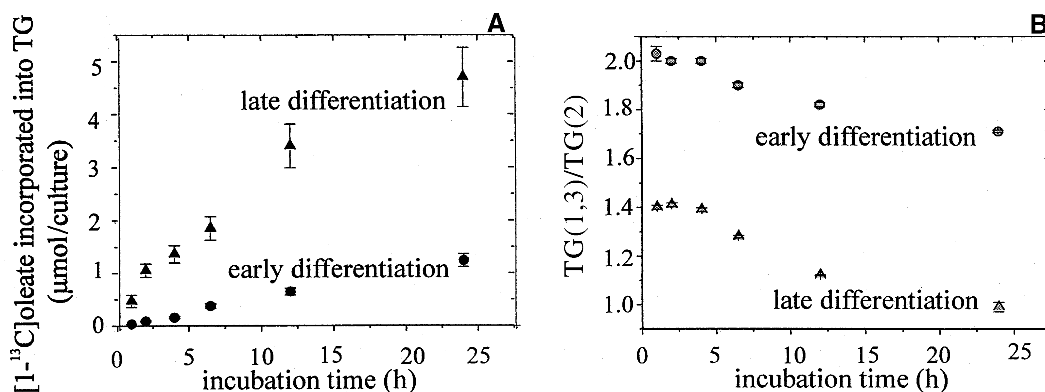

FIG. 2. (A) The incorporation of [1-^{13}C]oleate into triglycerides (TG) in cells at early [2 d after 120 μg/mL methylisobutylxanthine, 0.39 μg/mL dexamethasone, 100 μU/mL insulin (MDI) treatment] and late (6 d after MDI treatment) stages of differentiation as a function of incubation time. (B) The ratio of [1-^{13}C]oleate esterifed at the TG(1,3)/TG(2) positions as a function of incubation time. Data are presented as mean ± SE, *n* = 3.

(16). Palmitate, as the major end product of *de novo* synthesis (17), may have become increasingly available. Previous studies showed that palmitate is more likely than oleate to be esterified at the *sn*-1,3 position (8). Probably for these reasons, more [1-^{13}C]oleate was directed toward *sn*-2 position, resulting in a net decrease of the TG(1,3)/TG(2) ratio (Table 1).

Incorporation of [1-^{13}C]oleate increases with incubation time. To study the time-dependent incorporation (0–24 h) of [1-^{13}C]oleate into cellular lipids, we incubated cells at early (day 2) and advanced (day 6) stages of differentiation. Figure 2A shows that the incorporation of [1-^{13}C]oleate was dependent on incubation time, and total incorporation was greater in cells at advanced stages of differentiation. At the end of a 24-h incubation, the amount of [1-^{13}C]oleate detected in cellular TG was about 21% (2 d after MDI) and 72% (6 d after MDI) of the total [1-^{13}C]oleate added.

Analogous to the results shown in Table 1, we found that the incorporation of [1-^{13}C]oleate was similar at the TG(1,3) and TG(2) positions [TG(1,3)/TG(2) ≅ 2] in cells at early stages of differentiation. However, oleate was more likely to be esterified in the TG(2) position in cells at the advanced stage of differentiation. There was a gradual decrease in the TG(1,3)/TG(2) ratio as incubation time increased (Fig. 2B). This indicates that rearrangement of [1-^{13}C]oleate from the *sn*-1,3 to the *sn*-2 position may occur when the incubation time increases or the concentration of exogenous [1-^{13}C]-oleate decreases.

To determine the rate of substrate utilization, we incubated cells with unlabeled oleate (1 mM) and a trace amount of [1-^{14}C]oleate. We then measured the [1-^{14}C]oleate remaining in the medium to determine the concentration of exogenous oleate at different time points over a 24-h period (Fig. 3). The clearance of exogenous oleate was faster in cells at advanced stages of differentiation, and the labeled oleate was about 30% (2 d) and 85% (6 d) depleted at the end of a 24-h incubation. Both values are slightly greater than the amount of [1-^{13}C]oleate recovered in cellular TG (see above).

As previously reported (13), and as is evident from our data, the major metabolic fate of exogenous oleate is esterification into TG. Hence, the net rate of oleate usage can be approximately described by:

$$3 \text{ oleate} + \text{glycerol-3-phosphate} \rightarrow \text{TG} \qquad [1]$$

Because the cells used in this study were at a relatively early stage of differentiation compared to mature fat cells, the extent of accumulation of fatty acids into TG was far less than that of typical adipocytes (10). Hence, we can assume that there is no product inhibition in the reaction (Eq. 1), and the equilibrium constant is very large, so that the integrated form of the Henri-Michaelis-Menten equation (18) is applicable to describe the kinetics of oleate usage:

$$V_{max} = 2.3K_m \log \{[S_o]/[S]\} + ([S]_o - [S]) \qquad [2]$$

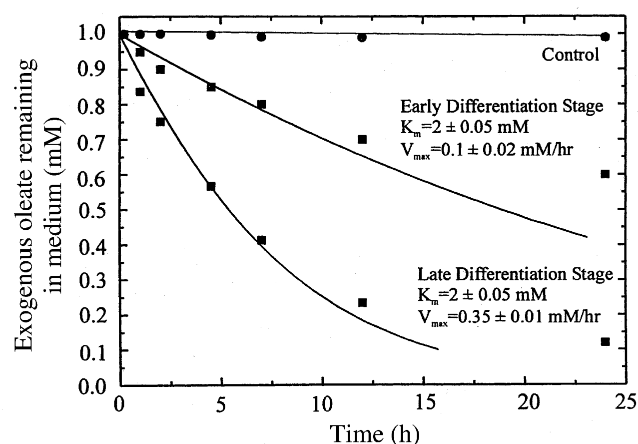

FIG. 3. The concentration of residual oleate in the culture medium after different incubation time intervals. Total oleate concentration was 1 mM at the beginning of incubation. Solid lines are the nonlinear least squares fit to the Henri-Michaelis-Menten equation. The last data point (24 h) was not included in the fit because the reaction mechanism might not be applicable after extended incubation.

TABLE 2
The Effects of Incubation with Linoleate (1 mM) and [1-^{13}C]Oleate (1 mM) on Differentiating 3T3L1 Adipocytes at Mid-Stage of Differentiation (4 d after MDI treatment)[a]

	Oleate	Oleate + linoleate
Total TG-derived FFA (μmol)	25.5 ± 5.1	26.7 ± 4.5
TG-incorporated [1-^{13}C]oleate (μmol)	2.6 ± 0.06	1.9 ± 0.04
% [1-^{13}C]Oleate /total TG-fatty acids	10.2 ± 0.3	7.1 ± 0.4
TG(1,3)/TG(2), ($P < 0.001$)	1.6 ± 0.2	2.7 ± 0.1

[a]Cells were incubated with the listed fatty acids in DMEM containing 10% FBS and insulin (100 μU/mL) for 5 h. The results are presented as mean ± SE per culture ($n = 3$). For abbreviations see Table 1.

where $[S]$ is the substrate (oleate) concentration with $[S]_o = 1$ mM, K_m is the pseudoequilibrium constant, and V_{max} is the limiting maximal velocity. Using a nonlinear least square-root fitting program, we calculated the values of K_m and V_{max} as indicated in Figure 3. For cells treated on day 2 and day 6, the K_m values were the same (~2 mM). V_{max} was substantially higher in the more differentiated cells. This is in accord with the general observations that both the mass and activities of lipogenic enzymes increase as cells progress to more advanced stages of differentiation (19).

Effects of linoleate on the incorporation of [1-^{13}C]oleate. As shown above, [1-^{13}C]oleate is more likely to be esterified at the *sn*-2 than *sn*-1,3 position, especially in cells at more advanced stages of differentiation or after longer periods of incubation. By exposing cells simultaneously to equimolar [1-^{13}C]oleate and linoleate (unlabeled), we found that the TG(1,3)/TG(2) ratio increased significantly in the presence of linoleate (Table 2). This alteration in positional labeling suggests competition between oleate and linoleate for storage in TG, especially into the *sn*-2 position.

Lipolysis and the reesterification of [1-^{13}C]oleate in cellular TG. We measured glycerol release in parallel with ^{13}C NMR lipid analysis in cells treated with lipolytic (ISO) and antilipolytic (insulin) agents. During 1–2 h incubations with ISO, glycerol release ranged from 1.1 to 5.3 μmol/culture (Table 3). During the first hour of incubation with ISO, addition of insulin suppressed glycerol release. Cells incubated with ISO lost about 10% of stored [1-^{13}C]oleate (Table 3, column A), whereas those incubated with ISO and insulin lost only ~1% (Table 3, column C). A second exposure to ISO stimulated glycerol release to the same extent in the presence or absence of insulin (cf. columns B and D). However, insulin still promoted slightly more retention of [1-^{13}C]oleate at both the *sn*-1,3 and *sn*-2 positions (Table 3, columns B and D). In all cases, free [1-^{13}C]oleate was not detected, and the retention of [1-^{13}C]oleate was higher at the *sn*-2 than *sn*-1,3 positions. In looking at the percent of [1-^{13}C]oleate in total stored FFA, we found that total TG contents before and after lipolytic treatment were similar (3.3 ± 0.4 mg/culture), implying active TG synthesis with simultaneous TG hydrolysis.

DISCUSSION

In the presence of high concentrations of glucose, most of the fatty acids stored in 3T3L1 adipocytes are synthesized *de novo*. These are mainly saturated and monounsaturated C_{14}–C_{18} fatty acids (20). In cells at early stages of differentiation, exogenous [1-^{13}C]oleate was the major substrate for TG synthesis (Table 1) when *de novo* synthesis was not yet very active (15). Additionally, at the beginning of incubation with oleate (Fig. 2B), the ratio of [1-^{13}C]oleate incorporated at the *sn*-1,3 and *sn*-2 positions was close to 2.0. Under conditions in which the quantity of *de novo* synthesized fatty acids would be predicted to increase (as incubation time increased, or in cells at more advanced stages of differentiation), relatively more [1-^{13}C]oleate became esterified at the *sn*-2 position (Table 1, Fig. 2B).

The greater propensity for [1-^{13}C]oleate to be esterified at the *sn*-2, rather than *sn*-1,3 position, could be a mechanism through which cells maintain an unsaturated acyl chain at the *sn*-2 position. Fatty acids with a higher extent of unsaturation than oleate appear to compete with oleate for esterification at the *sn*-2 position. When unlabeled linoleate was added to cells in combination with an equimolar amount of [1-^{13}C]oleate, we found that the incorporation of [1-^{13}C]oleate into TG was reduced by ~20%. However, the ratio of TG(1,3)/TG(2) increased by 69% (Table 2). This suggests that linoleate has a greater probability than oleate of being esterified at the *sn*-2 position. The total amount of stored TG did not change significantly, probably because the incubation was only for a rel-

TABLE 3
[1-^{13}C]Oleate Incorporation into TG Following Exposure to ISO and ISO/Insulin[a]

	Control	A	B	C	D
Glycerol, μmol/culture	—[c]	2.2 ± 0.3	5.1 ± 0.2	1.1 ± 0.1	5.3 ± 0.1
TG(1,3)/TG(2)	1.4 ± 0.1	1.3 ± 0.2	1.3 ± 0.1	1.4 ± 0.1	1.3 ± 0.2
% of [1-^{13}C]Oleate remaining at TG(1,3)	—	88 ± 2	83 ± 1	99 ± 2	88 ± 2
% of [1-^{13}C]Oleate remaining at TG(2)	—	94 ± 1	93 ± 1	100 ± 2	96 ± 1
% of [1-^{13}C]Oleate in total stored FFA[b]	9 ± 0.2	8 ± 0.4	8 ± 0.1	9 ± 0.8	8.5 ± 0.5

[a](A) [1-^{13}C]Oleate incorporation into TG in cells following exposure to ISO for 1 h; (B) after exposure for 2 h with a second dose of ISO at the end of the first hour; (C) following exposure to ISO/insulin for 1 h; (D) following exposure to ISO/insulin for the first hour, plus a second dose of ISO at the end of the first hour, and incubation extended for an additional hour. Results are presented as mean ± SE per culture ($n = 3$).
[b]Cells used were collected 3 d after MDI treatment.
[c]Control cultures were terminated at the beginning of lipolysis experiments, thus with no measurement of glycerol release. Basal glycerol release (without ISO) was 0.3 ± 0.1 μmol/culture after a 2-h incubation. See Table 1 for abbreviations.

atively short period after most of the stored TG had already been acquired from *de novo* lipid synthesis.

Incorporation of exogenous oleate into TG may occur from direct esterification into TG or degradation and reacylation of existing TG. While we are not able to discriminate between these two processes, we expect that the former plays an important role because of the rapid TG synthesis in differentiating cells and their relatively low net TG storage compared to mature fat cells. Biosynthesis of TG (and also phospholipids) begins with the acylation of glycerol-3-phosphate to lysophosphatidate and then to phosphatidate, which is further hydrolyzed into diacylglycerol (21). Diacylglycerol is the common precursor for both TG and phospholipids. The esterification at the *sn*-2 position by lysophosphatidate acyltransferase is relatively selective for unsaturated fatty acids (21). For these reasons, most phospholipid molecules contain palmitate or stearate in the *sn*-1 position and primarily unsaturated fatty acids in the *sn*-2 position (22). However, this selectivity is not absolute, and the fatty acid composition of the newly synthesized TG depends on the fatty acids available in the cell. Unsaturated fatty acids, when present, are more likely than saturated fatty acids to be esterified to the *sn*-2 position. They can also be esterified to *sn*-1,3 positions, especially when they are the major fatty acids available for TG synthesis. Our results agree with this paradigm, and further indicate that polyunsaturated fatty acids are more likely than monounsaturated fatty acids to be esterified at the *sn*-2 position.

When cells preincubated with [1-^{13}C]oleate were subjected to lipolysis stimulated by ISO, the extent of glycerol release (Table 3) indicated that about 55% of the stored TG was hydrolyzed after a 1-h incubation, about eightfold above basal glycerol release. This was somewhat greater than the typical four- to fivefold stimulation by ISO reported previously (23,24). This discrepancy is probably related to the different compositions of the basal media used in the different studies. A second exposure to ISO further increased glycerol release. It must be pointed out that the amount of glycerol released represents the amount of TG molecules that have been hydrolyzed. In the meantime, new TG molecules are being constantly synthesized from both endogenous and exogenous fatty acids and glucose. The endogenous fatty acids include those released from TG hydrolysis or obtained *via de novo* synthesis.

In these experiments, BSA was removed after incubation, and lipolysis proceeded in the absence of an extracellular "sink" for the released fatty acids [i.e., BSA (8,25)]. This condition prevents the removal of the released fatty acids. The NMR determination of esterified [1-^{13}C]oleate represents the sum of molecules that have not been cleaved and those that have been cleaved and reesterified. With the presence of a high concentration of glucose, utilization of fatty acids for oxidation is limited (26). Our finding that 83–96% of [1-^{13}C]-oleate remained esterified in TG indicates that a majority of the released fatty acids were reesterified. The continuous recycling of fatty acids maintains or even increases substrate availability for lipolysis.

The addition of insulin during the first hour of lipolysis reduced glycerol release by ~50%. [1-^{13}C]Oleate was found at both *sn*-1,3 and *sn*-2 positions with little change compared to the control. Taken together, these results suggest that the rate of TG hydrolysis was reduced and nearly all the released fatty acids were rapidly reesterified. A second dose of ISO overcame the inhibitory effect of insulin on lipolysis, resulting in glycerol release to an extent similar to that found in cells incubated in the absence of insulin. The amount of [1-^{13}C]-oleate in the TG molecules was still higher than in cells incubated without insulin (Table 3), suggesting that although the insulin/ISO ratio was not sufficient to suppress TG hydrolysis, it still enhanced the reesterification of released fatty acids.

Another interesting observation shown in Table 3 is that the loss of [1-^{13}C]oleate from the *sn*-1,3 position was always greater than that from the *sn*-2 position, in agreement with a previous report (8). The chance of having all three glycerol carbons in the same molecule esterified with [1-^{13}C]oleate is rather low, especially when other fatty acids are obtained through *de novo* synthesis (15). GC analysis of selected samples showed that palmitate in cellular TG (in cells incubated with oleate for 5 h) accounts for ~30% of the total stored fatty acids (not shown). Palmitate has a lower affinity for the *sn*-2 position compared to oleate (8). Therefore, for those TG molecules with [1-^{13}C]oleate at the *sn*-2 position, the *sn*-1,3 position would have a high probability of being occupied by a saturated acyl chain such as palmitate. If this is true, our observation and the observation of others (8) that [1-^{13}C]oleate at the *sn*-1,3 position was hydrolyzed rapidly indicates that unsaturated *sn*-1,3 chains are hydrolyzed more rapidly than saturated *sn*-1,3 chains. This is in accord with the prediction that TG molecules containing acyl chains with high polarity are hydrolyzed more rapidly than those with lower polarity (27,28). Because the hydrolysis of *sn*-1,3 chains is the rate-limiting step in lipolysis (29,30), cellular TG enriched with unsaturated fatty acids may have a higher fatty acid turnover rate than those enriched with saturated fatty acids, reducing the probability of fat cell hypertrophy.

In this work, we found that oleate was preferentially esterified into TG at the *sn*-2 position. The extent to which it was esterified was dependent on the stage of differentiation and incubation time. When linoleate was added together with oleate, more oleate was found to be esterified to the *sn*-1,3 position, probably as a result of competition between oleate and linoleate for the *sn*-2 position. Without providing albumin to remove fatty acids during lipolysis, most of the [1-^{13}C]oleate was found to be re-esterified into TG, especially when insulin was present. These results provide new insights concerning the incorporation of oleate into acylated lipids, and contribute to the general understanding of fatty-acid metabolism and energy storage in fat cells.

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The Effect of Conjugated Linoleic Acid on the Antioxidant Enzyme Defense System in Rat Hepatocytes

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ABSTRACT: Short-term effects of physiological concentrations of conjugated linoleic acid (CLA) on membrane integrity, metabolic function, cellular lipid composition, lipid peroxidation, and antioxidant enzymes were examined using rat hepatocyte suspension cultures. Incubation with CLA (5–20 ppm) for 3 h decreased the ability of hepatocyte plasma membranes to exclude trypan blue by approximately 25%, and caused leakage of cytosolic lactate dehydrogenase (LDH) into the medium. The significant decrease ($P < 0.02$) in hepatocyte viability as measured by LDH leakage during cell incubation with 10 and 20 ppm CLA was not associated with significant changes in cellular ATP content. Protein synthesis in hepatocytes was elevated ($P < 0.05$) in the presence of 5 and 10 ppm CLA, but at a higher concentration (20 ppm), protein synthesis was similar to that of control cells. Gluconeogenesis was maintained in cells incubated with lower concentrations of CLA (5 and 10 ppm) but was decreased ($P < 0.02$) at the higher concentration. Incubation with 20 ppm CLA for 3 h did not affect the specific activity of 3-hydroxy-3-methylglutaryl coenzyme A reductase, the rate-limiting enzyme of cholesterol synthesis. Both *cis*-9,*trans*-11/*trans*-9,*cis*-11, and *cis*-10,*trans*-12/*trans*-10,*cis*-12 isomers of CLA were incorporated to a similar level into hepatocytes. Levels ranged from 3.9 to 4.1%, respectively, of total fatty acids in neutral lipids, and from 0.7 to 0.8%, respectively, of total fatty acids in phospholipids. Cellular lipid peroxidation remained unchanged in the presence of CLA (5–20 ppm), despite significant inhibition ($P < 0.05$) of superoxide dismutase. Catalase activity was maintained near control levels in the presence of 5 and 10 ppm CLA but was significantly decreased in the presence of 20 ppm CLA. Glutathione peroxidase activity was significantly decreased in the presence of 10 ppm CLA. The apparent sensitivity of the antioxidant enzyme defense system of liver cells to CLA, coupled with the lack of effect of CLA on lipid peroxidation in cells, suggests that cytotoxic effects of CLA as described by LDH leakage and decreased gluconeogenesis were not mediated by a prooxidant action in hepatocytes.

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Abbreviations: CLA, conjugated linoleic acid; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; GPx, gluthathione peroxidase; HBSS, Hanks' balanced salt solution; HMGCoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; LDH, lactate dehydrogenase; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acids; SOD, superoxide dismutase; TBARS, thiobarbituric acid-reactive substances.

Conjugated linoleic acid (CLA) refers to a group of positional and geometric isomers of linoleic acid that exist naturally in dairy products and meat (1). Of the various CLA isomers detected in the lipid fractions of many different kinds of food, the *cis*-9,*trans*-11 isomer is the most abundant, constituting up to 90% of the total CLA content of dairy products (1). A quantitative comparison of the isomeric distribution of CLA in tissue lipids following the feeding of a commercial mixture of CLA isomers to pigs revealed that liver and heart tissues accumulated approximately 50% of total dietary CLA in the form of triglycerides and phospholipids with *cis*-9,*trans*-11 CLA being the primary isomer incorporated into liver phospholipids, as well as into liver and heart triglycerides with *cis*-11,*trans*-13 CLA as the primary isomer incorporated into heart phospholipids (2,3).

Administration of CLA to animal models of carcinogenesis has been shown to delay and reduce the onset of chemically induced tumors at various sites such as skin (4), mammary glands (5), colon (6), and forestomach (7). Experiments carried out in laboratory animals have shown CLA can inhibit chemically initiated cancer at several stages of the carcinogenic process (8,9). CLA has therefore prompted considerable interest as a potentially powerful cancer chemopreventive agent that possesses the ability to prevent carcinogenesis in different tissues by interfering either with mutagenesis or subsequent postmutagenic steps.

None of the animal feeding studies, some of which involved feeding high doses of CLA for prolonged periods, produced any signs of toxicity. A recent toxicological assessment of CLA in male rats based on determination of organ weights and hematological profiles of control and CLA-fed rats indicated a lack of toxicity and supported the determination of its generally-regarded-as-safe status (10).

An important aspect of the toxicological assessment of CLA must be determination of its effect on liver metabolism and the key role the liver plays in its uptake and metabolism. Mechanisms of toxicity induced by a variety of hepatotoxins include free-radical formation, covalent binding, reduction in cellular glutathione pools, disruption of Ca^{2+} homeostasis, disruption of tissue-repair mechanisms, and inhibition of cellular energy production (11). Current methods to evaluate such hepatotoxicity include measurement of the synthesis and secretion of albumin by liver, synthesis of cholesterol and

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lipoproteins, gluconeogenesis, cellular ATP content, membrane leakage of cytosolic enzymes such as lactate dehydrogenase (LDH), DNA, and protein synthesis (11).

Data on the impact of CLA on organs such as the liver, the major organ of lipid metabolism, are only now beginning to emerge (12,13). Despite an initial claim that feeding rats CLA produced no observable effects on the amount of thiobarbituric acid-reactive substances (TBARS) in liver (5), a recent study has predicted a possible hepatocarcinogenic effect of this dietary fatty acid in mice (13). Dietary CLA induced the accumulation of peroxisome-specific enzymes and ornithine decarboxylase activity in the liver in a manner suggesting a role for CLA as a peroxisomal proliferator in mice. The importance of CLA in hepatocarcinogenesis awaits a study of its effect on the steroid hormone receptor, peroxisome proliferator-activated receptor α (PPARα). Hepatocytes are a useful model for evaluating hepatotoxicity, PPARα-regulated growth responses, and hepatocarcinogenesis (14).

Owing to the involvement of oxidative events in carcinogenesis, protection against hepatic oxidative stress is an important aspect for considering both the safety and the cancer chemopreventive potential of CLA. Cancer chemopreventive agents that reduce the rate at which mutations arise are effective largely through their ability to induce the expression of antioxidant and detoxification proteins (15). Such an adaptive response enables the cell to eliminate metabolic damage caused by oxidative or chemical stress. The aim of this study was to determine if acute exposure of normal rat hepatocytes to physiological concentrations of CLA altered the specific activities of the antioxidant enzymes, superoxide dismutase, catalase, and glutathione peroxidase.

MATERIALS AND METHODS

Materials. CLA, a synthetic mixture comprising four positional isomers [29.5% *cis*-9,*trans*-11/*trans* 9,*cis*-11; 29% *cis*-10,*trans*-12/*trans*-10,*cis*-12; 21.7% *cis*-11,*trans*-13/*trans*-11,*cis*-13; 12.3% *cis*-8,*trans*-10/*trans* 8,*cis*-10, and minor amounts of all *trans* (3.6%) and all *cis* (2.9%) isomers (1)] was purchased from Nu-Chek-Prep (Elysian, MN). Waters silica Sep-Pak cartridges were purchased from AGB Scientific (Dublin, Ireland). Collagenase from *Clostridium histolyticum* type 1A, Hanks' balanced salt solution (HBSS), Hams F12, Percoll, trypan blue solution, glucose oxidase enzyme kit 510-DA, hydrogen peroxide, thiobarbituric acid, trichloroacetic acid, and hydroxymethylglutaryl coenzyme A were purchased from Sigma Ltd. (Poole, United Kingdom). 3-Hydroxy-3-methyl [3-^{14}C] glutaryl coenzyme A reductase (HMGCoA) (50 mCi/mmol), [5-^{3}H] mevalonic acid lactone (33 Ci/mmol), and [4,5-^{3}H] leucine (45–85 Ci/mmol) were purchased from Amersham Life Science Ltd. (Amersham, United Kingdom). Ransod and Ransel kits for the determination of superoxide dismutase (SOD) and gluthathione peroxidase (GPx) activity, respectively, were purchased from Randox Laboratories Ltd. (Armagh, Northern Ireland). All other reagents were purchased from BDH (Poole, United Kingdom).

Isolation of hepatocytes. Hepatocytes were isolated from male Sprague-Dawley rats according to a method recommended by the European Center for the Validation of Alternative Methods (16) and the Hepatocyte User Group. Animals were sacrificed by cervical dislocation and the liver was perfused *in situ* with a Ca^{2+}-free solution of HBSS, pH 7.4, containing 0.1 mM EGTA. The second step of the perfusion employed a solution of collagenase [0.03% (wt/vol)] in HBSS, pH 7.4 containing 1 mM calcium. The liver was excised and then passed through a sieve to separate the cells and to remove extracellular material. After centrifugation ($50 \times g$, 2 min) and Percoll treatment to remove damaged cells (17), the viable cell yield was determined using 0.1% (vol/vol) trypan-blue exclusion and LDH leakage (17).

CLA incubations. A 2000 ppm solution of CLA was prepared in ethanol and aliquots representing 5, 10, and 20 ppm CLA (equivalent to 17.8, 35.5, and 71 μM) as final concentrations in culture medium were transferred to empty and dry six-well plates. Ethanol was evaporated from wells prior to the addition of hepatocytes (~10^6 viable cells) for incubation in Ham's F12 nutrient medium for 3 h at 37°C, 5% CO_2, and >80% relative humidity. Control wells to which only ethanol was added were treated similarly.

CLA uptake into liver lipid fractions. Total lipids of hepatocytes were extracted using the extraction method of Folch *et al.* (18). Hepatocyte cultures were centrifuged at $800 \times g$ for 5 min and the supernatant was discarded. Chloroform/methanol solution [20 mL of 2:1 (vol/vol)] was added prior to sonication and after filtration through Whatman No. 1 filter paper; 5 mL of distilled water was added to the extract which was then centrifuged at $800 \times g$ for 20 min. The upper aqueous phase was discarded, and the lower organic phase containing the lipid fractions was evaporated to dryness. The lipids were redissolved in 1 mL of chloroform in preparation for the separation of the lipid fractions.

Separation of lipid fractions and fatty acid analysis. The extracted lipids were injected onto a Waters Sep-Pak silica cartridge and the neutral lipids eluted with 20 mL chloroform at a flow rate of 25 mL/min, followed by 10 mL of a 98:2 (vol/vol) chloroform/methanol solution to elute the monoglycerides. Finally, the phospholipids were eluted with 30 mL methanol. These fractions were dried under nitrogen, resuspended in 1 mL chloroform and stored at −80°C under nitrogen until required for analysis. Fatty acid methyl esters (FAME) of neutral and phospholipid fractions were prepared using acid-catalyzed methanolysis as described previously (19). Fatty acids were separated using a Supelcowax-10 capillary column (19) (Supelco Inc., Bellefonte, PA) (60 m × 0.32 mm i.d., 0.25 μm film thickness) and analyzed using a Varian 3500 gas–liquid chromatograph (GLC) (Varian, Harbor City, CA) fitted with a flame-ionization detector. CLA was identified by retention time with reference to a CLA mix, generously provided by the Food Research Institute, University of Wisconsin (Madison, WI). Fatty acids (other than CLA) were identified by their retention times with reference to standard FAME (Sigma Chemical Co., St. Louis, MO) and quantified

as described previously (20). The quantity of each fatty acid was expressed as a percentage of total fatty acids.

ATP measurement. Cellular ATP was determined using a coupled hexokinase/glucose 6-phosphate dehydrogenase enzyme assay (21).

Gluconeogenesis activity. After isolation of hepatocytes, 1×10^6 cells from fasted rats were incubated for 3 h at 37°C, 5% CO_2, and 80% relative humidity in 5 mL of a glucose-free medium containing 78.8 mM NaCl, 3.1 mM KCl, 0.8 mM KH_2PO_4, 0.8 mM $MgSO_4$, 1.7 mM $CaCl_2$, 16.7 mM $NaHCO_3$, 0.5% bovine serum albumin, 20 mM lactate, and 2 mM pyruvate. Glucose was measured using glucose oxidase enzyme assay kit (Sigma).

Protein synthesis. After isolation of hepatocytes, 1×10^6 cells were pulsed with L-[4,5-^{3}H]leucine (5 µCi) for 3 h at 37°C and 5% CO_2. Following incubation, cells were washed three times with phosphate-buffered saline (PBS) and the supernatant was discarded. One milliliter of trichloroacetic acid (TCA) [10% (wt/vol)] was added, and the samples were maintained on ice for 15 min. After centrifugation for 10 min at 750 × *g*, the pellet was washed three times with TCA [10% (wt/vol)] and finally resuspended in 100 µL of Protosol tissue solubilizer (Amersham Pharmacia Biotech, Little Chalfont, Bucks, United Kingdom). These solutions were digested for 30 min at 60°C before transfer to scintillation vials with 3 mL methanol and 10 mL scintillation fluid and counted in a Beckman LS-6500 scintillation counter.

Determination of enzyme activity. SOD and GPx activity were determined using Ransod and Ransel kits (Randox Laboratories Ltd., Armagh, Northern Ireland) respectively. The units of GPx activity are defined as µmol NADPH oxidized/min at pH 7.4 and 30°C. One unit of SOD activity is that quantity of enzyme that inhibits by 50% the conversion of xanthine to a formazon dye by xanthine oxidase. Catalase activity was determined as described (22) and expressed in Bergmeyer units which are defined as µmol [H_2O_2] decomposed min^{-1} mL^{-1} at pH 7 and 25°C.

Determination of lipid peroxidation using the TBARS method. Measurement of TBARS was performed according to Esterbauer and Cheeseman (23) and reported as nmol MDA/mg protein.

Statistical analysis. All experiments were performed in triplicate. Mean and standard error of triplicate assays performed on three to four animals (where indicated) were analyzed by Student's *t*-test.

RESULTS

Viability. The effect of CLA on hepatocyte viability as determined by both trypan blue exclusion and measurement of LDH activity in medium after 3 h is shown in Figure 1. Approximately 20–25% of the CLA-treated hepatocyte population was unable to exclude trypan blue. In using LDH leakage as an enzymatic-based measure, a significant decrease in viability ($P < 0.02$) was observed in cells treated with 10 and 20 ppm CLA.

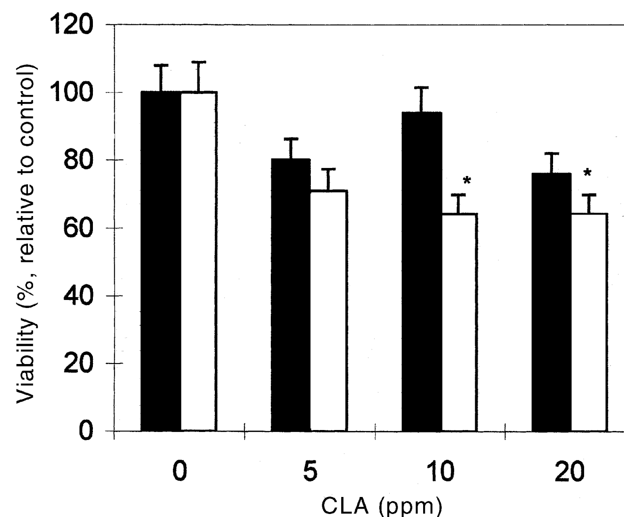

FIG. 1. The effect of conjugated linoleic acid (CLA) (5–20 ppm) on hepatocyte viability. Data are expressed as mean value ± SEM (n = 3). *$P < 0.02$ relative to control. ■, Trypan blue; □, lactate dehydrogenase.

CLA uptake into liver lipid fractions. The effect of short-term incubation of hepatocytes with 20 ppm CLA on the quantity of each fatty acid in neutral lipid and phospholipid fractions is shown in Table 1. Both neutral lipids and phospholipids contained similar amounts of palmitic and *trans*-18:1 acids. Phospholipids contained significantly higher ($P <$ 0.05) amounts of stearic and arachidonic acid and lower amounts of oleic, linoleic, and CLA isomers *cis*-9,*trans*-11/*trans* 9,*cis*-11 and *cis*-10,*trans*-12/*trans*-10,*cis*-12, compared with neutral lipids. No effect was seen on palmitic, stearic, oleic, *trans*-18:1, linoleic, linolenic, and arachidonic acids of neutral lipid fractions after exposure of hepatocytes to CLA (20 ppm). Incubation with 20 ppm CLA led to an approximate 2.4-fold increase in the quantity of *cis*-9,*trans*-11/*trans*-9,*cis*-11 (4.1 ± 0.4% total fatty acids, $P < 0.01$), and an

TABLE 1
Effect of Short-Term Incubation of Rat Hepatocytes with CLA (20 ppm) on Fatty Acid Concentrations in Lipid Fractions[a]

	Neutral lipids		Phospholipids	
Fatty acid	Control	CLA	Control	CLA
16:0	25.1 ± 0.2	24.9 ± 1.5	24.1 ± 0.2	25.5 ± 1.3
18:0	10.9 ± 0.4	9.2 ± 0.2	17.2 ± 1.8[a]	22.0 ± 1.5
$18:1^{\Delta 9}$	16.9 ± 0.6	15.6 ± 0.8	4.2 ± 0.7[a]	6.7 ± 0.6
trans 18:1	3.8 ± 0.1	3.8 ± 0.2	4.9 ± 1.2	6.4 ± 0.8
$18:2^{\Delta 9,12}$	27.1 ± 1.7	27.6 ± 1.2	21.7 ± 1.0[a]	18.1 ± 1.6
$18:3^{\Delta 9,12,15}$	1.5 ± 0.2	1.4 ± 0.3	2.0 ± 0.2	0.0*
c,t/t,c-9,11-18:2	1.7 ± 0.1	4.1 ± 0.4[b]	0.0*	0.8 ± 0.07
c,t/t,c-10,12-18:2	1.4 ± 0.4	3.9 ± 0.6[a]	0.0*	0.7 ± 0.1
$20:4^{\Delta 5,8,11,14}$	9.2 ± 1.0	9.1 ± 1.1	26.0 ± 2.4[a]	20.7 ± 1.7

[a]Values are means ± SEM (n = 3) expressed as percentage total fatty acid. Means with different superscript roman letters are significantly different from control, where [a]$P < 0.05$ relative to corresponding control; [b]$P < 0.01$ relative to corresponding control; *not detected in measurable quantities. Palmitic 16:0; stearic 18:0; oleic $18:1^{\Delta 9}$; linoleic $18:2^{\Delta 9,12}$; linolenic $18:3^{\Delta 9,12,15}$; CLA isomers *c,t/t,c*-10,12-18:2 and *c,t/t,c*-9,11-18:2; arachidonic acid $20:4^{\Delta 5,8,11,14}$; CLA, conjugated linoleic acid.

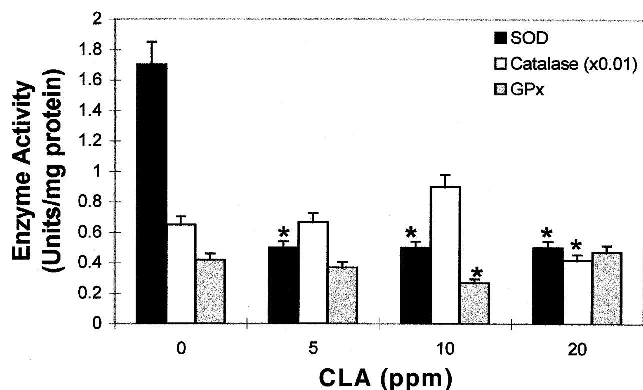

FIG. 2. The effects of CLA (5–20 ppm) on specific activities of superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx) in rat hepatocytes. Data are expressed as mean value ± SEM (n = 4). *P < 0.05 relative to control. See Figure 1 for other abbreviation.

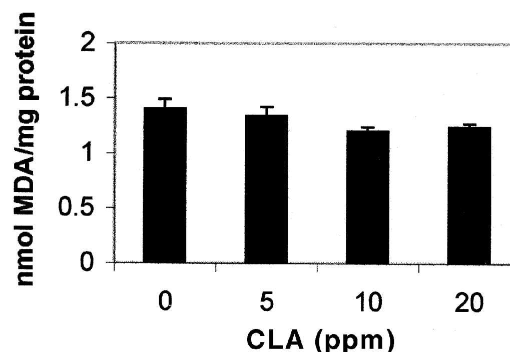

FIG. 3. The effect of CLA (5–20 ppm) on lipid peroxidation level as measured by thiobarbituric acid-reactive substances (TBARS, ■). Data are expressed as a mean value ± SEM (n = 4). MDA, malondialdehyde; see Figure 1 for other abbreviation.

approximate 2.8-fold increase in the quantity of *cis*-10, *trans*-12/*trans*-10,*cis*-12 isomer (3.9 ± 0.6% total fatty acids, $P < 0.05$) in neutral lipid fractions. Despite lower uptake of both CLA isomers into phospholipid fractions (0.7–0.8% total fatty acids), there was a trend toward a reduced level of phospholipids containing linoleic, linolenic, and arachidonic acids in hepatocyte suspension cultures exposed to CLA.

ATP, gluconeogenesis, protein and cholesterol synthesis. The data showing the effects of CLA on functional metabolic activities of liver cells are shown in Table 2. Cellular ATP levels were not significantly altered in cells exposed to increasing concentrations of CLA despite an apparent increase upon incubation with 10 ppm CLA. Gluconeogenesis was significantly reduced (25 ± 1 µg/mg protein, $P < 0.02$) in cells exposed to 20 ppm CLA relative to control cells, but there was no effect at lower concentrations of CLA. Incubation of cells with 5 and 10 ppm CLA led to approximate 1.6- and 2-fold increases, respectively, in protein synthesis ($P < 0.05$) relative to control cells. At 20 ppm CLA, however, the rate of ^{3}H-leucine uptake into protein (240 ± 50 dpm $h^{-1} \cdot 10^5$ cells^{-1}) was similar to that of the control (180 ± 5 dpm $h^{-1} \cdot 10^5$ cells^{-1}). The specific activity of HMGCoA reductase in CLA-treated cells (60 ± 5 pmol mevalonate formed min^{-1}·mg protein^{-1}) was similar to that of control cells (55 ± 10 pmol mevalonate formed min^{-1}·mg protein^{-1}).

The effect of CLA on antioxidant enzymes and lipid peroxidation. The effect of CLA on specific activities of SOD, catalase, and GPx is shown in Figure 2. Cellular SOD activity was significantly decreased from 1.7 ± 0.2 U/mg to approximately 33% of control levels (0.5 ± 0.01 U/mg , $P < 0.05$) upon exposure of cells to CLA in the range 5–20 ppm. While incubation with 5 and 10 ppm CLA had no significant effect on the specific activity of catalase, activity was decreased ($P < 0.05$) by approximately 36% in the presence of 20 ppm CLA. Incubation with 10 ppm CLA resulted in decreased GPx activity ($P < 0.05$) relative to the control (Fig. 2). CLA in the range 5–20 ppm was found to have no significant effect on lipid peroxidation as measured by TBARS (Fig. 3).

DISCUSSION

Hepatocyte suspension cultures were used as a model for short-term studies on the effects of CLA on membrane integrity and a number of liver cell "housekeeping" functions.

TABLE 2
Effect of CLA on ATP, Gluconeogenesis, Protein, and Cholesterol Synthesis[a]

		CLA (ppm)		
	Control	5	10	20
ATP (pmol/10^5 cells)	4.1 ± 1.0	1.9 ± 1.1	14.1 ± 9.4	4.0 ± 0.42
Glucose (µg/mg protein)	40 ± 2	45 ± 3	54 ± 10	25 ± 1[a]
Protein (dpm ^{3}H leucine incorporation $h^{-1} \cdot 10^5$ cells^{-1})	180 ± 5	300 ± 40[b]	350 ± 40[b]	240 ± 50
HMGCoA reductase (pmol ^{14}C mevalonate·min^{-1} mg^{-1})	55 ± 10	n.d.	n.d.	60 ± 5

[a]Values are means ± SEM (n = 3). Means with different superscript roman letters are significantly different from control. [a]$P < 0.02$; [b]$P < 0.05$. n.d., Not determined; HMGCoA reductase, 3-hydroxy-3-methylglutaryl coenzyme A reductase; see Table 1 for other abbreviation.

Measurement of trypan blue exclusion and LDH leakage are alternative means of measuring the viability of cells. Trypan blue exclusion is the quickest method for identifying cell damage, but it does have serious limitations. Uneven cell distribution over the slide, statistically small samples, and albumin binding of the dye may give misleadingly low values for percentages of cells stained with trypan blue. Our study showed an increase in dye uptake by cells that were incubated with CLA, suggesting a decline in viability. However, the decline did not reach statistical significance. In view of the relatively large standard error of the mean ATP values reported, it would be imprudent to predict that the extent of dye exclusion observed was the result of an energy-dependent maintenance of plasma membrane potential. For this reason, the extent of enzyme leakage (LDH) from the cytoplasm of isolated hepatocytes into culture medium occurring over a 3-h incubation period was considered. This approach to viability testing avoids the statistical problems with the trypan blue technique arising from the small number of cells examined and the possibility of dye binding caused by increased protein synthesis (Table 1). An increase in leaked-LDH activity in medium as a percentage of total LDH activity of the extract was observed when cells were incubated with 10 and 20 ppm CLA, indicating approximately 35% cellular disruption.

An examination of the metabolic competency of the CLA-treated cells showed some anabolic activity, as was evident from the increased radiolabeled precursor uptake into protein in the presence of 5 and 10 ppm CLA. At 20 ppm CLA, the increase in protein synthesis was not as remarkable and may have been a metabolic index of a decreased buffering capacity by cells when exposed to 20 ppm CLA. Protein synthesis is pH dependent, with leucine incorporation into protein being depressed by lower pH levels (17). Particular metabolic pathways such as gluconeogenesis are widely believed to be far more susceptible to toxic agents than processes maintaining plasma membrane integrity. We observed a maintenance of gluconeogenic capacity in cells treated with 5 and 10 ppm CLA, suggesting a lack of toxicity upon exposure of hepatocytes to 5 and 10 ppm CLA. By contrast, a significant decrease in gluconeogenesis was observed only when cells were incubated with 20 ppm CLA, which, in association with LDH leakage, may be evidence that the higher concentration of CLA mediated a decline in cellular integrity. However, in view of the lack of effect on the activity of HMGCoA reductase, the rate-limiting enzyme of cholesterol biosynthesis, it is obvious that more *in vitro* tests should be carried out to examine further the toxicity profile of CLA at concentrations of 20 ppm or more in hepatocytes.

Despite recent findings that diets containing 1.0 and 1.5% CLA were associated with hepatic peroxisome proliferation and induction of ornithine decarboxylase (13), no study has yet shown cytotoxic effects of CLA in liver cells comparable to those observed in some cancer cell lines (24–26). The observed effects of CLA on gluconeogenesis, protein synthesis, ATP status, lipid oxidation, and antioxidant enzymatic activity must be considered as relative only to the control incubations carried out in the absence of CLA. A definitive conclusion of the effects of CLA on these end points should await the inclusion of a positive control (equimolar concentration of linoleic acid) in the experimental design.

The concentrations of CLA (5–20 ppm) used in this study (equivalent to 17.8–71 μM) were similar to those used by Shultz *et al.* in an earlier cell culture study (26). A previous *in vivo* study carried out by Belury *et al.* (13) showing the effects of CLA on hepatic metabolism in mice was conducted using doses of 0.5–1.5% CLA in diet. The addition of 1.5% CLA to rat diets is equivalent to administering ~225 mg CLA (assuming an average daily intake of 15 g of food). Allowing for 55% absorption of CLA into lymph, as estimated by Sugano *et al.* (31), this would suggest up to 124 mg CLA may be taken up by the liver in the portal vein. In our study, 1×10^6 cells representing approximately 10 mg whole liver were exposed to 100 μg CLA (a 20-ppm dose in a 5-mL well plate). In taking into account the total weight of rat liver (10 g), this was approximately 100 mg CLA, and was therefore close to physiological concentrations.

Previous studies reported trace levels of CLA in phospholipid (0–0.1%) and neutral lipid (0.06–0.2%) fractions of mammary glands and mouse liver (12,27,28). Ip *et al.* (27) showed that feeding rats a 1% CLA diet increased the CLA content of mammary tissue phospholipids to 0.4%, and of neutral lipids to 3.5% total fatty acids (27). Similar levels of incorporation were observed in liver fractions from CLA-fed mice (12,28). Of the two predominant CLA isomers present in synthetic CLA mixtures used in animal-feeding studies to date, the *cis*-9,*trans*-11/*trans*-9,*cis*-11 isomer was incorporated to a greater extent (up to 0.4%) than the *trans*-10,*cis*-12 isomer (up to 0.3%) into the liver phospholipids of pigs (3), rats (27), and mice (12,28). Our *in vitro* study showed not only approximate equal distribution of both isomers, but substantially more of both isomers in hepatocyte phospholipids (up to 0.8%) and in neutral lipids (up to 4.1%), compared with dietary-feeding studies. In light of evidence that CLA isomers may be metabolized by desaturation and elongation (29, 30), the equal distribution of *cis,trans/trans,cis* -9,11 and *cis,trans/trans,cis*-10,12 isomers in hepatocytes suggests that both isomers, while having accumulated to a similar extent, were slowly metabolized under the experimental conditions described.

In view of the myriad of beneficial biological effects attributed to CLA observed *in vivo* and *in vitro*, it is plausible that protection against lipid peroxidation may be a mechanism by which CLA preserves the phospholipid milieu of cell membranes for subsequent eicosanoid metabolism or membrane-mediated signaling events. In this study, we have shown that both CLA isomers were readily incorporated into cell membrane phospholipids and neutral lipids. It is of note that short-term incubations of hepatocytes with 20 ppm CLA resulted in detectable levels of both *cis*-9,*trans*-11/*trans* 9,*cis*-11 and *cis*-10,*trans*-12/*trans*-10,*cis*-12 isomers in the phospholipid fraction, in addition to slightly higher levels of stearic and oleic acids compared with control incubations

while lowering levels of oxidation-susceptible linoleic, linolenic, and arachidonic acids. As an antioxidant, the presence of CLA in phospholipid membranes would be expected to reduce or at least maintain the amount of TBARS produced in cells during culture. In our study, the level of lipid peroxidation in CLA-treated cells was found to remain similar to control levels [1.4 ± 0.09 nmol malondialdehyde (MDA)/mg protein]. These data are consistent with a previous study (32), where rat liver was reported to have 1.1 ± 0.18 nmol MDA/mg protein.

However, the apparent insensitivity of liver cells to CLA-mediated changes in lipid peroxidation may be a reflection of the ample antioxidant status of hepatocytes. Induction of oxidative stress is expected to lead to an increase in the level of activity of the antioxidant defense enzymes and finally to lipid peroxidation if the endogenous defense system is overloaded. SOD is known to be the first line of antioxidant defense dismuting superoxide radicals to H_2O_2 and water. In this study, SOD was decreased in cells exposed to CLA suggesting that generation of $O_2^{\cdot -}$, the major initiator of the oxygen radical cascade that feeds into the lipid peroxidation chain reaction, did not occur in CLA-treated cells. The observation that 20 ppm CLA caused a significant decrease in catalase activity while having no significant effect on GPx activity may be supportive of a possible quenching effect of CLA on potential reactive oxygen species generated as a result of CLA metabolism in liver cells. Although this study has shown an antioxidant enzyme-sparing effect in hepatocytes, it does not exclude possible effects of CLA in maintaining levels of other membrane-bound antioxidants such as α-tocopherol as reported previously (33). The apparent sensitivity of the antioxidant enzyme defense system of liver cells to CLA coupled with the lack of effect of CLA on lipid peroxidation in cells serve as indirect evidence that CLA may not be a prooxidant in normal liver cells. Longer-term incubations of hepatocyte suspension cultures with physiological concentrations of CLA or of spheroid aggregate-culture systems for between 48 and 96 h may be useful to investigate the effects of chronic exposure to CLA by diet.

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Characterization of the *ybdT* Gene Product of *Bacillus subtilis*: Novel Fatty Acid β-Hydroxylating Cytochrome P450

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ABSTRACT: We have characterized the gene encoding fatty acid α-hydroxylase, a cytochrome P450 (P450) enzyme, from *Sphingomonas paucimobilis*. A database homology search indicated that the deduced amino acid sequence of this gene product was 44% identical to that of the *ybdT* gene product that is a 48 kDa protein of unknown function from *Bacillus subtilis*. In this study, we cloned the *ybdT* gene and characterized this gene product using a recombinant enzyme to clarify function of the *ybdT* gene product. The carbon monoxide difference spectrum of the recombinant enzyme showed the characteristic one of P450. In the presence of H_2O_2, the recombinant *ybdT* gene product hydroxylated myristic acid to produce β-hydroxymyristic acid and α-hydroxymyristic acid which were determined by high-performance liquid chromatography (HPLC) and gas chromatography–mass spectrometry. The amount of these products increased with increasing reaction period and amount of H_2O_2 in the reaction mixture. The amount of β-hydroxyl product was slightly higher than that of α-hydroxyl product at all times during the reaction. However, no reaction products were detected at any time or at any concentration of H_2O_2 when heat-inactivated enzyme was used. HPLC analysis with a chiral column showed that the β-hydroxyl product was nearly enantiomerically pure *R*-form. These results suggest that this P450 enzyme is involved in a novel biosynthesis of β-hydroxy fatty acid.

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β-Hydroxy fatty acids are usually found as their CoA derivatives in the fatty acid β-oxidation pathway, where β-hydroxy acyl-CoAs are produced from the corresponding acyl-CoAs by sequential reaction of acyl-CoA dehydrogenase and Δ2-enoyl-CoA hydratase. Gram-negative bacteria have β-hydroxy fatty acids as acyl moieties of cell wall lipids such as lipid A and ornithine lipid (1), whereas gram-positive bacteria such as *Bacillus* produce acylpeptides containing β-hydroxy fatty acid, as antibiotic compounds (2). Whether this acyl moiety is derived from the β-hydroxy intermediate in the β-oxidation pathway or is produced in another metabolic pathway is unclear.

In contrast, α-hydroxy fatty acids are components of sphingolipids of various species. We have studied fatty acid α-hydroxylase from *Sphingomonas paucimobilis*, a bacterium with large amount of sphingoglycolipid containing α-hydroxymyristic acid as an acyl moiety. Fatty acid α-hydroxylase is a unique enzyme which introduces an oxygen atom from H_2O_2 into fatty acid to produce α-hydroxy fatty acid (3). We recently cloned the fatty acid α-hydroxylase gene from this bacterium (4). Sequence analysis of the cloned gene and spectral analysis of the recombinant enzyme indicated that fatty acid α-hydroxylase from *S. paucimobilis* was a cytochrome P450 (P450) enzyme, which is designated as $P450_{SP\alpha}$ here. P450 enzymes have several highly conserved regions in their amino acid sequences associated with their secondary structure (5). $P450_{SP\alpha}$ also has the conserved sequences, especially in helix-K. However, a few sequences of $P450_{SP\alpha}$ are unique as compared to other bacterial P450s: (i) the conserved Thr in helix-I, which is believed to be important for O_2 activation (6), was absent; (ii) the presence of an aromatic region (7); and (iii) the consensus sequence in the heme-binding region, which was modified by insertion of amino acids (4).

Recently, the complete genome sequence of *B. subtilis* (8) was released into the DDBJ/GenBank/EMBL database. By homology search, we found that the deduced amino acid sequence of the *ybdT* gene (accession number, AB006424) product, which is a 48-kDa protein of unknown function, was significantly homologous to that of $P450_{SP\alpha}$. Moreover, the *ybdT* gene product appeared to have sequences highly homologous to those of $P450_{SP\alpha}$ in helix-I, aromatic region, and heme-binding region. Thus, we hypothesized that *ybdT* encodes fatty-acid hydroxylating P450, and in this study, we characterized its gene product.

MATERIALS AND METHODS

Materials. Bacillus subtilis IFO14144 was obtained from the Institute for Fermentation (Osaka, Japan). *Escherichia coli* BL21 and KOD DNA polymerase were purchased from Toyobo Co., Ltd. (Tokyo, Japan). The expression plasmid pGEX-4T-1 and glutathione-Sepharose 4B were purchased from

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Abbreviations: ADAM, 9-anthryldiazomethane; GC–MS, gas chromatography–mass spectrometry; HPLC, high-performance liquid chromatography; P450, cytochrome P450; $P450_{BS\beta}$, fatty acid β-hydroxylating P450 from *Bacillus subtilis*; $P450_{SP\alpha}$, fatty acid α-hydroxylating P450 from *Sphingomonas paucimobilis*; TMS, trimethylsilyl.

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Amersham-Pharmacia. 9-Anthryldiazomethane (ADAM) was purchased from Funakoshi Co., Ltd. (Tokyo, Japan). 3-[(3-cholamidopropyl)-Dimethylammonio]-1-propanesulfonate (CHAPS) was purchased from Nakarai Tesque (Kyoto, Japan). Diazomethane was synthesized from *N*-nitroso-*N*-methylurea (Sigma Chemical Co., St. Louis, MO). α-Hydroxymyristic acid and β-hydroxymyristic acid were purchased from Sigma Chemical Co. Other reagents was purchased from Wako Pure Chemicals (Osaka, Japan).

Cloning of the ybdT *gene, and expression and purification of a glutathione* S*-transferase-*ybdT *gene product fusion protein. Bacillus subtilis* was cultured in Luria broth at 37°C. Genomic DNA was isolated by standard procedure (9). The *ybdT* gene was amplified by polymerase chain reaction (PCR) using the following primers: 5′-CGGGATCCATGAATGAGCAGATTCCACATG-3′ (the sense primer including the start codon of *ybdT* gene) and 5′-CCGCTCGAGGACAACAAAATGGTATCAGAAG-3′ (the antisense primer, complementary to the sequence downstream from the *ybdT* gene). After amplification, a 1.4 kbp single band was determined by agarose-gel electrophoresis. The amplified DNA fragment was isolated from the agarose gel and digested by *Bam* HI and *Xho* I. The *Bam* HI-*Xho* I fragment was ligated into a *Bam* HI-*Xho* I-digested pGEX4T-1, and the resulting expression plasmid including the *ybdT* gene was transfected into *E. coli* BL21. Sequencing of the cloned polymerase chain reaction fragment indicated that the nucleotide sequence of the isolated gene from *B. subtilis* IFO14144 was completely identical to the reported sequence of *ybdT*. Expression and purification of a glutathione *S*-transferase-*ybdT* gene product fusion protein were performed by a modification of the method previously described (4). Briefly, cells were disrupted with sonication in 0.1 M Tris-HCl (pH 7.5), 20% glycerol, and 1 mM dithiothreitol, and the supernatant was obtained by centrifugation at 100,000 × *g* for 60 min. Glutathione *S*-transferase-*ybdT* gene product fusion protein was bound to glutathione-Sepharose and eluted with 0.1 M Tris-HCl (pH 7.5), 20% glycerol, 0.1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), 10 mM reduced glutathione, and 1 mM dithiothreitol.

Enzyme assay and determination of reaction product. Fatty acid hydroxylation activity was determined by a modification of the method for assaying $P450_{SP\alpha}$ activity (4). The reaction mixture contained 0.1 M potassium phosphate buffer (pH 5.9), 0.2 mM H_2O_2, 60 μM myristic acid, and the enzyme preparation in a total volume of 0.2 mL. Incubation was carried out at 37°C for the indicated periods. The reaction was terminated by the addition of 20 μL of 2 N HCl, and then the products and the substrate were extracted with ethyl acetate. The extract was washed with distilled water, and the ethyl acetate layer was evaporated. For high-performance liquid chromatography (HPLC) analysis, the resulting residue was treated with ADAM. HPLC analysis of ADAM-derivatized fatty acids was performed by a minor modification of the method described previously (10).

For gas chromatography–mass spectrometry (GC–MS), the products were treated with diazomethane and then sequentially treated with *N,O*-bis(trimethylsilyl)trifluoroacetamide. GC–MS was performed essentially as described previously (3). For GC, samples (1 μL) were introduced onto the column which was operating at 250°C. A GC oven temperature program was then applied: the initial temperature of 150°C was held for 4 min, and then the temperature was increased linearly to 270°C by 4°C/min. Helium was used as the carrier gas at a flow rate of 0.5 mL/min. The GC–MS interface temperature was 250°C. Electron-impact MS was performed with an ion source temperature of 250°C, emission current of 0.37 mA, and electron energy of 40 eV.

Analysis of optical configuration of α-hydroxy fatty acids. The optical configuration of the hydroxyl products was determined by HPLC with an OA-3100 column (Sumika, Osaka, Japan) according to the method of Nakagawa *et al*. (1). After the reaction, the hydroxymyristic acids were treated with 3,5-dinitrophenyl isocyanate (Sumika) in the presence of a small amount of dry pyridine at room temperature overnight. The solvents used for the HPLC analysis was methanol containing 50 mM ammonium acetate and 10% distilled water. Flow rate was 0.5 mL/min.

RESULTS AND DISCUSSION

As shown in Figure 1, the amino acid sequence of the *ybdT* gene product indicated as BSβ showed significant homology to that of $P450_{SP\alpha}$ (overall identity, 44%). Helix-I, helix-K to the aromatic region, and the heme-binding region were highly homologous. As described in the introduction, many P450 have the conserved Thr in helix-I. However, both the *ybdT* gene product and $P450_{SP\alpha}$ lack the conserved Thr, suggesting that the *ybdT* gene product does not require O_2 activation for its catalytic activity, as $P450_{SP\alpha}$ does not (4). In helix-K, the Glu-Xaa-Xaa-Arg motif which is conserved in all P450 was also conserved in the *ybdT* gene product. The aromatic region was proposed by Gotoh and Fujii-Kuriyama (7). This region was originally found in eukaryotic P450, but not in bacterial P450 with a few exceptions, one of which was $P450_{SP\alpha}$. The *ybdT* gene product also has an aromatic region similar to that of $P450_{SP\alpha}$ (75% identical). Moreover, the consensus motif of the heme-binding region was modified by insertion of amino acids, similar to $P450_{SP\alpha}$ (4), although the heme-binding Cys was found at position 363. On the basis of these observations, we assumed that the *ybdT* gene product would show similar enzymatic properties to $P450_{SP\alpha}$, and thus characterized the *ybdT* gene product using a recombinant enzyme.

We constructed a fusion gene of glutathione *S*-transferase gene and the *ybdT* gene in pGEX 4T-1, and then the fusion protein was expressed in *E. coli*. The CO difference spectrum of the fusion protein is characteristic of P450, which showed a peak at 446 nm (Fig. 2). Thus, we refer to the *ybdT* gene product as $P450_{BS\beta}$ (fatty acid β-hydroxylating cytochrome P450 from *B. subtilis*) below. Next, we investigated whether the recombinant $P450_{BS\beta}$ enzyme could hydroxylate fatty acid in the presence of H_2O_2 similarly to $P450_{SP\alpha}$. HPLC analysis of products using myristic acid as the substrate

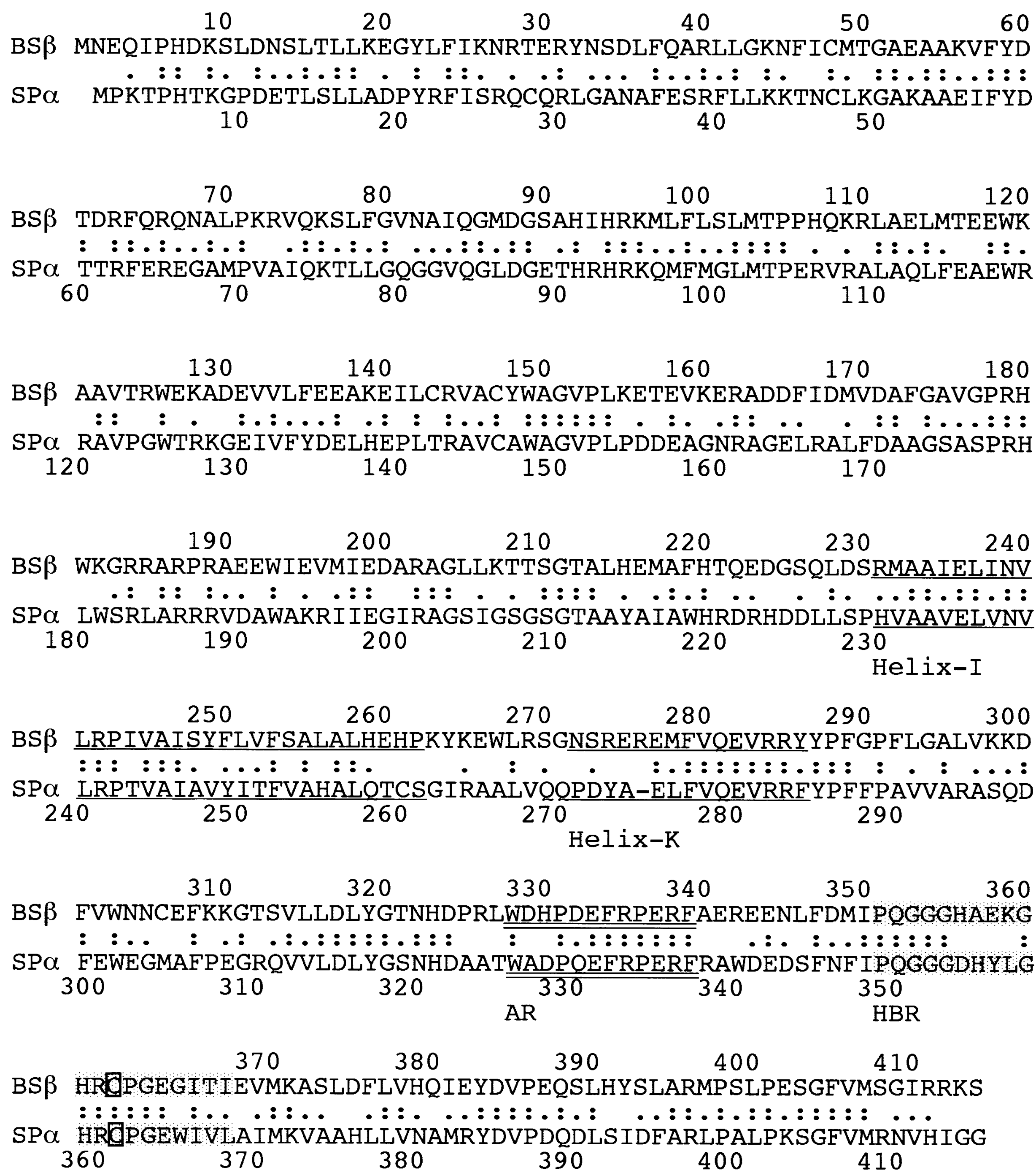

FIG. 1. Comparison of amino acid sequences of fatty acid β-hydroxylating cytochrome P450 from *Bacillus subtilis* ($P450_{BS\beta}$) and fatty acid α-hydroxylating cytochrome P450 from *Sphingomonas paucimobilis* ($P450_{SP\alpha}$). $P450_{BS\beta}$ and $P450_{SP\alpha}$ are indicated as BSβ and SPα, respectively. Helix-I and helix-K are underlined. The aromatic region (AR) is double-underlined. The heme-binding region (HBR) is shaded. Heme-binding cysteines are boxed. Dots and double dots indicate similar amino acids and identical amino acids between $P450_{BS\beta}$ and $P450_{SP\alpha}$, respectively.

showed newly produced peaks at retention times of 12.5 and 15.2 min after reaction (compare Fig. 3A and 3B). The retention times of these peaks corresponded with those of authentic β-hydroxymyristic acid (12.5 min) and α-hydroxymyristic acid (15.2 min), respectively. To further characterize these products, we performed GC–MS analysis for methyl, trimethylsilyl (TMS)-products. One of these products showed a fragmentation pattern of the methyl, TMS-derivative of β-hydroxymyristic acid (Fig. 4A). The ions at m/z 175 and 257 are formed by cleavage between C_3 and C_4 and between C_2 and C_3, respectively. The ion at m/z 175 is characteristic for the hydroxylation of fatty acid at the C_3-position. The ion at m/z 315 (M − 15) is formed by cleavage between CH_3 and Si of the TMS moiety. The fragmentation pattern of the other prod-

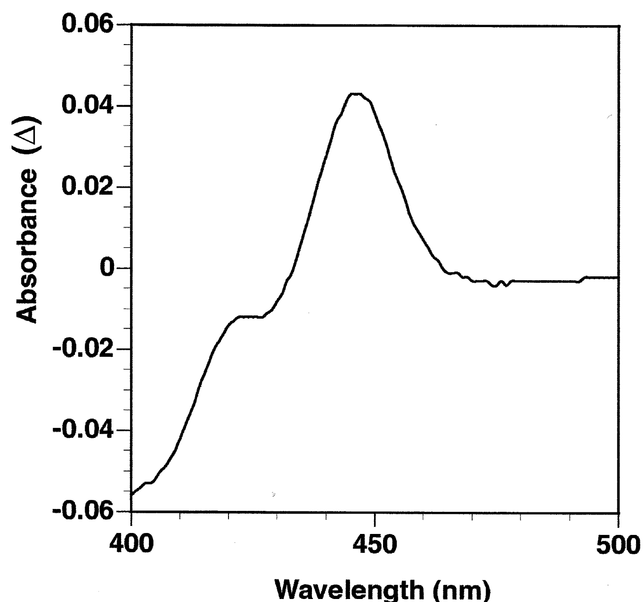

FIG. 2. CO difference spectrum of $P450_{BS\beta}$. $P450_{BS\beta}$ was dissolved in 0.1 M potassium phosphate buffer (pH 7.2) containing 20% glycerol and 1 mM dithiothreitol. The final concentration of protein was 0.28 mg/mL. For abbreviation see Figure 1.

uct corresponded to that of the methyl, TMS-derivative of α-hydroxymyristic acid (Fig. 4B). The ion at *m/z* 315 also was seen. The ion at *m/z* 271 is the characteristic fragment ion of an α-hydroxy fatty acid that has lost the carboxylmethyl group of *m/z* 330 (M). Furthermore, fragmentation patterns of these products were nearly identical to those of methyl, TMS-derivatives of authentic α-hydroxymyristic acid and β-hydroxymyristic acid (data not shown). Based on these results, we concluded that the reaction products were β-hydroxymyristic acid and α-hydroxymyristic acid.

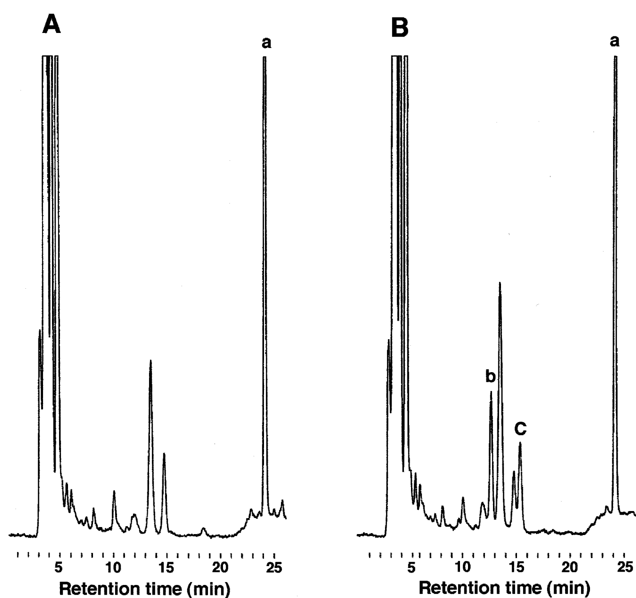

FIG. 3. Identification of products by high-performance liquid chromatography (HPLC). Incubation periods were 0 min (A) and 4 min (B). Peak **a** is that of myristic acid as the substrate. The retention times of peaks **b** and **c**, newly produced after the reaction, corresponded with those of β-hydroxymyristic acid and α-hydroxymyristic acid, respectively.

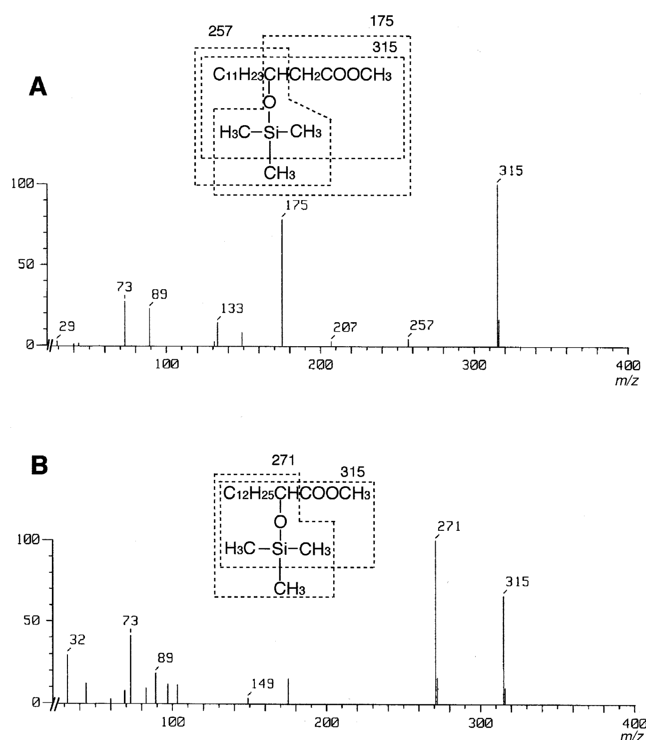

FIG. 4. Determination of methyl, trimethylsilyl (TMS)-derivatives of products by gas chromatography–mass spectrometry. Methyl, TMS-derivatives of two products found in Figure 3 showed fragmentation patterns of β-hydroxymyristic acid (A) and α-hydroxymyristic acid (B).

To determine whether these products were formed enzymatically, we investigated the effects of incubation period, the amount of H_2O_2 added to the reaction mixture, and heat treatment of the enzyme on the amount of products formed. As shown in Figure 5A, the total amount of products increased in a time-dependent manner. The amount of β-hydroxymyristic acid was slightly greater than that of α-hydroxymyristic acid at all sampling times. The amounts of β-hydroxymyristic acid, α-hydroxymyristic acid, and total products increased with the concentration of H_2O_2 in the reaction mixture and approached a plateau at a concentration of 200 μM (Fig. 5B). Other peroxides such as cumene hydroperoxide, *t*-butylhydroperoxide, *t*-butylperoxybenzonate, and *m*-chloroperoxybenzoic acid had no effect on the activity of $P450_{BS\beta}$ (data not shown). Conversely, no product was detected at any time or at any concentration of H_2O_2 when heat-treated enzyme was used. Turnover rate was approximately 300 nmol/min/nmol P450, which was comparable to that of $P450_{SP\alpha}$ (4). These results indicated that formation of these products was due to an enzymatic reaction of $P450_{BS\beta}$.

We demonstrated that $P450_{BS\beta}$ showed fatty acid β-hydroxylation and α-hydroxylation activities and specifically required H_2O_2 for its activity, similar to $P450_{SP\alpha}$ (11). Based on the specific requirement for H_2O_2 and high turnover rate in the pres-

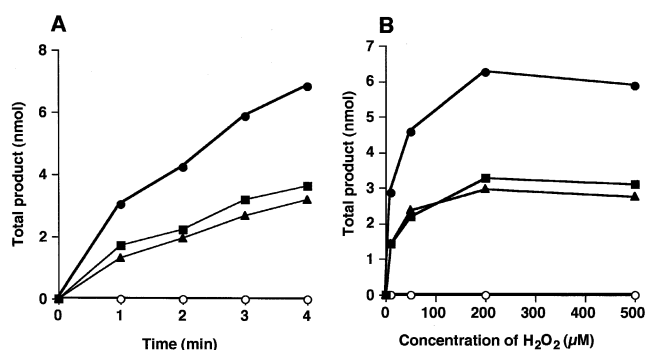

FIG. 5. Changes in the amount of product formed as a result of varying the incubation period (A) and varying the amount of H_2O_2 (B). Amount of the enzyme preparation in the reaction mixture was 1.5 μg (containing 5.7 pmol of P450). (A) Closed and open circles represent the amounts of product formed during the indicated incubation periods when nontreated and heat-treated (100°C, 10 min) enzyme were used, respectively. The concentration of H_2O_2 was 0.2 mM. Closed squares and triangles represent the amounts of β-hydroxyl product and α-hydroxyl product, respectively, when nontreated enzyme was used. (B) The amounts of products formed in the presence of various concentrations of H_2O_2 were measured. The incubation period was 4 min. Symbols are the same as those in (A).

ence of H_2O_2, it may be more appropriate to refer to $P450_{SP\alpha}$ and $P450_{BS\beta}$ as "peroxygenases" (12) rather than "monoxygenases." To our knowledge, this is the first direct evidence that β-hydroxy fatty acid is produced by such a "peroxygenase."

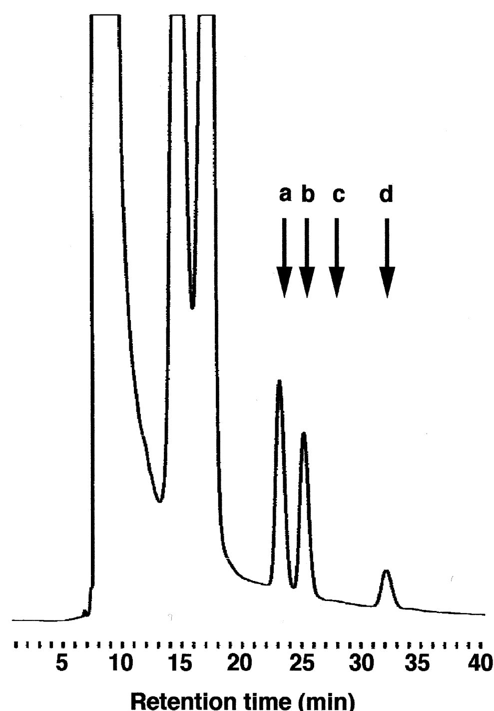

FIG. 6. HPLC analysis of optical configuration of the products. Arrows show the retention times of authentic *R*-enantiomer (**a**, 23.5 min) and *S*-enantiomer (**c**, 28.0 min) of β-hydroxymyristic acid and authentic *R*-enantiomer (**b**, 25.7 min) and *S*-enantiomer (**d**, 32.6 min) of α-hydroxymyristic acid, respectively. For abbreviation see Figure 3.

Configuration analyses indicated that β-hydroxymyristic acid of this acylpeptide was an *R*-enantiomer (D-enantiomer) (13). This configuration is due to favoring the *R*-enantiomer as the fatty acid substrate in acyltransferation step of the acylpeptide biosynthesis (14,15). By HPLC analysis using a chiral column, we observed that β-hydroxymyristic acid formed by the reaction of $P450_{BS\beta}$ is nearly enantiomerically pure *R*-form (Fig. 6). *S*-Form of β-hydroxymyristic acid could not be detected, but *S*-form of α-hydroxymyristic acid was about 22% of the total amount of α-hydroxyl product. In yeast, Venter *et al.* (16) demonstrated that 3-hydroxy-polyenoic fatty acid was *R*-form and suggested that the *R*-form of β-hydroxy fatty acid is formed by other metabolic reactions such as direct oxygenation by P450, because the normal β-hydroxyl intermediate in β-oxidation is the *S*-form. Thus, at least β-hydroxy fatty acid produced by the reaction of $P450_{BS\beta}$ may be utilized to synthesize acylpeptides, antibiotic compounds produced by *Bacillus* species, although the physiological roles of α-hydroxyl fatty acid in this bacterium remain to be elucidated.

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Identification of Acylated Glycoglycerolipids from a Cyanobacterium, *Synechocystis* sp., by Tandem Mass Spectrometry

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ABSTRACT: Acylated glycoglycerolipids were identified in the total lipid extract from cyanobacterium *Synechocystis* sp. PCC 6803. These compounds have a palmitoyl group esterified to the hydroxyl group at the C-6 position of the terminal glycosyl moiety of digalactosyl monoacylglycerol and digalactosyl diacylglycerol. Their structural elucidation was accomplished by tandem mass spectrometry coupled with fast atom bombardment ionization. Acylated digalactosyl monoacylglycerol has a structure of 1-hydroxy-2-palmitoyl-3-*O*-[(6-*O*-palmitoyl)-α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol. This compound has not been reported previously.

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Glycoglycerolipids, such as monogalactosyl, digalactosyl, and sulfoquinovosyl diacylglycerols (MGDG, DGDG, and SQDG, respectively), have been regarded as the major lipid components of the photosynthetic membrane in plants, algae, and various bacteria (1,2). These glycoglycerolipids account for more than 90% of the total glycerolipids in the intact cells of *Synechocystis* sp. PCC 6803 (3). They may also play a critical role in photosynthesis owing to their exclusive distribution in the chloroplasts. Furthermore, glycoglycerolipids possessing pharmacological activity (4,5) or unique structural features (6–10) have been isolated and characterized from various organisms.

Cyanobacteria provide excellent models for the study of oxygenic photosynthesis (3). Recently, the structural identification of individual molecular species of major glycerolipids, such as MGDG, DGDG, SQDG, and phosphatidylglycerol, which were purified from the total lipid extract of cyanobacterium *Synechocystis* sp. PCC 6803, was accomplished using tandem mass spectrometry (MS/MS) coupled with fast atom bombardment (FAB) (11). Collision-induced dissociation (CID) of sodium-adducted molecular ions ($[M + Na]^+$ or $[M + 2Na - H]^+$) gives rise to numerous product ions that provide information on the structure of a polar head and fatty acyl groups, including the double-bond positions in fatty acyl chains and regiospecificity of the acyl linkages (11,12). Additionally, four *sn*-2 acyl lysoglycerolipids corresponding to their monoacylglycerolipids, which contain exclusively a palmitoyl group at the *sn*-2 position, were also isolated simultaneously.

Here we describe the structural characterization of acylated digalactosyl monoacylglycerol (DGMG) and DGDG isolated from *Synechocystis* sp. PCC 6803, using FAB–CID–MS/MS. To our knowledge, acylated DGMG has not been reported previously, although acylated DGDG, MGDG, and monogalactosyl monoacylglycerol (MGMG) were isolated from leaf homogenates and nitrogen-fixing cyanobacteria before (6–9).

EXPERIMENTAL PROCEDURES

Isolation of acylated DGMG and DGDG. Cyanobacterium *Synechocystis* sp. PCC 6803 was cultured as described previously (11). Briefly, the cells in BG11 medium were cultivated at 28°C for 3 to 4 d and illuminated continuously at an intensity of 1000 lux with a fluorescent lamp. The culture was harvested in the mid-log phase of growth by centrifugation at 12,000 × *g* and 4°C for 15 min. The harvested cells were homogenized in 300 mL of chloroform/methanol/water (1:1:1, by vol). Total lipids were extracted from the homogenized cells by the method of Bligh and Dyer (13). The extracted lipids were separated by two-dimensional thin-layer chromatography on silica plates (Whatman, Hillsboro, OR) according to the method of Douce *et al.* (14). The solvent systems were chloroform/methanol/water (65:25:4, by vol) as solvent for dimension I and chloroform/acetone/methanol/acetic acid/water (10:4:2:2:1, by vol) for dimension II. Lipids were located by spraying the plate with a solution of 0.01% primuline in 80% acetone, followed by visualization under ultraviolet light. Two spots corresponding to unknown lipids A and B were detected between MGDG and MGMG and recovered in small quantities.

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Abbreviations: CID, collision-induced dissociation; DGDG, digalactosyl diacylglycerol; DGMG, digalactosyl monoacylglycerol; FAB, fast atom bombardment; MGDG, monogalactosyl diacylglycerol; MGMG, monogalactosyl monoacylglycerol; MS/MS, tandem mass spectrometry; SQDG, sulfoquinovosyl diacylglycerol.

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Mass spectrometry. All mass spectrometric analyses were performed using a JMS-HX110/110A tandem mass spectrometer (JEOL, Tokyo, Japan) of four-sector instrument with an E_1B_1-E_2B_2 configuration as described previously (15). Briefly, the ion source was operated at 10 and −10 kV accelerating voltages in the positive- and negative-ion modes, respectively. Ions were produced by FAB using the cesium ion beam, which was generated from the ion gun and accelerated to 12 keV. The sample was dissolved in chloroform/methanol (1:1, vol/vol) at about 5 μg/μL and then mixed with 1 μL 3-nitrobenzyl alcohol (Sigma, St. Louis, MO) for the positive-ion spectra and triethanolamine (BDH, Poole, Dorset, United Kingdom) for the negative-ion spectra on the FAB probe tip. CID of the sodium-adducted and deprotonated molecular ions (i.e., $[M + Na]^+$ and $[M - H]^-$) selected with first mass spectrometer (MS-1; E_1B_1) occurred in the collision cell located between B_1 and E_2 and floated at 3.0 and −3.0 kV, respectively. The resultant product ions were analyzed by B/E scan method of second mass spectrometer (MS-2; E_2B_2). The collision gas, helium, was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 70%.

RESULTS AND DISCUSSION

Positive-ion FAB mass spectra of unknown lipids A and B, which were found between MGDG and MGMG on two-dimensional thin-layer chromatography shown in Figure 1, display abundant peaks corresponding to $[M + Na]^+$ ions at *m/z* 915 and 1175, as shown in Figure 2A and 2B, respectively. Also, their negative-ion FAB mass spectra (not shown) show deprotonated molecular ions ($[M - H]^-$) at *m/z* 891 and 1151. Thus, their molecular masses are 892 and 1152 Da. Exact mass measurements for the ions of *m/z* 915 and 1175 revealed the elemental compositions $C_{47}H_{88}O_{15}Na$ (observed, 915.6019: theoretical, 915.6021) and $C_{65}H_{116}O_{16}Na$ (observed, 1175.8145: theoretical, 1175.8161), respectively. The negative-ion FAB–CID–MS/MS spectra of glycoglycerolipids were helpful in determining the fatty acid composition in previous work (12). Namely, as shown in Figure 3A, the CID–MS/MS spectrum of $[M - H]^-$ ion of lipid A indicates a dominant carboxylate ion at *m/z* 255 corresponding to a palmitate (16:0). Furthermore, the neutral loss of a palmitoyl group as the ketene analog (R=C=O) and free fatty acid (RCOOH) yields abundant peaks at *m/z* 653 and 635, respectively. Also, further loss of another palmitoyl group of the ion at *m/z* 653 results in the product ions at *m/z* 415 and 397. The ion of *m/z* 397 can also be generated by further loss of another palmitoyl group of the product ion at *m/z* 635 as the ketene analog. This fragmentation pattern is similar to that observed in the negative-ion CID spectrum of DGDG, which was investigated in our previous work (12).

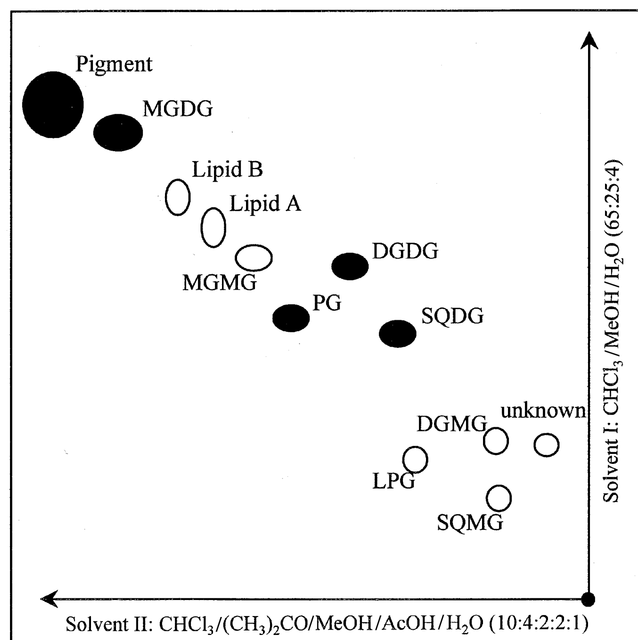

FIG. 1. A schematic picture of separation of total lipids from *Synechocystis* sp. PCC 6803 by two-dimensional thin-layer chromatography (TLC). Dark circles indicate the four major diacylglycerolipids. The four minor *sn*-2 acyl lysoglycerolipids corresponding to their monoacylglycerolipds are also represented. White circles labeled as Lipid A and B correspond to unknown lipids in small amounts. Their structural determination is described in the text.

From these results, the unknown lipid A is established as containing two palmitoyl groups and a disaccharide as a polar head group. From the dominant carboxylate anions at *m/z* 255 and 277 appearing in the negative-ion CID spectrum of an unknown lipid B, this compound contains palmitoyl and 18:3 acyl groups. The product ions formed by the neutral losses of carboxylic acid and ketene analog of these fatty acyl groups are also observed. However, by comparing the fragmentation pattern observed in the CID–MS/MS spectra of $[M + Na]^+$ ions (*m/z* 915 and 1175) of unknown lipids A and B as shown in Figure 4A and 4B, respectively, with that of DGDG reported previously, the structural difference between unknown lipids and DGDG was identified. To confirm this difference more explicitly, we compared the feature of these spectra with that of the CID spectrum of $[M + Na]^+$ ion (*m/z* 677) of DGMG species, which was isolated from the same cyanobacterium and contained a palmitoyl group at the *sn*-2 position. Figure 4C shows the positive-ion CID spectrum of the $[M + Na]^+$ ion of DGMG. Although the CID–MS/MS spectrum of $[M + Na]^+$ ion was very complicated owing to a variety of fragmentations, they were helpful in determining the structure of unknown lipids, based on the comparison between these spectra.

In the CID spectrum of the $[M + Na]^+$ ion (*m/z* 915) of lipid A, charge-remote fragmentation occurring along a palmitoyl chain yields a series of homologous product ions from *m/z* 899 to 731 ($^3I_2$), due to consecutive loss of C_nH_{2n+2} from the terminus of a palmitoyl group. The product ions resulting from the cleavage within the terminal sugar ring occur at *m/z* 543 ($^{1,5}X_1$) and 557 ($^{0,2}X_1$). These are very informative about the structural feature. As shown in Figure 4C, the same product ions in the CID–MS/MS spectrum of $[M + Na]^+$

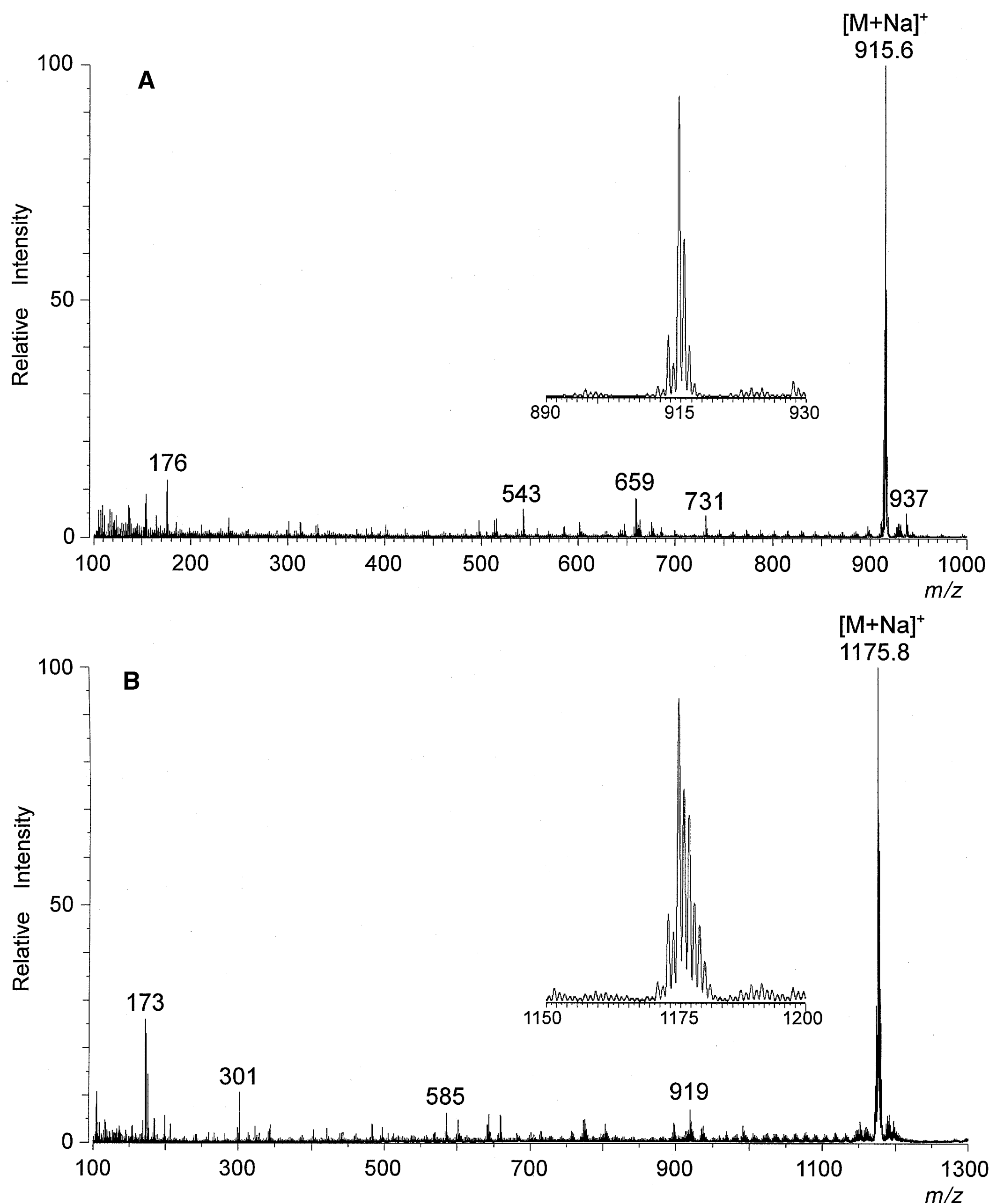

FIG. 2. Positive-ion fast atom bombardment (FAB) mass spectra of unknown (A) lipid A and (B) lipid B isolated from the cyanobacterium *Synechocystis* sp. PCC 6803. The inserts indicate the expanded region in which the sodium-adducted molecular ions are observed. The ions of *m/z* 915 and 937 in A correspond to $[M + Na]^+$ and $[M + 2Na - H]^+$ of lipid A, respectively, whereas the ion of *m/z* 1175 in B is the $[M + Na]^+$ ion of lipid B.

ion of DGMG are observed at the same *m/z* value. These results lead to the conclusion that one of two palmitoyl groups in lipid A is attached to the glycerol backbone and the other to the carbon on the terminal sugar ring. Fragmentation of the glycosidic bond, with loss of the nonreducing terminal portion as a neutral fragment, which is observed at *m/z* 513 ($Y_1 - 2H$) and 515 (Y_1), provides further evidence for this result. On the other hand, the concomitant loss of two groups, which are linked to the C-4 and C-5 positions of a terminal sugar unit, *via* 1,2-elimination, produces the common product ion at *m/z* 629 in both spectra. Thus, a palmitoyl group must be esterified to a hydroxyl group of the C-4 or C-6 position on the sugar ring. The remainder of the peaks except for those at *m/z* 169 and 185 in Figure 4A are up-shifted by 238 Da in comparison with the corresponding one observed in Figure 4C, including the $^{0,4}A_2$ and $^{3,5}A_2$ ions which provide information on the 1→6 linkage between the two sugar rings. The product ions of *m/z* 169 and 185 may be formed by second fragmentation of the B_1' and B_1 ions *via* the ketene loss of the palmitoyl group on the terminal sugar ring, respec-

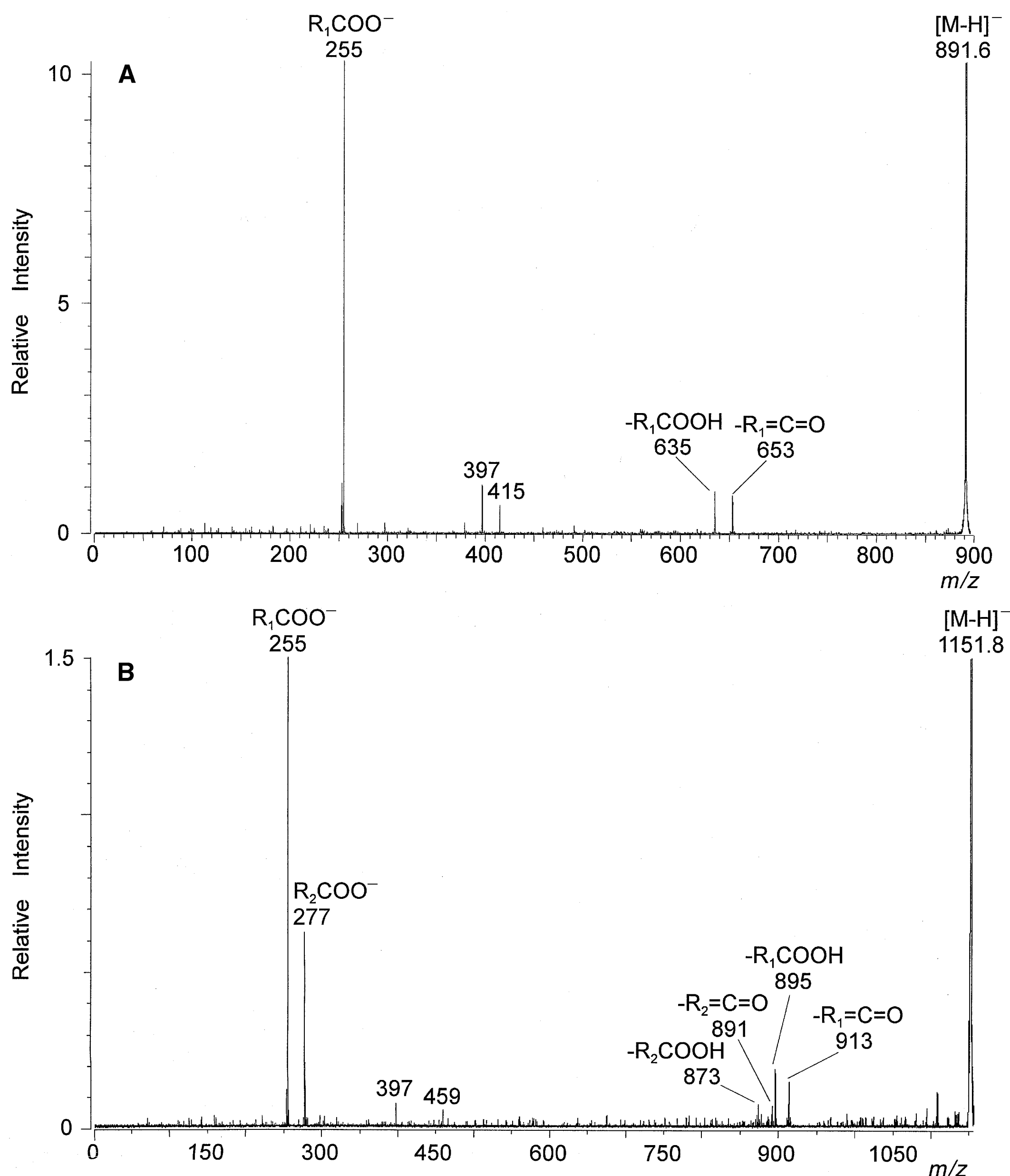

FIG. 3. Negative-ion FAB collision-induced dissociation (CID) tandem mass (MS/MS) spectra of $[M - H]^-$ ions of unknown (A) lipid A and (B) lipid B. The ions at *m/z* 255 and 277 correspond to a palmitate (16:0) and 18:3 carboxylate anions, respectively. For abbreviation see Figure 2.

tively. The fragmentation pathways observed in CID of the sodium-adducted molecular ion are illustrated in Figure 5.

All of the acylated DGDG, MGDG, and MGMG that have been found so far had a fatty acyl group linked to the C-6 position of the terminal sugar ring (5–8). Consequently, the palmitoyl group of this new acylated galactolipid is also linked to the C-6 position. Thus, since DGMG isolated from the same cyanobacterium was synthesized by specific enzymatic hydrolysis of the *sn*-1 fatty acyl group of DGDG species and then had a structure of 1-hydroxy-2-palmitoyl-3-*O*-[α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol, as mentioned in a previous work (11), acylated DGMG has a structure of 1-hydroxy-2-palmitoyl-3-*O*-[(6-*O*-palmitoyl)-α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol. This compound is a novel lipid which has been not reported previously.

When the CID spectrum of $[M + Na]^+$ ion (*m/z* 1175) of lipid B shown in Figure 4B is compared with that of lipid A shown in Figure 4A, the product ions such as A, B, B′, C-2H and E, which do not contain the fatty acyl groups on glycerol backbone, are observed at the same *m/z*. Thus, lipid B also contains a palmitoyl group on the C-6 position of the terminal sugar ring. However, the product ions such as X, Y-2H, Y, G_2 and $^3I_2$ are up-shifted by 260 Da corresponding to a mass difference of an 18:3 acyl group ester-linked on the glycerol backbone. Furthermore, the spectral pattern of a series of ho-

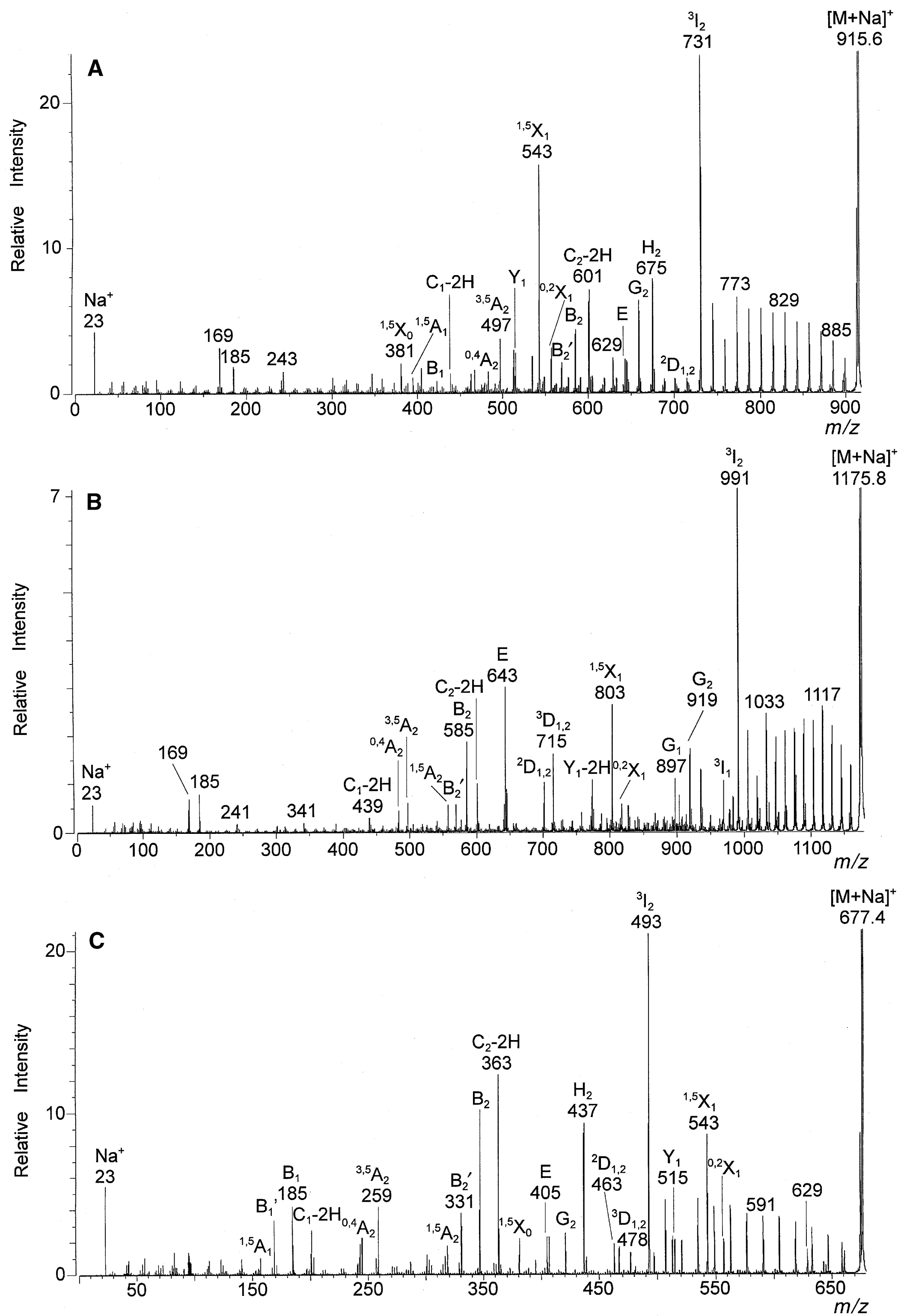

FIG. 4. Comparison of positive-ion FAB–CID–MS/MS spectra of [M + Na]$^+$ ions; (A) an unknown lipid A of *m/z* 915, (B) an unknown lipid B of *m/z* 1175, and (C) digalactosyl monoacylglycerol of *m/z* 677.

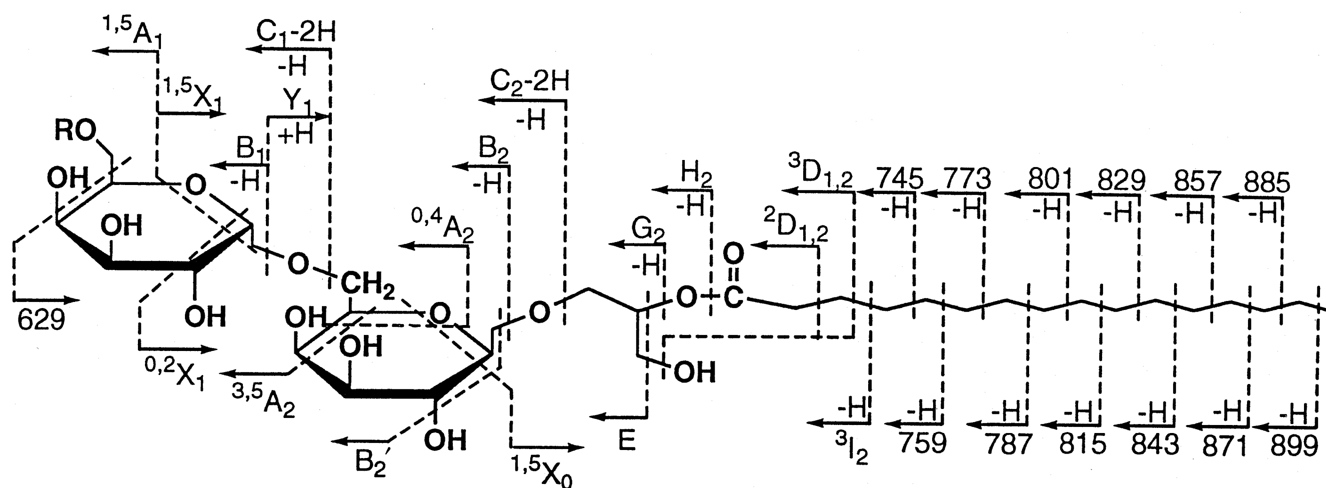

FIG. 5. Proposed structure of a new glycoglycerolipid. The fragmentation pathways are also shown. The nomenclature proposed by Domon and Costello (16) was adopted with minor changes and addition of the new notation for the cleavages of the fatty acyl groups, as described previously (3,11). R is a palmitoyl group at the C-6 position of the terminal sugar unit. The resultant product ions contain a sodium ion.

mologous ions appearing in high-mass region provides information about the double-bond positions in the fatty acyl groups. The homologous ions can be divided into two groups: *m/z* 1089, 1075, 1061, 1047, 1033, 1019, 1005, and 991 ($^3I_2$), due to the saturated palmitoyl chain (16:0), and *m/z* 1091(=, where = represents the double-bond position), 1077, 1063, 1051(=), 1037, 1023, 1011(=), 997, 983 and 969 ($^3I_1$), due to the triply unsaturated γ-linolenoyl chain (18:3). The ions at *m/z* 1159, 1145, 1131, 1117, and 1103 are common to the cleavage of both fatty acyl groups. Also, based on the previous result that the G_2 ion due to the loss of *sn*-2 fatty acyl group was always larger than the G_1 ion due to the loss of an *sn*-1 fatty acyl group (11), γ-linolenoyl and palmitoyl groups are positioned at *sn*-1 and *sn*-2, respectively. Thus, since corresponding DGDG species ($[M + Na]^+ = 937$) isolated from the same cyanobacterium had a structure of 1-γ-linolenoyl-2-palmitoyl-3-*O*-[α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol, acylated DGDG of *m/z* 1175 has a structure of 1-γ-linolenoyl-2-palmitoyl-3-*O*-[(6-*O*-palmitoyl)-α-D-galactopyranosyl-(1→6)-β-D-galactopyranosyl]-*sn*-glycerol. In addition, according to the results obtained by the same structural analyses for other molecular species, the species of *m/z* 1177 and 1179 have the same structure as a species of *m/z* 1175 except for containing linoleoyl (18:2) and oleoyl (18:1) groups at the *sn*-1 position, respectively. The distribution and fatty acid compositions of these molecular species are similar to those of DGDG molecular species, which was reported previously (11).

As proposed by Heinz and coworkers (7,8), the synthesis of acylated DGMG and DGDG involves the transfer of an acyl group catalyzed by an enzyme such as acyltransferase. Furthermore, they concluded that this enzyme had no positional specificity with respect to transferring the acyl group from the *sn*-1 or *sn*-2 positions of the donor molecules but specifically transferred the acyl group to the C-6 position of the acceptor molecule (6,7). However, as described previously (11), the majority of glycerolipids isolated from *Synechocystis* sp. PCC 6803 contained a palmitoyl group at the *sn*-2 position. Moreover, since the positive-ion FAB mass spectra shown in Figure 2A and 2B indicate the exclusive presence of *m/z* 915 and molecular ion distributions similar to that of DGDG, respectively, these new acylated DGMG and DGDG should be formed by specifically transferring the palmitoyl group from the *sn*-2 position of other galactolipids to the C-6 position of the terminal sugar ring of DGMG and DGDG.

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Δ5-Olefinic Acids in the Seed Lipids from Four *Ephedra* Species and Their Distribution Between the α and β Positions of Triacylglycerols. Characteristics Common to Coniferophytes and Cycadophytes

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ABSTRACT: The fatty acid compositions of the seed lipids from four *Ephedra* species, *E. nevadensis, E. viridis, E. przewalskii,* and *E. gerardiana* (four gymnosperm species belonging to the Cycadophytes), have been established with an emphasis on Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA). Mass spectrometry of the picolinyl ester derivatives allowed characterization of 5,9- and 5,11-18:2; 5,9,12-18:3; 5,9,12,15-18:4; 5,11-20:2; 5,11,14-20:3; and 5,11,14,17-20:4 acids. Δ5-UPIFA with a Δ11-ethylenic bond (mostly C_{20} acids) were in higher proportions than Δ5-UPIFA with a Δ9 double bond (exclusively C_{18} acids) in all species. The total Δ5-UPIFA content was 17–31% of the total fatty acids, with 5,11,14-20:3 and 5,11,14,17-20:4 acids being the principal Δ5-UPIFA isomers. The relatively high level of *cis*-vaccenic (11-18:1) acid found in *Ephedra* spp. seeds, the presence of its Δ5-desaturation product, 5,11-18:2 acid (proposed trivial name: ephedrenic acid), and of its elongation product, 13-20:1 acid, were previously shown to occur in a single other species, *Ginkgo biloba,* among the approximately 170 gymnosperm species analyzed so far. Consequently, Ephedraceae and Coniferophytes (including Ginkgoatae), which have evolved separately since the Devonian period (~300 million yr ago), have kept in common the ability to synthesize C_{18} and C_{20} Δ5-UPIFA. We postulate the existence of two Δ5-desaturases in gymnosperm seeds, one possibly specific for unsaturated acids with a Δ9-ethylenic bond, and the other possibly specific for unsaturated acids with a Δ11-ethylenic bond. Alternatively, the Δ5-desaturases might be specific for the chain length with C_{18} unsaturated acids on the one hand and C_{20} unsaturated acids on the other hand. The resulting hypothetical pathways for the biosynthesis of Δ5-UPIFA in gymnosperm seeds are only distinguished by the position of 11-18:1 acid. Moreover, ^{13}C nuclear magnetic resonance spectroscopy of the seed oil from two *Ephedra* species has shown that Δ5-UPIFA are essentially excluded from the internal position of triacylglycerols, a characteristic common to all of the Coniferophytes analyzed so far (more than 30 species), with the possibility of an exclusive esterification at the *sn*-3 position. This structural feature would also date back to the Devonian period, but might have been lost in those rare angiosperm species containing Δ5-UPIFA.

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Gymnosperms are generally divided into two main taxonomic groups: the Coniferophytes and the Cycadophytes (1,2). Despite the fact that not all authors agree on this classification, what is important is that extant species belonging to these two distinct groups have evolved independently since the Devonian period for approximately 300 million years (1,2). The monophyletic taxon status of gymnosperms is doubtful (1). Takagi and Itabashi (3) have analyzed in detail the seed lipids from 21 gymnosperm species, including two Cycadophytes, whereas Wolff and coworkers have established the fatty acid composition of seed lipids from approximately 170 Coniferophyte species of the families Pinaceae, Cupressaceae, Taxodiaceae, Sciadopityaceae, Taxaceae, Cephalotaxaceae, Podocarpaceae (4–14; Wolff, R.L., Pédrono, F., and Marpeau, A.M., unpublished results), and more recently, of the family Araucariaceae (Wolff, R.L., Christie, W.W., Pédrono, F., and Marpeau, A.M., unpublished results).

Both Japanese and European researchers agreed on the systematic presence of Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA or Δ5-olefinic acids) in all analyzed gymnosperm seeds. The same peculiarity would also hold true for gymnosperm leaves (15). The following structures of Δ5-UPIFA have been characterized in gymnosperm plants: 5,9-18:2 (taxoleic); 5,11-18:2; 5,9,12-18:3 (pinolenic); 5,9,12,15-18:4 (coniferonic); 5,11-20:1; 5,11,14-20:3 (sciadonic), and 5,11,14,17-20:4 (juniperonic) acids, all ethylenic bonds being in the *cis* configuration. The content and profile of individual Δ5-UPIFA depend on the family, the genus, or even the species considered (5–8). The same Δ5-UPIFA (and others, e.g., 5,13-22:2 acid) have been observed in the seeds of about a dozen angiosperm species, which may additionally

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Abbreviations: Ag-TLC, argentation thin-layer chromatography; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TAG, triacylglycerol; UPIFA, unsaturated polymethylene-interrupted fatty acid.

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contain Δ5-monoenoic acids, and *trans* Δ5-UPIFA are known in two or three angiosperm species (16–26).

The first report on the fatty acid composition of the seed lipids from a Cycadophyte species was probably that of Kleiman *et al*. (27), which was published more than 30 yr ago. The species under analysis was *Ephedra campylopoda*, and it was shown that the seeds contained several Δ5-UPIFA, i.e., 5,11-18:2; 5,11-20:2; 5,11,14-20:3, and 5,11,14,17-20:4 acids. The position of the ethylenic bonds as established by a complex combination of preparative or analytical gas–liquid chromatography (GLC) of the fatty acid methyl esters (FAME), fractionation according to the number of ethylenic bonds by argentation thin-layer chromatography (Ag-TLC), partial hydrazine reduction, and ozonolysis. The double bond location in eicosatetraenoic acid was later confirmed by GLC–mass spectrometry (MS) of its monomethoxy eicosanoates (28). With few discrepancies, the Δ5-olefinic acids identified in *E. campylopoda* seed lipids were the same as those characterized a few years earlier by Schlenk and Gellerman (29) in *Ginkgo biloba* seeds and leaves. Litchfield (30) analyzed the seed lipids from another Ephedraceae, *E. nevadensis* (known as Nevada Mormon tea), at approximately the same time, and though the exact location of the ethylenic bonds in each acid was not determined, he concluded that *E. nevadensis* seed fatty acids apparently resembled those identified in *E. campylopoda* by Kleiman *et al*. (27).

In the 1980s, some authors published supplementary data for the seeds and/or leaves of a few other Cycadophytes, i.e., *Welwitschia mirabilis* (31), *Gnetum scandens* (32), *Gnetum gnemon* (33), and *Macrozamia communis* (34). No Δ5-olefinic acids were reported for these species, but surprisingly, some of them were found to contain cyclopropene acids. Takagi and Itabashi (3) found several Δ5-UPIFA in the seeds of *Cycas revoluta*, though in low amounts, as in *E. sinica* dried stalks.

In the present study, FAME were prepared from *E. nevadensis*, *E. viridis*, *E. przewalskii*, and *E. gerardiana* seed lipids and analyzed by capillary GLC on three fused-silica capillary columns with different polarities, one coated with a 100% cyanopropyl polysiloxane stationary phase (CP-Sil 88; Chrompack, Middelburg, The Netherlands), a second one with a polyethylene glycol stationary phase (DB-Wax; J&W Scientific, Folsom, CA), and a third one with a 1:1-cyanopropyl-phenyl polysiloxane stationary phase (Silar-5CP; Chrompack). Identifications of peaks were performed by comparison of their equivalent chain lengths on the DB-Wax column (isothermal) or their retention times on the CP-Sil 88 and the Silar-5CP columns (temperature programming) with the corresponding parameters of FAME prepared with known sources of individual Δ5-UPIFA. However, owing to the botanical and taxonomic distance between Coniferophytes and Cycadophytes, the absolute FAME structures were also established by GLC–MS of the picolinyl esters prepared from *E. nevadensis* seed lipids.

Additionally, we studied the seed oil from the latter species and from *E. viridis* by ^{13}C nuclear magnetic resonance (NMR) spectroscopy, which allows differentiation of Δ5-UPIFA present in the α and β positions of the triacylglycerols (TAG) because it is known that in all Coniferophyte families analyzed so far, the Δ5-UPIFA are almost completely excluded from the internal position of seed TAG (3,11,12, 35–43). It was therefore interesting to verify whether this structural feature had remained unchanged in the two "branches" of gymnosperms, even after a gap of 300 million years.

MATERIALS AND METHODS

Seeds, oil extraction, and FAME preparation. *Ephedra nevadensis* and *E. viridis* (green jointfir ephedra) seeds were purchased from Lawyer Nursery, Inc. (Plains, MT). Seeds from *E. przewalskii* were collected in the vicinity of Ulan Bator, Mongolia, and seeds from *E. gerardiana* were from the Botanical Garden, University of Hamburg. The extraction of lipids from whole seeds and the preparation of FAME were performed as described in detail elsewhere for other gymnosperm seeds (4,5). All FAME preparations were done in duplicate and analyzed twice on the DB-Wax column and once on the CP-Sil 88 and the Silar-5CP columns. As a rule, FAME were prepared and analyzed on the DB-Wax column immediately after lipid extraction.

Analytical GLC. FAME were analyzed in a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped with a DB-Wax column (30 m × 0.32 mm i.d., 0.5 µm film; J&W Scientific). The oven temperature was 190°C and the inlet pressure of the carrier gas (helium) was 140 kPa. Alternatively, a CP-Sil 88 column (50 m × 0.25 mm i.d., 0.2 µm film; Chrompack) was operated with temperature programming in a Carlo Erba HRGC chromatograph from 150 to 185°C at 4°C/min with H_2 at 100 kPa. The injector (split mode) and flame-ionization detector were maintained at 250°C for both columns. Quantitative data were calculated by SP 4290 integrators (Spectra Physics, San Jose, CA). Additionally, all FAME samples were analyzed on a Silar-5CP column (Chrompack) using a temperature program standardized for seed oil fatty acid "fingerprints" as described elsewhere (44).

Identification of FAME peaks. The seed lipids from selected conifer species (45) and from Ranunculaceae such as *Cimicifuga* spp. (46) were used as sources of Δ5-olefinic acid methyl esters with known structures to identify fatty acids from *Ephedra* seed lipids by GLC, either by coinjection, comparison of the equivalent chain lengths (DB-Wax column), or retention times (CP-Sil 88 and Silar-5CP). FAME prepared from meadowfoam seed oil were used to locate Δ5-monoenoic (16:1, 18:1, 20:1, and 22:1) acids on chromatograms.

Preparation of picolinyl esters (47). The free acids (1 mg), prepared by hydrolysis with 0.1 M ethanolic potassium hydroxide, were converted to acid chlorides by reaction with oxalyl chloride (0.5 mL) at ambient temperture overnight. Excess reagent was removed in a stream of nitrogen, and the product was reacted immediately with a solution (0.5 mL) of 3-hydroxymethylpyridine in dichloromethane [20 mg/mL; stored over beads of molecular sieve type 4A (Fisher Scien-

tific, Loughborough, United Kingdom)]. After 1 h at ambient temperature, the solvent was evaporated, the product taken in isohexane (5 mL), and washed with water (2 × 2 mL). The isohexane solution was dried over anhydrous sodium sulfate and evaporated; the product was dissolved in isohexane containing butyl-hydroxytoluene (50 ppm) for analysis by GLC–MS.

GLC–MS. The picolinyl ester derivatives were submitted to GLC–MS with a Hewlett-Packard 5890 Series II plus gas chromatograph attached to an HP model 5989 mass spectrometer. The latter was used in the electron impact mode at 70 eV with a source temperature of 250°C. The chromatograph was fitted with on-column injection and equipped with a capillary column of fused silica coated with Supelcowax 10 (25 m × 0.25 mm i.d., 0.25 μm film; Supelco UK, Poole, United Kingdom). After holding the temperature at 80°C for 3 min, the column was temperature programmed at 20°C/min to 160°C, then at 2°C/min to 280°C, where it was held for 5 min. Helium at 1 mL/min was the carrier gas.

^{13}C NMR spectroscopy. ^{13}C NMR spectra were obtained on a Bruker AM 300 spectrometer (Karlsruhe, Germany) operating at a frequency of 75.47 MHz. Samples were prepared in 5-mm tubes with approximately 100 mg of oil (from *E. nevadensis* and *E. viridis* seeds) in 0.5 mL deuteriochloroform that contained tetramethylsilane as reference and $Cr(acac)_3$ as relaxation agent (0.025 M). Spectra were acquired by a nuclear Overhauser effect-suppressed, inverse-gated, proton-decoupling technique and employed a 90° excitation pulse, a 5-s pulse delay, and a sweep width of 20 kHz (32 K data points). The 90° pulse width was 3.9 μs. The number of scans was 1000 per spectrum.

RESULTS AND DISCUSSION

The phyletic position of Ephedraceae. A hypothetical phyletic relationship between Ephedraceae and Conipherophytes is shown in Figure 1 (adapted from Ref. 2). This figure is only intended to help visualize and emphasize the considerable distance between these plant groups on a geological time scale. It is believed that the Gnetatae, to which the Ephedraceae would belong along with the Gnetaceae and the Welwitschiaceae (with only one species, *W. mirabilis*), are the closest living relatives of the angiosperms, though they have common roots with the Coniferophytes (1,26,48). However, the extant Gnetatae might not be the ancestors of angiosperms, because no fossil records of this group have been found before the Tertiary period, while angiosperms certainly existed in the Jurassic (49). Yet, as mentioned above, Δ5-olefinic acids are also present in the seeds of a few rather archaic angiosperm families (22–24).

Fatty acid characterization and composition. Fatty acids are most easily identified by MS in the form of picolinyl esters or 4,4-dimethyloxazoline derivatives (50,51), and the former were used here. Some key diagnostic ions are listed in Table 1. As picolinyl esters, conventional monoenoic and methylene-interrupted dienoic fatty acids are recognized by characteristic gaps of 26 amu in the mass spectra for fragmentation on either side of the double bond and by abundant ions adjacent to the double bond. Picolinyl esters of fatty acids with a 5,9-double bond system can be recognized by a prominent ion at m/z = 219, representing cleavage between carbon atoms 7 and 8; other ethylenic bonds are located as for more conventional unsaturated fatty acids (50,51). An isolated double bond in position 5 can be located by the appropriate gap of 26 amu, but a prominent ion at m/z = 232 for a fragmentation adjacent to the double bond is often a more useful diagnostic guide. For example, spectra of picolinyl esters of 5-20:1 and 5,13-22:2 acids have been published previously (52). Mass spectra of 4,4-dimethyloxazoline derivatives of the same Δ5-UPIFA prepared from the seed lipids of various conifer species and of *Cimicifuga* spp. have been published previously (45,46).

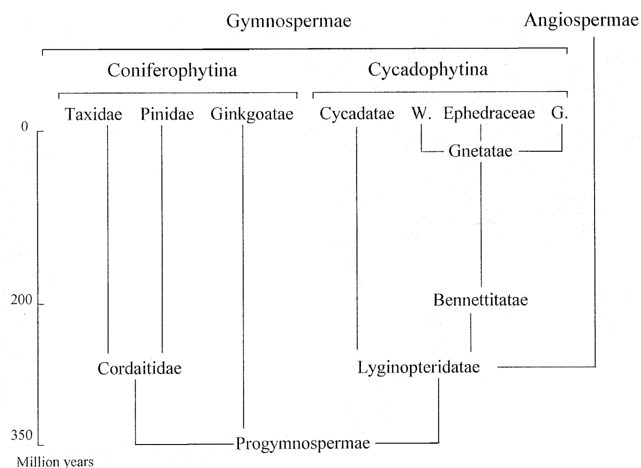

FIG. 1. Hypothetical phylogenic relationship between Ephedraceae and Coniferophytes [W., Welwitschiaceae; G., Gnetataceae) (Adapted from Ref. 2)].

TABLE 1
Characteristic Ions in the Mass Spectra of Picolinyl Ester Derivatives of Unsaturated Fatty Acids in *Ephedra nevadensis* Seed Oil and Their Structures

Fatty acid picolinyl esters	M^+ m/z (intensity, %)	Other diagnostic ions m/z (intensity, %)
9-16:1	345 (75)	220 (15), 274 (38), 288 (47)
9-17:1	349 (40)	220 (14), 274 (25), 288 (32)
9-18:1	373 (55)	220 (13), 274 (43), 288 (43)
11-18:1	373 (46)	248 (11), 302 (44), 316 (37)
5,9-18:1	371 (22)	219 (25), 260 (12), 272 (10)
5,11-18:1	371 (14)	232 (19), 286 (9), 300 (11)
9,12-18:2	371 (100)	260 (22), 274 (17), 300 (12)
5,9,12-18:3	369 (40)	219 (27), 258 (25), 272 (10)
9,12,15-18:3	369 (77)	300 (39), 314 (14), 340 (17)
5,9,12,15-18:4	367 (17)	219 (16), 258 (14), 298 (14)
11-20:1	401 (45)	248 (10), 302 (30), 316 (23)
13-20:1	401 (33)	276 (10), 330 (28), 344 (23)
5,11-20:2	399 (24)	232 (14), 260 (6), 286 (8)
11,14-20:2	399 (70)	248 (15), 288 (34), 302 (19)
5,11,14-20:3	397 (41)	232 (16), 286 (25), 300 (10)
11,14,17-20:3	397 (36)	368 (10), 328 (24), 288 (14)
5,11,14,17-20:4	395 (31)	232 (15), 286 (17), 326 (21)

With regard to Δ5-UPIFA, all species analyzed here have seed lipids that contain the whole series of such acids (Tables 1 and 2). No Δ5-monoenoic acids were observed on routine chromatograms of FAME (the three capillary columns used here allow distinction of the isomeric *cis*-5 and *cis*-9 18:1 acids; results not shown). With the possible exception of *E. sinica* stalk lipids (3), this appears to be a general feature of gymnosperm lipids. However, in a few species distinct from *Ephedra*, small peaks with the same retention time of 5-18:1 acid have been observed on Silar-5CP columns, which require further investigation and identification (Aitzetmüller, K., unpublished observations). In contrast to most gymnosperm species, Δ5-monoenoic acids have been observed in the seeds of a number of angiosperm families, sometimes in great amounts (22,53), and 5,11,14-20:3 acid also has been observed at levels up to 30% in a few angiosperm seed oils (53).

Our results clearly show that 5,9- and 5,11-18:2 acids, though in minor amounts, coexist in *Ephedra* spp. seed lipids (Table 2), with a higher level of the latter as compared to the former. As dicussed elsewhere for FAME prepared from *G. biloba* seed lipids (13), analyses of FAME prepared from *Ephedra* seed lipids on the DB-Wax capillary column allow only a partial resolution of the 5,9- and 5,11-18:2 acids, whereas baseline resolution of these two isomers is obtained on the CP-Sil 88 and Silar-5CP capillary columns. Identification (by their retention times) and quantitation of these acids were thus made with the latter columns. These identifications were supported by MS of the picolinyl esters separated on a Supelcowax capillary column (similar to the DB-Wax) (Table 1).

The same octadecadienoic acids that were formally identified in *G. biloba* seeds (13) were identified by GLC–MS of the picolinyl esters, and our observations confirm the tentative identifications of these acids by Takagi and Itabashi (3) in *E. sinica* dried stalks, based on calculated equivalent chain lengths. In the study by Kleiman *et al*. (27) on *E. campylopoda* seed lipids, only the 5,11-18:2 isomer was identified by chemical means. For practical purposes, it would appear that *Ephedra* seeds are more useful than *G. biloba* seeds in preparing 5,11-18:2 acid, because their oil content is *ca*. 11%

TABLE 2
Fatty Acid Composition (wt% of total fatty acids[a]) of Four *Ephedra* spp. Seed Lipids (this study) and Comparison with *E. campylopoda*

Fatty acid structure[b]	Species				
	E. nevadensis	*E. viridis*	*E. przewalskii*	*E. gerardiana*	*E. campylopoda*
14:0	0.1	0.1	0.7	0.1	Trace
16:0	6.1	6.2	6.9	6.6	7.2
7-16:1	0.2	0.2	0.5	0.1	
9-16:1	0.9	0.4	0.7	0.7	0.4
aiso-17:0	Trace	Trace	0.2	—[c]	—
17:0	0.03	0.03	0.2	0.1	—
9-17:1	0.04	0.03	0.1	Trace	—
18:0	2.3	2.5	3.4	2.5	7.9
9-18:1	34.2	29.2	24.6	17.1	26.8
11-18:1	12.2	9.7	7.3	11.2	6.2
9,12-18:2	10.6	13.5	9.3	8.7	9.3
9,12,15-18:3	7.9	9.5	16.8	10.5	7.8
20:0	0.3	0.3	0.5	0.4	0.1
11-20:1	0.4	0.5	0.6	0.5	0.1
13-20:1	0.3	0.22	0.2	0.5	0.1
11,14-20:2	1.2	2.0	0.7	2.6	1.4
7,11,14-20:3	Trace	Trace	—	—	—
11,14,17-20:3	0.9	1.5	1.5	3.3	2.2
22:0	0.2	0.2	0.3	0.2	—
5,9-18:2[d]	1.4	1.0	0.5	0.4	—
5,11-18:2[d]	4.0	1.9	2.0	1.7	2.0
5,9,12-18:3	0.1	0.2	Trace	Trace	—
5,9,12,15-18:4	0.2	0.2	0.5	0.5	—
5,11-20:2	1.6	1.5	1.1	1.5	1.2
5,11,14-20:3	5.6	6.6	4.4	7.5	5.4
5,11,14,17-20:4	8.9	11.7	8.8	19.2	21.9
Others	0.4	0.8	8.2[e]	4.2[e]	0.0
∑Δ5[f]	21.9	23.1	17.3	30.8	30.5

[a]Means of analyses of two methyl ester preparations.
[b]Double bonds are counted from the carboxylic group and are in the *cis* configuration.
[c]Not reported (Ref. 27), or not detected (present study).
[d]Proportions determined on the CP-Sil 88 or the Silar-5CP columns.
[e]May contain some unsaponifiable components (late-eluting peaks).
[f]Sum of Δ5-olefinic acids.

(relative to undehulled seeds) instead of only 1.5–2%, respectively. The 5,9,12-18:3 and 5,9,12,15-18:4 acids, which are the Δ5-desaturation products of linoleic and α-linolenic acids, are present in small amounts (0.1–0.5%), and were not reported previously in *Ephedra* seeds (27,28). However, an isomeric 18:3 acid (unknown structure) was noted on the chromatogram of FAME prepared from *E. nevadensis*, and published by Litchfield (30). The 5,11-20:2 acid (derived from 11-20:1 acid) also occurs in the seed lipids of the four *Ephedra* species (1.1–1.6%), but we could not detect the Δ5-desaturation product of 13-20:1 acid, the elongation product of *cis*-vaccenic acid. Both the 9,12-18:2 and the 9,12,15-18:3 acids are elongated to 11,14-20:2 and 11,14,17-20:3 acids, respectively, but none of these acids accumulated to a large extent (3.3% at most). They are apparently actively Δ5-desaturated to 5,11,14-20:3 and 5,11,14,17-20:4 acids, which are present at levels in the range 4.4–7.5% and 8.8–19.2%, respectively. The total Δ5-UPIFA reached 17.3% in *E. przewalskii*, 21.9% in *E. nevadensis*, 23.1% in *E. viridis*, and 30.8% in *E. gerardiana*. The latter value compares well with the proportion determined in *E. campylopoda* (30.5%) (Table 2). It is interesting to note that total Δ5-UPIFA is less than 33% in the five *Ephedra* species analyzed so far, such as the Conipherophyte species. For the latter species, this was explained by the fact that all Δ5-UPIFA are mostly esterified to only one position of TAG, the *sn*-3 position (37).

Among "habitual" fatty acids, the main component was oleic (9-18:1) acid (17–34%), accompanied by fairly high levels of 11-18:1 (*cis*-vaccenic or asclepic) acid (*ca*. 7–12%). Among Coniferophytes, only *G. biloba* seeds present a higher level of *cis*-vaccenic acid (17–18%) (3,54,55); other species generally contain less than 2% of this acid in their seed lipids. Following the octadecenoic acids, and in more or less equal abundance, are linoleic (9,12-18:2) and α-linolenic (9,12,15-18:3) acids (9–13% and 8–17%, respectively). The level of α-linolenic acid was between that present in Pinaceae and Taxaceae on the one hand, and that in Taxodiaceae and Cupressaceae on the other hand. The saturated fatty acids, principally 16:0 and 18:0 acids, were quite low (less than 10% in the species analyzed here), which is an habitual feature of most Coniferophytes. Other minor fatty acids were 14:0, possibly 15:0, 17:0, 9-17:1 [identified by its mass spectrum (Table 1)], and 22:0 acids. 14-Methylhexadecanoic acid, which is an ubiquitous branched fatty acid in Pinaceae seeds (56), and also present in *G. biloba* seeds (55), could not be positively identified in *Ephedra* seeds.

Hypotheses on Δ5-desaturases in gymnosperms. A metabolic pathway for the synthesis of habitual fatty acids and of Δ5-UPIFA was proposed initially by Itabashi and Takagi (57), who analyzed the fatty acid composition of *Taxus cuspidata* (Japanese yew) seed, aril, and leaf lipids. Later, Wolff *et al*. (5) proposed a similar scheme based on the analysis of the seed fatty acids from 28 species belonging to the families Pinaceae, Taxaceae, Taxodiaceae, Sciadopityaceae, and Cupressaceae. Both groups of researchers postulated the existence of a Δ5-desaturase that would use 9-18:1, 9,12-18:2, 9,12,15-18:3, and their elongation products, 11-20:1, 11,14-20:2, and 11,14,17-20:3 acids, as substrates. Additionally, in *G. biloba* seed lipids (13) and in *Ephedra* spp. (this study), the 11-18:1 acid also is visibly a substrate for the Δ5-desaturase and gives 5,11-18:2 acid. With regard to this acid, we propose the trivial name "ephedrenic" acid, to recall that it is a widespread fatty acid in *Ephedra* spp. seeds, though it also occurs in *G. biloba* seed lipids.

Our observations suggest that there might exist two Δ5-desaturases in Coniferophyte and Ephedraceae seeds, one specific for unsaturated fatty acids with a Δ11-ethylenic bond, and another one specific for unsaturated fatty acids with a Δ9 double bond (Figure 2). In the particular case of *Ephedra* spp. seeds, the former would present a high activity, whereas the latter would be considerably less active: Fatty acids with a 5,11-ethylenic pattern are considerably higher than those containing a 5,9 system. For example, an opposite situation is observed in Pinaceae and in some Taxaceae [e.g., *Taxus* species (11)], where the 5,9-system is generally prominent. *Pinus edulis* (9) and other pines from the subsection *Cembroides* (10) are exceptions. In these species, the proportion of 5,11,14-20:3 acid may be higher than that of the 5,9,12-18:3 acid, though both acids are exceptionally low in this subsection of *Pinus*. On the other hand, the 5,11-dienoic arrangement predominates in some other families such as Sciadopityaceae [with the single extant species *Sciadopitys verticillata* (58)], Cupressaceae, and Taxodiaceae (3,5,7,14). If one ignores the existence of the 5,11-18:2 acid in *G. biloba* and *Ephedra* spp. seeds, then one can also hypothesize that there may exist two desaturases, one specific for unsaturated C_{18} acids, and the other specific for unsaturated C_{20} acids (Fig. 2). A third possibility—at least in some Cycadophytes—would depend on different chain-elongation activities (from C_{18} to C_{20}), prior to Δ5-desaturation by only one Δ5-desaturase, which generally prefers Δ11- and/or C_{20} substrates over Δ9- and/or C_{18} substrates. This model could also explain the observed different Δ5-UPIFA levels. The Δ5-UPIFA would then simply depend on the relative amounts of the preformed Δ11- (e.g., 11-18:1) or C_{20} acid substrates that were available to attack a single Δ5-desaturase.

The problem in making a decision regarding these possibilities is linked to the existence of 5,11-18:2 acid, issued from 11-18:1 acid, which is both a C_{18} acid and a Δ11-unsaturated acid. Moreover, the 13-20:1 acid, a C_{20} monoenoic acid without a Δ11-ethylenic bond, is apparently not a substrate for the Δ5-desaturase(s). These two exceptions would favor the first hypothesis, i.e., a specificity of the Δ5-desaturases for either the Δ9- or the Δ-11 ethylenic bond. In view of the fact that no Δ5-monoenoic acids are present, one ethylenic bond in the latter positions is apparently necessary for the introduction of a Δ5-ethylenic bond. Whatever the number of Δ5-desaturases and their substrate specificities, it is likely that they exist in both "branches" of gymnosperms. This is clearly demonstrated for *Ephedra* spp. and *Cycas revoluta* (3), another Cycadophyte. Except for the elongation of 5,9,12-18:3 acid to 7,11,14-20:3 (bishomopinolenic) acid,

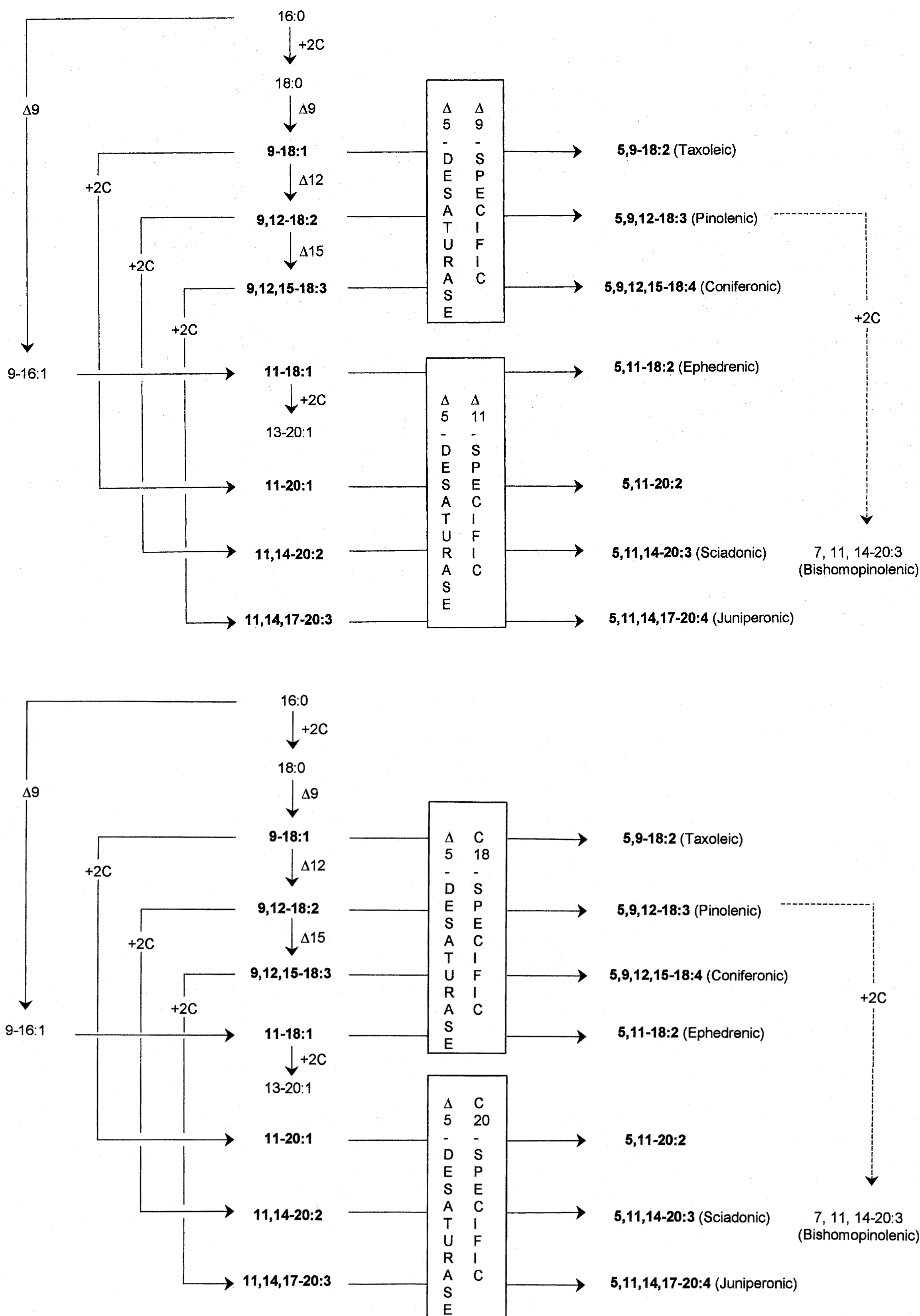

FIG. 2. Possible metabolic pathways for the biosynthesis of known unsaturated fatty acids occurring in gymnosperm seeds, and particularly in *Ephedra* spp. seeds. The upper figure is based on a specificity of Δ5-desaturases for the first ethylenic bond (Δ9 vs. Δ11), and the lower figure is based on a specificity for the chain length (C_{18} vs. C_{20}). The reaction symbolized by a dotted arrow is apparently limited to Pinaceae species, and does not seem to take place in *Ephedra* spp. seeds.

which was shown to occur in many species, but exclusively in the Pinaceae family (59), all other reactions shown in Figure 2 would occur simultaneously in *Ephedra* spp. seeds. The end products as well as the intermediate fatty acids have all been characterized in the present study.

When considering the ancestors of gymnosperms, possibly an extinct group of Pteridophytes (or derived from them, e.g., the "seed ferns"), or some still more remote ancestors, it is interesting to note that some extant species of the latter plant group (e.g., ferns) can synthesize arachidonic (5,8,11,14-20:4) and eicosapentaenoic (5,8,11,14,17-20:5) acids in their green parts (29). This implies that they contain one Δ6-desaturase specific for polyunsaturated C_{18} acids and one Δ5-desaturase specific for polyunsaturated C_{20} acids, which is a feature common to the more ancient group Bryophytes [e.g., mosses (29,60,61) and liverworts (61)], many microalgae and algae, some molds, and to most animals. However, the simultaneous presence of both Δ5- and Δ6-acids also has recently been observed in an angiosperm at levels between 3 and 5% of each (Aitzetmüller, K., unpublished results). The passage from Pteridophytes (or a related plant group) to gymnosperms might thus have been accompanied by a change of the C_{18}-specific (or Δ9-specific) Δ6-desaturase to a C_{18}-specific (or Δ9-specific) Δ5-desaturase, whereas the C_{20}-specific (or Δ11-specific) Δ5-desaturase would have kept its specificity. No fatty acids with a Δ6-ethylenic bond have been reported in any of the gymnosperm families analyzed so far, but conclusive results supported by MS indicate that the seeds from at least one species of the Araucariaceae family would contain both the Δ6-desaturase specific for polyunsaturated C_{18} acids and the Δ5-desaturase specific for polyunsaturated C_{20} acids. Their lipids contain γ-linolenic, stearidonic, arachidonic, and eicosapentaenoic acids, in addition to C_{20} Δ5-olefinic acids characteristic of other Coniferophytes (Wolff, R.L., W.W. Christie, F. Pédrono, and A.M. Marpeau, unpublished results). Because the interfamily relationships of Araucariaceae among Conipherophytes are mostly obscure, and because little is known as to which family could be more primitive, it is unclear whether the original status of polyunsaturated fatty acids in gymnosperms was a Δ6-unsaturated C_{18} status or a Δ5-unsaturated C_{18} status.

The possibility of the simultaneous presence of a C_{18}-specific Δ6-desaturase and of a C_{20}-specific Δ5-desaturase in a Spermaphyte is more than of academic interest. The logical metabolites of the combined action of both desaturases, with the intermediate intervention of a C_{18}-C_{20} elongase, are arachidonic and eicosapentaenoic acids. Surprisingly enough, arachidonic acid has been characterized in a few rare angiosperm species that are not known to contain the metabolic precursor γ-linolenic acid, whereas species containing the latter acid would not contain arachidonic acid (62). It is noteworthy that garlic and virgin wheat germ oil, have been reported to contain arachidonic acid (62).

Distribution of Δ5-UPIFA between the α- and β-chains of TAG. Several studies, using either chemical or enzymatic degradative procedures (3,36–39,43) or ^{13}C NMR spectroscopy (11,12,35,36,40–43), have shown that in all Coniferophyte seed lipids analyzed so far (more than 30 species belonging to 8 families and 15 genera; Table 3), Δ5-UPIFA are practically excluded from the β-position of TAG. This was corroborated by two studies, indicating that TAG molecular species with two Δ5-UPIFA were scarce [*Pinus pinaster* and *P. koraiensis* (63)] or undetectable [*P. koraiensis* (64)]. A more detailed study of the stereodistribution of Δ5-UPIFA has shown that they were principally esterified (more than 90%) to the *sn*-3 position [in *Taxus baccata*, *Larix decidua*, *Sciadopitys verticillata*, *Juniperus communis*, and the two preceding *Pinus* species (37)]. This results in a maximum observed content of Δ5-UPIFA in TAG of 33% of total fatty acids [in *Larix sibirica* seeds (65)], supporting the hypothesis that these acids would be exclusively esterified to the *sn*-3 position of TAG from

TABLE 3
Coniferophyte Species Showing a Definite Enrichment of Δ5-Unsaturated Polymethylene-Interrupted Fatty Acids in the α-Chains of Seed Triacylglycerols

Family	Species	Methods[a]	References[b]
Ginkgoaceae	*Ginkgo biloba*	GR	(3)
Podocarpaceae	*Podocarpus nagi*	GR	(3)
	P. andinus	NMR	(12)
Taxaceae	*Torreya nucifera*	GR	(3)
	T. grandis	NMR	(11)
	Taxus cuspidata	GR	(3)
	T. baccata	GR, NMR	(35–37)
	T. chinensis	NMR	(11)
Cephalotaxaceae	*Cephalotaxus drupaceae*	NMR	(12)
Pinaceae	*Pinus armandi*	—	(38)
	P. koraiensis	GR, NMR	(3,35–39)
	P. cembra	—	(38)
	P. sylvestris	NMR	(35,40)
	P. mughus	NMR	(35)
	P. nigra	NMR	(35)
	P. griffithii	NMR	(35)
	P. pinaster	GR, NMR	(35–37)
	P. pinea	GR	(36)
	Larix decidua	GR, NMR	(36,37)
	L. leptolepis	NMR	(41)
	L. sibirica	NMR	U.R.[c]
	Picea jezoensis	GR	(3)
	P. abies	NMR	(41)
	P. sitchensis	NMR	(41)
	Cedrus atlantica	NMR	(41)
	Abies concolor	NMR	(41)
	A. alba	NMR	(42)
Sciadopityaceae	*Sciadopitys verticillata*[d]	GR, NMR	(36,37,41)
Taxodiaceae	*Cryptomeria japonica*	GR	(3)
Cupressaceae	*Thuja occidentalis*	NMR	(41)
	Juniperus virginiana	NMR	(41)
	J. communis	GR	(36,38)
	Biota orientalis	PL, NMR	(43)

[a]GR, Grignard reagent; NMR, ^{13}C-nuclear magnetic resonance spectroscopy; PL, pancreatic lipase.
[b]References 3, 36, 37, 38, and 43 relate to purified triacylglycerols; data in Refs. 3, 11, 35, 40, and 41 were determined with total lipids, and those in Reference 42 were established with whole seeds. Reference 38, and experimental procedures therein, are in Japanese.
[c]Farines, M., and Wolff, R.L., unpublished results.
[d]Erroneously reported earlier as a species of the Taxodiaceae family (58).

Coniferophyte seeds.

One aim of the present study was to determine whether the systematic and specific esterification of Δ5-UPIFA to the *sn*-3 glycerol hydroxyl observed in the Coniferophyte branch also occurred in the Cycadophyte branch, even after a separation of 300 million years. As a first approach, ^{13}C NMR spectroscopy was applied to *E. nevadensis* and *E. viridis* seed crude oils. As detailed elsewhere (35,41), this technique not only allows calculation of Δ5-acid levels, but also distinction of Δ5 acids esterified to the α (*sn*-1/3) and β (*sn*-2) positions of TAG with specific reference to carbon atoms 1 (acyl) and 2. The *E. nevadensis* oil has three signals for both C_1 and C_2 corresponding to Δ5 acids in the α position and to the remaining acids in the α and β positions. *Ephedra viridis* oil shows the same major signals accompanied by some small signals which cannot be assigned with certainty. One of these may relate to Δ5 acids in the β position. The results are set out in Table 4. The levels of Δ5 acids measured by NMR (mol%)—both C_1 and C_2 signals—are in good agreement with the more accurate levels measured by GLC (wt%). Consequently, the Δ5 acids occur wholly or mainly in one or both of the α positions in *Ephedra* spp. seed TAG, and the level of these acids in the β position does not exceed 3% (experimental limit detection level).

Because ^{13}C NMR spectroscopy does not distinguish between the *sn*-1 and *sn*-3 positions, our conclusion is thus limited to the α positions. At this molecular level, Coniferophytes and Cycadophytes (at least those species containing Δ5-UPIFA) share in common the distribution of Δ5 acids in TAG. The present study provides some evidence that the unusual role for Δ5 unsaturation [or of the Δ5-desaturase(s)] in the acylation (or desaturation) of glycerol esters in gymnosperm seed lipids is of great antiquity. Apparently, the stereospecific distribution of Δ5-UPIFA in the seed TAG from species of the two gymnosperm branches has remained unchanged during the past 300 million years.

Few data are available for the intraglyceride distribution of Δ5-UPIFA in angiosperm species presenting such acids in their seed lipids. The best documented species are apparently *Limnanthes* species, i.e., *L. douglasii* (66) and *L. alba* (67). The former species was subjected to complete stereospecific analysis, and it was observed that Δ5-olefinic acids (mostly 5-20:1, 5-22-1, and 5,13-22:2 acids, none of which occur in gymnosperm seeds) were esterified to all three glyceride positions, although not in equal amounts. Δ5-Olefinic acids were more abundant in the α positions (almost equally distributed between the *sn*-1 and *sn*-3-positions) than in the β position (66). The TAG species in *L. alba* seeds have been investigated, and almost 51% of them contained three Δ5-olefinic acids (67).

From the absence of 5-18:1 and 5-20:1 acids in reports on gymnosperm seed lipids (which is a distinctive feature as compared to some angiosperms containing Δ5 acids), it may be inferred that the Δ5-desaturase(s) acts as a final step in the biosynthesis of C_{18} Δ5-UPIFA, that is after the introduction of Δ9-, Δ12-, and Δ15-ethylenic bonds, and of the C_{20} Δ5-UPIFA after elongation of oleic, linoleic, and α-linolenic to the corresponding bishomo derivatives. However, it cannot be decided whether the introduction of the Δ5-ethylenic bond occurs before or after incorporation of C_{18} and C_{20} unsaturated acids into TAG. In both cases, one should postulate the existence of either Δ5-desaturase(s) or an acylase specific for the *sn*-3 position of glycerol. This emphasizes some major differences between gymnosperms and angiosperms with regard to the biosynthesis of Δ5-acylated TAG. Within the Gnetatae, the Ephedraceae seed TAG composition and structure are gymnospermous biochemical characteristics apparently not shared by Gnetaceae and Welwitschiaceae (48), which may help clarify the much controversed taxonomy and phylogeny of Gnetatae (1).

We also have found a different tentative classification of gymnosperms by Meyen (68). This author suggests another taxonomic and phyletic position for the family Ephedraceae, which he includes in the order Ephedrales. This order is grouped with Ginkgoales and seven other extinct orders in the class Ginkgoopsida. The reason for this grouping is the primary platyspermy of the seeds, possibly "of utmost importance for tracing gymnosperm phylogeny" (1). Coincidentally, *Ephedra* (this study) and *Ginkgo biloba* (13) are the two single genera among the 170 gymnosperm species we analyzed that contain both taxoleic and ephedrenic acids in sig-

TABLE 4
Total Content of Δ5 Acids in the Seed Oil from Two *Ephedra* Species as Determined by ^{13}C NMR Spectroscopy and by GLC, and Their Content in the α and β Positions[a]

		Fatty acid content[b]					
		α and β positions				Total	
Species	Signal	Δ5 (α)[c]	Δ5 (β)	Other (α)	Other (β)	Δ5 (NMR)	Δ5 (GLC)
Ephedra nevadensis	C1	23.9	—	40.9	35.2	23.9	21.9
	C2	23.4	—	43.6	33.0	23.4	21.9
E. viridis	C1	25.9	2.5[d]	36.3	35.3	25.9 (28.4)[e]	23.1
	C2[f]	23.6	2.6[d]	40.5	31.5	23.6 (26.2)	23.1

[a]NMR, nuclear magnetic resonance; GLC, gas–liquid chromatography.
[b]NMR data, mol%; GLC data, wt%.
[c]Total Δ5 acids at the indicated position.
[d]Uncertain assignments.
[e]Total values in parenthesis include the uncertain 2.5/2.6%.
[f]Also an unassigned signal of 1.8%.

nificant amounts.

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Positional Analysis of Triglycerides and Phospholipids Rich in Long-Chain Polyunsaturated Fatty Acids

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ABSTRACT: Four sources of long-chain polyunsaturated fatty acids (LCP) differing in their chemical structure (triglycerides or phospholipids) and in their origin (tuna triglycerides, fungal triglycerides, egg phospholipids, and pig brain phospholipids) were analyzed to determine the distribution of the component fatty acids within the molecule. Lipase and phospholipase A_2 hydrolysis was performed to obtain 2-monoacylglycerols and lysophospholipids, respectively, which allowed us to determine the distribution of fatty acids between the *sn*-2 and *sn*-1,3 positions of triglycerides or between the *sn*-1 and *sn*-2 position of phospholipids. Fatty acids in the LCP sources analyzed were not randomly distributed. In tuna triglycerides, half of the total amount of 22:6n-3 was located at the *sn*-2 position (49.52%). In fungal triglycerides, 16:0 and 18:0 were esterified to the *sn*-1,3 (92.22% and 91.91%, respectively)18:1 and 18:2 to the *sn*-2 position (59.77% and 62.62%, respectively), and 45% of 20:3n-6 and only 21.64% of 20:4n-6 were found at the *sn*-2 position. In the lipid sources containing phospholipids, LCP were mainly esterified to the phosphatidylethanolamine fraction. In egg phospholipids, most of 20:4n-6 (5.50%, *sn*-2 vs. 0.91%, *sn*-1) and 22:6n-3 (2.89 vs. 0.28%) were located at the *sn*-2 position. In pig brain phospholipids, 22:6n-3 was also esterified to the *sn*-2 (13.20 vs. 0.27%), whereas 20:4n-6 was distributed between the two positions (12.35 vs. 5.86%). These results show a different fatty acid composition and distribution of dietary LCP sources, which may affect the absorption, distribution, and tissue uptake of LCP, and should be taken into account when supplementing infant formulas.

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Long-chain polyunsaturated fatty acids (LCP) play an important role in the structure and function of cellular membranes and are precursors of important lipid mediators. Moreover, some LCP, especially 22:6n-3, account for a large proportion of the polyunsaturated fatty acids in neuronal membranes. Studies with animals have shown an association between very low levels of 22:6n-3 in the retina and central nervous system and measures of visual and behavioral outcomes (1,2). Therefore, several studies have been carried out in preterm and term infants to evaluate the influence of dietary 22:6n-3, and both 20:4n-6 and 22:6n-3 on visual acuity and cognitive development (3). These reports and the observation that infants fed conventional formula have lower erythrocyte and plasma levels of the n-6 and n-3 LCP led us to consider that infants may benefit from dietary supplementation with 22:6n-3 or both 20:4n-6 and 22:6n-3.

A number of highly unsaturated dietary lipid sources are currently available for supplementing infant formulas with LCP, namely: egg yolk lipids, fish oils, and oils from unicellular organisms (i.e., fungi and algae). These lipid sources differ in their structure [triglycerides (TG) or phospholipids (PL); position of fatty acids in their backbone], fatty acid composition, and the presence of other components.

Lipase-catalyzed hydrolysis of triglycerides (TG) occurs in the mouth, stomach, and small intestine. The products of hydrolysis are free fatty acids (FFA) from the *sn*-1 and *sn*-3 positions and *sn*-2-monoacylglycerols (2-MG), which are readily absorbed, reesterified into triglycerides, and secreted into the lymph in the form of chylomicrons (4). The distribution of fatty acids in the outer and *sn*-2 positions within the molecule governs the lumenal partition between the free and 2-MG forms (5). Under normal dietary conditions, dietary PL constitute a minor portion of the PL presented to the gut for intestinal absorption; the majority are of biliary origin. Biliary PL (primary phosphatidylcholine, PC) are absorbed from the lumen as lysophosphatidylcholine (lysoPC) and are reacylated to PC for the formation of chylomicrons (6). A number of studies have focused on the importance of fatty acids esterified to the *sn*-2 position of TG (7–9). Fat and mineral absorption was higher in rat and infants fed diets containing structured triglycerides with 16:0 mainly in the *sn*-2 position (7). Linoleic acid was also better absorbed when it was esterified to the *sn*-2 position (8). Palmitic acid in the *sn*-2 position may also have some effects on cholesterol metabolism (9). Little information is available about the positional distribution of fatty acids in PL sources and its influence on the absorption and distribution of fatty acids. Recent studies carried out in preterm infants fed breast milk or formula with LCP derived from PL or from TG showed that absorption of 22:6n-3 and n-3 LCP was greater from PL-

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Abbreviations: EFA, essential fatty acids; FFA, free fatty acids; HPTLC, high-performance thin-layer chromatography; LCP, long-chain polyunsaturated fatty acids; LysoPC, lysophosphatidylcholine; LysoPE, lysophosphatidylethanolamine; 2-MG, 2-monoacylglycerols; MONO, monounsaturated fatty acids; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipids; SAT, saturated fatty acids, TG, triglycerides; TLC, thin-layer chromatography.

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LCP formula than from breast milk or TG-LCP formula (10). On the other hand, data from animal studies suggest that in the enterocytes, dietary fatty acids provided as TG are predominantly incorporated into chylomicrons, whereas dietary fatty acids provided as PL seemed to be predominantly incorporated into intestinal very low density lipoproteins. If such differences exist, the LCP source could influence the distribution and bioavailability of dietary LCP (11).

Stereospecific analysis of TG has received much attention by many authors in the last few years (12–14). Some methods involving chemical and enzymatic reactions have been reported to analyze positional fatty acid distribution. The chemical method using the Grignard degradation is one of the most reliable methods for obtaining representative diacylglycerols, but it does not give reliable results for the composition of 2-MG because of acyl migration during the reaction (13). Therefore, this method is usually combined with an alternative procedure to obtain the *sn*-2 composition, such as lipase hydrolysis, hydrolysis of PL derived from diacylglycerides, and separation of chiral isomers (15,16). This means *sn*-1, *sn*-2, and *sn*-3 fatty acid composition can be determined separately. Lipase hydrolysis does not give the complete structure of the TG molecule, but it offers reliable information about the *sn*-2 position, which is physiologically the most important. It has been reported that LCP such as 20:4n-6, 20:5n-3, and 22:6n-3 are resistant to lipase hydrolysis when linked to the outer position of the glycerol molecule (13). However, when prolonged incubation times are used, leading to nearly complete hydrolysis, representative 2-MG are obtained (12). Phospholipase A_2 hydrolysis has been used not only to study positional distribution of fatty acids in fungi (17) and bacteria (18) but also to analyze TG structure together with the Grignard reaction (12).

In the present study, we measured lipid and fatty acid composition and fatty acid distribution between the inner and outer positions of TG and PL molecules in four LCP dietary lipid sources currently available for use in infant formulas.

EXPERIMENTAL PROCEDURES

Samples. Two sources of LCP-enriched TG were analyzed: fungal TG and tuna TG. In addition, two sources of LCP-enriched PL were analyzed: one from egg lipids (egg PL) and the other from pig brain (pig brain PL).

Materials. *Rhizopus arrhizus* lipase (EC 3.1.1.3) and bee venom phospholipase A_2 (EC 3.1.1.4) were obtained from Boehringer Mannheim (Mannheim, Germany). TLC plates coated with a 0.25-mm thin layer of silica gel G60 and acetyl chloride were purchased from Sigma Chemicals Co. (St. Louis, MO). High-performance thin-layer chromatography (HPTLC) precoated silica gel 60 plates were purchased from Merck (Darmstadt, Germany).

HPTLC analysis. HPTLC was used to analyze the lipid composition of each lipid source. Plates were washed with chloroform/methanol/water (60:35:1.5, by vol) and activated by heating for 30 min at 100°C. Five µg of oils was placed on the HPTLC plates and developed using chloroform/methanol/acetic acid/water (65:50:1:4, by vol) for polar lipids (19), and hexane/isopropyl ether/acetic acid (75:25:1.5, by vol) for neutral lipids (20). After migration, plates were treated with a solution of 3% copper acetate in 8% *o*-phosphoric acid and heated at 180°C for 10 min. The relative percentage of each lipid was measured by photodensitometry in a Shimadzu CS 9000 scanning densitometer (Kyoto, Japan).

Fatty acid composition of oils. The total fatty acid compositon of the lipid sources were obtained by direct transmethylation according to the method of Lepage and Roy (21). The fatty acid composition of the main PL classes in the sources containing PL were measured after separation of species by TLC (developing solvent: chloroform/methanol/acetic acid/water, 60:40:1:2, by vol) and methylation.

Lipase hydrolysis of TG. The lipase reaction method of Fischer *et al.* (22), with minor modifications was used to hydrolyze the TG sources. Six mg of the lipid sources was sonicated for 10 min with Tris-HCl buffer (40 mM, pH 7.2) containing 50 mM sodium borate (to reduce positional migration of fatty acids) and 0.5% sodium taurocholate. One hundred to 120 units of lipase was added to the mixture and incubated at 22°C for up to 1 h with continuous shaking. The reaction was stopped by the addition of 0.5 mL 0.1 N acetic acid. Lipids were extracted from the assay with chloroform/methanol (2:1, vol/vol). After evaporation of the solvent under N_2, lipid extracts were applied to TLC plates and developed with hexane/isopropyl ether/acetic acid (75:25:1.5, by vol). Bands were visualized by iodine vapors and the band corresponding to 2-MG (*sn*-2) was scraped into test tubes. The lipid fractions were methylated as above (21).

Phospholipase A_2 hydrolysis of phospholipids. Phospholipase A_2 reaction was performed according to a modified procedure of Griffith *et al.* (23). Five mg of the sample was dissolved in 1 mL of diethylether, and 1 mL borate buffer (100 mM, pH 8.9) was added. The mixture was then sonicated for 15 min. Eighty to 200 units of phospholipase A_2 was added to the mixture and incubated with continuous shaking for 30 min at 37°C. Diethylether was then evaporated under N_2, and samples were extracted with chloroform/methanol (2:1, vol/vol). To obtain the different lipid fractions, lipid extracts were subjected to single-dimension double development with hexane/isopropanol/acetic acid (75:25:1.5, by vol) followed by chloroform/methanol/acetic acid/water (65:50:1:4, by vol). The spots were visualized with iodine vapors and the bands corresponding to lysoPC, lysophosphatidylethanolamine (lysoPE), and free fatty acids (FFA) were scraped and collected into tubes and methylated (21). Bee venom phospholipase A_2 hydrolyzes not only PC but also phosphatidylethanolamine (PE), phosphatidylinositol, and phosphatidylserine (24).

Gas–liquid chromatography. Fatty acid methyl esters were separated and quantified by gas–liquid chromatography, using a Hewlett-Packard 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) equipped with a flame-ionization detector and a 60 m × 0.32 mm internal diameter SP-2330 capillary column (Supelco, Bellefonte, PA). Nitrogen at a flow rate of 1 mL/min was used as the carrier gas, and the

split ratio was 1:40. Temperature programming started at 165°C for 3 min, then was raised 2°C/min to 195°C, held for 2 min at 195°C, increased again at 3°C/min to 211°C, held for 6 min at 211°C, and then decreased at 15°C/min to return to 165°C. The injector and detector temperatures were 275°C. Peaks were identified by comparison with known standards (Sigma, St. Louis, MO).

Calculation. Results were expressed as the means of three replicates in mole percentage of total fatty acids. The fatty acid composition of the *sn*-1,3 position in tryglycerides was calculated from the overall fatty acid composition, and from that of the *sn*-2 position, using the formula: [*sn*-1,3] = 1.5[TG] − 0.5[*sn*-2]. The fatty acid composition of the *sn*-2 position in PL was calculated from the overall fatty acid composition of each PL class and from that of the *sn*-1 position using the formula: [FFA] = 2[PL] − [LysoPL]. The contribution of each PL class to the FFA fractions was corrected by their percentage in the original source. Both *sn*-1,3 in TG and *sn*-2 in PL were calculated from the other positions because they were obtained in the FFA fraction after the corresponding hydrolysis. We verified that, by means of structured standards, the composition of this fraction was less reliable because it was contaminated by acyl migration.

RESULTS AND DISCUSSION

Characterization of the lipid sources. The percentage of each lipid fraction in the original dietary lipid sources was assessed by HPTLC. Tuna TG and fungal TG were composed mainly of TG (>99%), and therefore were subjected to lipase hydrolysis without previous purification. Egg PL was composed of 86.76% PC and 11.13% PE. Pig brain PL was a complex mixture of PL in which PC and PE represented 24.43 and 43.75% of the total PL, respectively.

The overall fatty acid compositions of these lipid sources are shown in Figure 1 and in Tables 1–3. Tuna TG were characterized by a high content of 22:6n-3 and a low ratio of 20:5n-3 to 22:6n-3 (0.29). Saturated and monounsaturated fatty acids were also present, with percentages exceeding 30 and 20% mol of the total fatty acids, respectively. On the contrary, fungal TG contained a high proportion of 20:4n-6, without detectable amounts of 22:6n-3. The availability of oils containing a high proportion of n-6 and n-3 LCP, separately, provides the advantage of varying the n-6/n-3 ratio in infant formulas. Egg PL had a 20:4n-6/22:6n-3 ratio of 1.36. Pig brain PL contained a higher proportion of LCP compared to the egg PL source, with a 20:4n-6/22:6n-3 ratio of 1.31.

Positional analysis of TG. Table 1 gives the fatty acid composition of the TG molecule in the lipid sources containing LCP and that of the *sn*-2 and *sn*-1,3 positions. Likewise, Figure 2 shows the percentages of the total amount of selected fatty acids which were located at the *sn*-2 position (calculated according to the formula % *sn*-2 = [(*sn*-2) × 100]/3 TG). If random distribution among the three positions of the TG molecule was expected, 33.33% of the total amount of a considered fatty acid would be located at the *sn*-2 position. How-

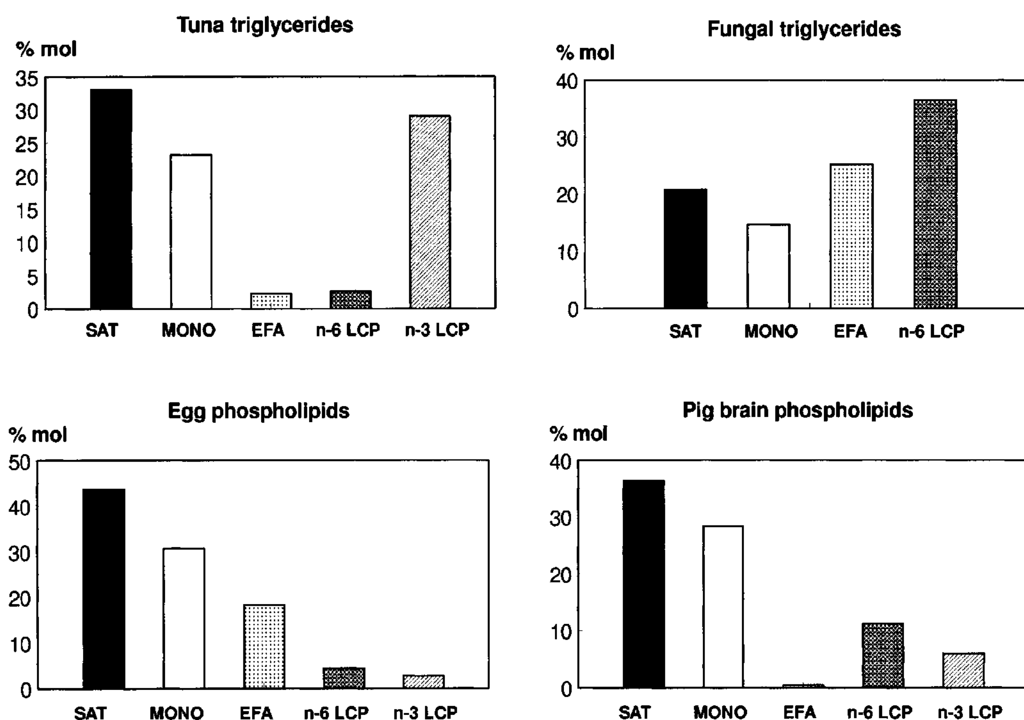

FIG. 1. Fatty acid profile of long-chain polyunsaturated fatty acid (LCP)-enriched dietary lipid. SAT, saturated fatty acids; MONO, monounsaturated fatty acids; EFA, essential fatty acids.

TABLE 1
Fatty Acid Composition of Intact Triglycerides, *sn*-2 and *sn*-1,3 Positions of Triglycerides in LCP-Enriched Dietary Lipid Sources (mol %)[a]

	Tuna TG			Fungal TG		
Fatty acid	TG	*sn*-2	*sn*-1,3	TG	*sn*-2	*sn*-1,3
14:0	4.53	5.12	4.24	—	—	—
16:0	23.25	26.06	21.84	14.49	338	20.05
18:0	5.29	4.66	5.61	6.26	1.52	8.64
18:1n-9	23.21	12.59	28.52	14.65	26.27	8.84
18:2n-6	1.68	2.8	1.12	22.84	42.91	12.8
18:3n-6	0.25	—	0.37	2.5	2.28	2.6
18:3n-3	0.6	0.94	—	2.39	2.64	2.26
18:4n-6	1.48	1.28	1.57	—	—	—
20:2n-6	0.33	—	0.49	0.42	0.54	0.36
20:3n-6	0.21	—	0.32	3.25	4.5	2.63
20:4n-6	2.1	3.12	1.59	28.37	18.42	33.35
20:5n-3	6.53	5.15	6.79-	—	—	—
22:4n-6	0.3	0.59	0.16	4.18	—	6.27
24:1n-9	0.94	1.31	0.76	—	—	—
22:6n-3	22.54	31.13	18.24	—	—	—

[a]TG, fatty acid composition of the total triglyceride; *sn*-2, fatty acid composition of the *sn*-2 monoglycerides obtained after lipase hydrolisis; *sn*-1,3, fatty acid composition of *sn*-1 and *sn*-3 positions obtained as followed [*sn*-1,3] = 1.5[TG] – 0.5[*sn*-2]; LCP, long-chain polyunsaturated fatty acids.

ever, the *sn*-2 position in tuna TG contained 49.52% of the total 22:6n-3. Eicosapentaenoic acid and palmitic acid were almost equally distributed between the *sn*-2 and *sn*-1,3 positions. In fungal TG, 16:0 and 18:0 were mainly esterified to the *sn*-1,3 positions, whereas the opposite was found for 18:1 and 18:2. Forty-five percent of 20:3n-6 and only 21.64% of 20:4n-6 were found at the *sn*-2 position.

Only one work is available on the stereospecific distribution of highly unsaturated oils (12). This study showed the analysis of six polyunsaturated oils containing triglycerides, two of which were quite similar in the total fatty acid and *sn*-2 fatty acid composition to the tuna TG and fungal TG reported here, and were also of tuna and fungal origin.

It has been reported that polyunsaturated fatty acids are distributed within the TG molecule of human milk in a highly specific manner, with 20:4n-6 and 22:6n-3 being located at the *sn*-2 and *sn*-3 positions (13). Although LCP linked to the outer position of the TG molecule are resistant to pancreatic hydrolysis, breast-fed infants may efficiently hydrolyze TG containing LCP due to gastric lipase and bile salt-stimulated lipase. In contrast to human milk, infant formulas do not contain any lipase activity, and consequently the hydrolysis of the *sn*-1 and *sn*-3 ester bonds of TG may decline. In the case of appropriate lipase hydrolysis, LCP absorbed as 2-MG or FFA may undergo different metabolic processes (25). Some researchers suggest a possible substantial blood absorption pathway for polyunsaturated fatty acids with reference to their hydrosolubility properties (26). On the other hand, it has been reported that 20:5n-3 and 22:6n-3 were more readily and efficiently absorbed when the TG administered had a specific intramolecular structure, with medium-chain fatty acids located in the *sn*-1 and *sn*-3 positions and n-3 LCP located in the *sn*-2 position, compared with TG having a random fatty acid distribution among the three positions (27). Our results showed that nearly 50% of 22:6n-3 and more than 20% of 20:4n-6 were esterified to the *sn*-2 position. Fatty acids in the *sn*-2 position may have some unique properties in terms of facilitating fatty acid intestinal absorption and improving

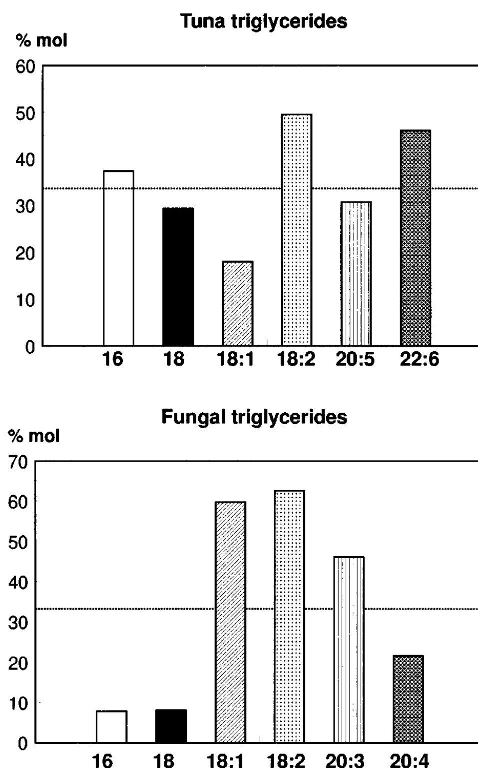

FIG. 2. The *sn*-2 fatty acid composition in triglyceride LCP-enriched lipid sources. The horizontal broken line indicates the proportion of each fatty acid expected (33.33%) on the basis of random distribution. See Figure 1 for abbreviation.

TABLE 2
Fatty Acid Composition of Intact PL, PC, and PE Fractions and the Corresponding Lysophospholipids and Free Fatty Acids in Egg PL (mol%)[a]

				sn-1[b]		
Fatty acids	PL	PC	PE	LysoPC	LysoPE	*sn*-2[c]
16:0	31.56	40.27	23.04	70.49	36.18	9.8
16:1n-9	1.21	1.03	0.7	0.34	—	1.64
18:0	12.1	13.11	26.6	26.26	58.62	0.63
18:1n-9	29.44	27.1	19.17	2.52	2.88	48.65
18:2n-6	17.73	14.75	13.43	0.19	0.77	28.29
18:3n-3	0.56	0.26	0.38	0.1	0.36	0.41
20:2n-6	0.25	0.25	0.34	—	—	0.43
20:3n-6	0.26	0.18	0.42	—	—	0.31
20:4n-6	3.73	1.96	9.26	—	0.28	5.5
22:4n-6	0.22	—	1.06	—	—	0.25
22:6n-3	2.74	1.08	5.6	0.1	0.91	2.89

[a]PL, PC and PE: fatty acid composition of the total phospholipids, phosphatidylcholine and phosphatidylethanolamine fractions, respectively.
[b]Fatty acid composition of the *sn*-1 position obtained from the corresponding lysophospholipid fraction (lysoPC and lysoPE) after phospholipase A_2 hydrolysis.
[c]Fatty acid composition of the *sn*-2 position obtained as follows [FFA]=2[PL] − [LysoPL].

mineral balance (7). However, the influence of this fatty acid distribution on LCP absorption and metabolic fate when supplementing infant formulas needs further research.

Positional analysis of phospholipids. Tables 2 and 3 give the fatty acid composition of intact PL, the fatty acid composition of the major PL fractions, and the fatty acid composition of the products after phospholipase A_2 hydrolysis in egg PL and pig brain PL. The fatty acid composition of lysoPC and lysoPE represents that of the *sn*-1 position in PC and PE, respectively. The fatty acid composition of FFA represents that of the *sn*-2 position in the complete PL. As shown in Tables 2 and 3, the PC fraction contained more 16:0 and 18:1n-9 and less 18:0 than PE in PL from eggs. In pig brain PL, PC contained more 16:0, 18:1n-9, and 18:0 than PE. LCP were mainly esterified to the PE fraction of PL sources, both from egg and pig brain, and were located at the *sn*-2 positions, because they were nearly absent from lysophospholipids. Arachidonic acid in pig brain PL was also present in the *sn*-1 position. On the contrary, saturated fatty acids were mainly esterified to the *sn*-1 position, but oleic acid to the *sn*-2. These results agree with the general assumption that, in tissues, saturated fatty acids were linked to the *sn*-1 and polyunsaturates to the *sn*-2 position of PL.

TABLE 3
Fatty Acid Composition of Intact PL, PC, and PE Fractions and the Corresponding Lysophospholipids and Free Fatty Acids in Pig Brain PL (mol%)[a]

				sn-1[a]		
Fatty acids	PL	PC	PE	LysoPC	LysoPE	*sn*-2[b]
16:0	15.85	45.5	5.01	51.6	12.69	13.76
16:1n-9	0.57	0.41	—	—	—	0.36
18:0	20.54	21.37	14.38	36.97	60.62	—
18:1n-9	23.51	2.36	21.77	11.43	—	49.66
18:2n-6	0.47	0.82	—	—	—	—
18:3n-3	0.54	0.08	1.25	—	3.59	—
20:4n-6	7.43	1.19	10.97	—	5.86	12.35
24:0	1.49	—	—	—	—	—
22:4n-6	2.96	—	9.44	—	3.72	10.75
24:1n-9	4.28	0.85	1.8	—	13.25	—
22:6n-3	5.66	0.42	9.2	—	0.27	13.20
DMA 16:0	3.36	—	6.18	—	[c]	—
DMA 18:0	4.9	—	8.19	—	[c]	—
DMA 18:1n-9	2.72	—	4.16	—	[c]	—
DMA 18:1n-7	3.91	—	5.92	—	[c]	—

[a]Fatty acid composition of the *sn*-1 position obtained from the corresponding lysophospholipid fraction (lysoPC and lysoPE) after phospholipase A_2 hydrolysis.
[b]Fatty acid composition of the *sn*-2 position obtained as follows: [FFA] = 2[PL] − [lysoPL].
[c]Lyso(alkenyl)PE was lost on the thin-layer chromatography plate. For abbreviations see Table 2.

As far as we know, no study has been made about the positional distribution of PL in sources currently used in infant nutrition. Henderson *et al.* (18) and Kendrick and Ratledge (17) used similar methods to analyze the positional distribution of fatty acids in PL isolated from species of bacteria and fungi which contained LCP. Phospholipids enriched in LCP are an available source of these fatty acids and some of them are used currently to supplement infant formulas. Data of animal studies suggest that in the enterocyte, dietary fatty acids provided as TG are predominantly incorporated into chylomicrons. On the contrary, fatty acids provided as PL seem to be predominantly incorporated into very low density particles synthetized at the enterocytes (28). It has been recently reported that infants fed LCP-supplemented formula from egg PL absorbed 22:6n-3 as efficiently as did breast-fed infants (10,11), and better than did infants fed a formula containing LCP-TG (10).

In conclusion, there are many differences in the chemical structure and fatty acid composition and distribution of the currently available dietary sources of LCP for infant formulas. These differences may affect the absorption, distribution, and tissue uptake of these important fatty acids and should be taken into account when supplementing infant formulas.

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Preparation, Separation, and Confirmation of the Eight Geometrical *cis/trans* Conjugated Linoleic Acid Isomers 8,10- Through 11,13-18:2

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ABSTRACT: Conjugated linoleic acid (CLA) mixtures were isomerized with *p*-toluenesulfinic acid or I_2 catalyst. The resultant mixtures of the eight *cis/trans* geometric isomers of 8,10-, 9,11-, 10,12-, and 11,13-octadecadienoic (18:2) acid methyl esters were separated by silver ion–high-performance liquid chromatography (Ag^+–HPLC) and gas chromatography (GC). Ag^+–HPLC allowed the separation of all positional CLA isomers and geometric *cis/trans* CLA isomers except 10,12-18:2. However, one of the 8,10 isomers (8*cis*,10*trans*-18:2) coeluted with the 9*trans*,11*cis*-18:2 isomer. There were differences in the elution order of the pairs of geometric CLA isomers resolved by Ag^+–HPLC. For the 8,10 and 9,11 CLA isomers, *cis,trans* eluted before *trans,cis*, whereas the opposite elution pattern was observed for the 11,13-18:2 geometric isomers (*trans,cis* before *cis,trans*). All eight *cis/trans* CLA isomers were separated by GC on long polar capillary columns only when their relative concentrations were about equal. Large differences in the relative concentration of the CLA isomers found in natural products obscured the resolution and identification of a number of minor CLA isomers. In such cases, GC–mass spectrometry of the dimethyloxazoline derivatives was used to identify and confirm coeluting CLA isomers. For the same positional isomer, the *cis,trans* consistently eluted before the *trans,cis* CLA isomers by GC. High resolution mass spectrometry (MS) selected ion recording (SIR) of the molecular ions of the 18:1, 18:2, and 18:3 fatty acid methyl esters served as an independent and highly sensitive method to confirm CLA methyl ester peak assignments in GC chromatograms obtained from food samples by flame-ionization detection. The high-resolution MS data were used to correct for the nonselectivity of the flame-ionization detector.

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The distribution of conjugated linoleic acid (CLA) isomers was examined in a number of natural products, including milk, cheese, meat, adipose tissue, and tissues from animals fed commercial CLA mixtures, using the newly developed silver ion–high-performance liquid chromatography (Ag^+–HPLC) system (1–4). The resolution of the CLA isomers was further enhanced by using up to six Ag^+–HPLC columns in series (5). The initial results indicated that Ag^+–HPLC resolved positional *cis/trans* CLA isomers, but not pairs having the same double-bond positions with opposite geometrical configuration. In cheese lipids, however, we observed what appeared to be two 11,13-octadecadienoic (18:2) acids with *cis,trans* and *trans,cis* double-bond configurations through gas chromatography–electron ionization mass spectrometry (GC–EIMS) (4).

It is occasionally difficult to match the Ag^+–HPLC data based on ultraviolet (UV) detection at 234 nm with GC results obtained using 100-m polar capillary columns and a flame-ionization detector (FID) for some lipid extracts. Extraneous peaks may arise in the Ag^+–HPLC chromatogram from conjugated fatty acids of different chain lengths eluting in the same region (5), or in the GC chromatogram from non-CLA fatty acid methyl esters (FAME). These two methods are complementary, but peculiar results should be thoroughly investigated using additional techniques to avoid misidentifications.

To confirm the identity of positional and geometrical CLA isomers, we have prepared all possible *cis/trans* CLA isomers of 8,10-, 9,11-, 10,12- and 11,13-18:2 by reaction of a commercial CLA mixture with *p*-toluenesulfinic acid (6) or I_2 (7). The mixture of isomers was then evaluated by GC and Ag^+–HPLC under optimal chromatographic conditions. High resolution GC–EIMS was used to deal with the problem of extraneous peaks in GC–FID chromatograms.

MATERIALS AND METHODS

A commercial CLA mixture containing four major positional CLA isomers as their methyl esters was purchased from Nu-Chek-Prep Inc. (Elysian, MN). The methyl esters of 9*cis*,11*trans*-18:2 (98%), 10*trans*,12*cis*-18:2 (98%) and technical grade 9*cis*,11*trans*-18:2 (75–78%) were obtained from

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Abbreviations: Ag^+–HPLC, silver ion–high-performance liquid chromatography; *cis/trans*, refers to all CLA isomers with either a *cis,trans* or a *trans,cis* configuration; CLA, conjugated linoleic acid; DMOX, 4,4-dimethyloxazoline; FAME, fatty acid methyl esters; FID, flame-ionization detector; GC–EIMS, gas chromatography–electron ionization mass spectrometry; SIR, selected ion recording; UV, ultraviolet.

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Matreya Inc. (Pleasant Gap, PA). *p*-Toluenesulfinic acid, I_2, and 2-amino-2-methyl-1-propanol (95%) were purchased from Aldrich Chemical Company Inc. (Milwaukee, WI). Hexane and acetonitrile were UV grade.

The 10*trans*,12*cis*-18:2 FAME was reacted with *p*-toluenesulfinic acid as previously described (6). Each of the commercial mixtures of CLA isomers and 9*cis*,11*trans*-18:2 was isomerized in the presence of I_2 as catalyst (7). Briefly, the CLA isomers were dissolved in hexane, a crystal of I_2 was added, and the mixture was allowed to stand in sunlight for 5 h. The reaction products were washed with 0.1 M sodium thiosulfate solution and the hexane solution was washed with water, dried with sodium sulfate, followed by removal of hexane using a rotary evaporator.

Ag^+–HPLC analyses were performed using a Waters 510 solvent delivery system (Waters Associates, Milford, MA), a Waters 717 plus Autosampler with a 200 µL injection loop, a Waters 486 tunable absorbance UV detector set at 234 nm, and a Waters software program (Millennium™ version 2.15.01). ChromSpher 5 Lipids analytical (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size) silver-impregnated columns were used (Chrompack, Bridgewater, NJ). The solvent flow was set at 1.0 mL/min. The best resolutions were obtained with isocratic operation and a mobile phase of 0.1% acetonitrile in hexane. The mobile phase mixture was prepared fresh each day, stirred vigorously before use, and stirred continuously with a magnetic stirrer. The flow was started for 0.5 h prior to sample injection. Typical injection volumes were 5–15 µL.

The GC (model 5890; Hewlett-Packard, Palo Alto, CA) was equipped with a flame-ionization detector (FID) and an automatic injection system (model 1090, Hewlett-Packard). Analyses were performed using a fused-silica capillary column (CP-Sil 88; 100 m × 0.25 mm i.d. × 0.2 µm film thickness; Chrompack Inc., Raritan, NJ). The column was held at 70°C for 4 min after injection, the temperature was programmed at 13°C/min to 175°C, held at 175°C for 27 min, then temperature programmed at 4°C/min to 215°C, and held at 215°C for 31 min. Hydrogen was used as the carrier gas, at a split ratio of 20:1. The detector and injector were set at 250°C.

A high-resolution mass spectrometer (Autospec Q, Micromass, Wythenshawe, England) coupled through a line-of-sight interface to a GC (model 5890 series II GC, Hewlett-Packard) provided GC–MS data. The static resolution of the instrument was adjusted to 10,000 at the *m/z* 293 ion of perfluorokerosene. Data acquisition recorded the selected ion profiles of *m/z* 292.2402, 294.2559, 296.2715 for 200 ms per scan using *m/z* 292.9824 of perfluorokerosene as the lock mass. Total scan time was 0.8 s/scan. Data were acquired from 35 to 60 min during the GC–MS analysis.

The hydrogen carrier gas at a head pressure of 25 psi resulted in a measured linear velocity of 35 cm/s through a 100 m × 0.2 mm i.d. 0.2 µ phase cyanopropyl polysiloxane capillary column (CP-Sil 88, Chrompack) with the septum purge set at 3 mL/min and the split flow at 100 mL/min. Injector and transfer line temperatures were 220°C, while the ion source temperature was 250°C. The GC oven was initially held at 75°C for 2 min after splitless injection. This was followed by temperature programming to 180°C at 5°/min, held at 180° for 30 min and then increased to 220°C at 5°/min, and held at 220°C for 20 min.

RESULTS AND DISCUSSION

Isomerization of CLA. Both *p*-toluenesulfinic acid and I_2-catalyzed isomerization of CLA led to the formation of extensive *trans,trans* and all possible *cis,trans* and *trans,cis* 8,10-, 9,11-, 10,12-, and 11,13-18:2 isomers; the ratio of the two geometric *cis/trans* isomers formed was 1:1 (Table 1). The *p*-toluenesulfinic acid-catalyzed isomerization conditions (dioxane, 105°C) resulted in less than 0.5% double-bond migration (6). The I_2-catalyzed isomerization reaction was generally complete after 2 h (Fig. 1). Thereafter, the isomer distribution remained unchanged. Isomerization led to a marked increase in the *trans,trans* content at the expense of both the *cis/trans* and *cis,cis* CLA isomers. For example, the composition of the original 75–78% technical grade 9*cis*,11*trans*-18:2 from Matreya Inc. was 79.2% *cis/trans*, 15.1% *cis,cis,* and 5.7% *trans,trans*. After isomerization, the composition of the product mixture was 13.4% *cis,trans*, 13.4% *trans,cis*, 2.4% *cis,cis*, and 70.8% *trans,trans*. Similar changes in CLA isomer distribution were observed with the other CLA mixtures investigated.

Ag^+–HPLC separation of the isomerized CLA mixture. Ag^+–HPLC, using three columns in series, separated the 11,13-, 9,11-, and 8,10-18:2, but not the 10,12-18:2 geometric isomers (Fig. 2B) in contrast to the original CLA mixture known to contain only four *cis/trans* CLA isomers (1–5) shown in Figure 2A. Using up to six Ag^+–HPLC columns in series gave essentially the same elution sequence as that observed using three Ag^+–HPLC columns, except with improved resolution; the 10,12-18:2 geometric isomers still remained unresolved (data not shown). The separation of the 9,11-18:2 geometric isomers and the lack of separation of the 10,12-18:2 geometric isomers were further demonstrated by

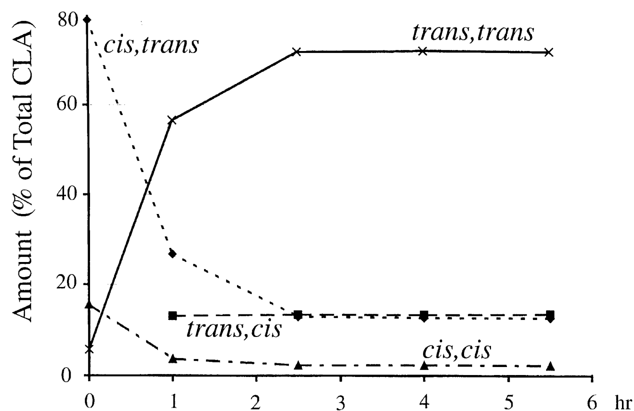

FIG. 1. Variation in the relative concentrations of the 9,11-18:2 geometric isomers during I_2-catalyzed isomerization. Equilibrium was reached in less than 3 h.

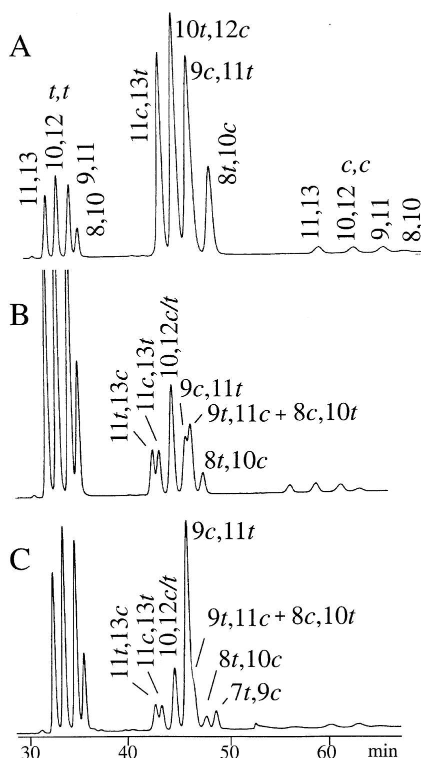

FIG. 2. Silver ion–high performance liquid chromatographic (Ag$^+$–HPLC) separation of a commercial conjugated linoleic acid (CLA) fatty acid methyl ester (FAME) mixture (A) before, and (B) after isomerization using three Ag$^+$–HPLC columns. (C) The same isomerized commercial CLA FAME mixture spiked with the total FAME mixture from a cheese test sample.

isomerization followed by Ag$^+$–HPLC analysis of the individual commercially available CLA isomers (data not shown). Individual 8,10- and 11,13-18:2 could not be tested separately, because these CLA isomers were not commercially available. The separation of the 8,10-18:2 geometric isomers was demonstrated by quantitative consideration of the Ag$^+$–HPLC chromatogram (Table 1). Isomerization resulted in an equal abundance of geometric *cis/trans* CLA isomers from any given pair of positional CLA isomers; the sum of both geometric *cis/trans* isomers compared well with the relative composition present in the original CLA mixture. The exception was 8,10-18:2. One of the 8,10-18:2 *cis/trans* isomers was well resolved and present at only half the relative concentration in the original mixture, whereas the 9*trans*,11*cis*-18:2 peak was greater by the amount of half the 8,10-18:2 isomer concentration (Table 1). This clearly suggested that one of the 8,10-18:2 *cis/trans* isomers coeluted with 9*trans*,11*cis*-18:2.

The elution order of the geometric *cis/trans* CLA pairs was established by comparison of the isomerized mixture (Fig. 2B) with the original CLA mixture (Fig. 2A) and by co-injection of the isomerized commercial CLA mixture with cheese lipids (Fig. 2C). The 9*cis*,11*trans*-18:2 isomer, known to be the major CLA isomer in cheese (3,4,9), eluted before the opposite 9*trans*,11*cis*-18:2 isomer (Fig. 2C). The two geometric *cis/trans* isomers of 11,13-18:2 also separated as two peaks,

TABLE 1
Relative Concentration of *cis/trans* CLA Isomers in the Commercial CLA Preparation and Its Isomerized Products

CLA isomer	Isomerized CLA	Commercial CLA
11*trans*,13*cis*	0.121	
11*cis*,13*trans*	0.122	
Total 11,13	0.244	0.238
Total 10,12	0.320	0.322
9*cis*,11*trans*	0.159	
9*trans*,11*cis*	0.213[a]	
Total 9,11	0.372	0.312
8*trans*,10*cis*	0.064	0.128

[a]The contribution of the 9*trans*,11*cis* is 0.149 if the assumed contribution for 8*cis*,10*trans*-18:2 of 0.064 (as indicated by the relative concentration of 8*trans*,10*cis*-18:2) is subtracted from the found relative concentration (0.213 – 0.064 = 0.149). CLA, conjugated linoleic acid.

but the elution order was reversed compared to that of the 9,11-18:2 pair. The second 11,13-18:2 peak corresponded to 11*cis*,13*trans*-18:2 shown previously to be present in commercial CLA preparations (1–5; see also Fig. 2), while the first eluting 11,13-18:2 peak, 11*trans*,13*cis*-18:2, was generally more abundant in natural dairy products (4). The identity of the resolved 8,10-18:2 isomer was 8*trans*,10*cis*-18:2 based on comparison to the commercial CLA mixture which is known to contain this geometric isomer (1–5). The other 8,10 geometric *cis/trans* isomer, 8*cis*,10*trans*-18:2, coeluted with 9*trans*,11*cis*-18:2 (Table 1). Therefore, the elution order of the 8,10- and 9,11-18:2 geometric *cis/trans* isomers (*cis,trans* before *trans,cis*) was observed to be opposite to the elution order of the 11,13-18:2 geometric *cis/trans* isomers (*trans,cis* before *cis,trans*). The reason for the difference in elution order of the geometric *cis/trans* CLA isomers, or the nonresolution of the 10,12-18:2 isomer under identical conditions is not known. There may be a structural difference, which is reminiscent of the alternating physical properties, such as melting point, of monounsaturated fatty acids (10).

In a recent report, an opposite elution order for the 9,11-18:2 *cis/trans* isomers was shown using two Ag$^+$–HPLC columns and a mobile phase of 0.5% acetonitrile in hexane (8). These two separations differed essentially in the concentration of acetonitrile. Furthermore, a claim was made in that report (8) that the resolution of CLA isomers could be improved by increasing the acetonitrile in hexane. Both concerns were investigated further.

Increasing the content of acetonitrile to 0.2, 0.3, and 0.4% reduced the retention time (volume) for the *cis/trans* CLA isomers from 40 min to 23, 19, and 16 min, respectively, using three Ag$^+$–HPLC columns in series. The peaks became sharper but their resolution was degraded. At 0.2% acetonitrile, the *cis/trans* 11,13-18:2 isomers did not resolve any longer, and at 0.3% acetonitrile, the *cis/trans* 9,11-18:2 isomer pair appeared as one broad peak (data not shown). Reducing the acetonitrile content to 0.05% improved the separation of the 11,13-18:2 pair but not that of the 9,11-18:2 pair, with a concomitant doubling of the elution time. There was no indication of a reversal of the elution sequence of the 9,11-18:2 *cis/trans* isomers with changes in the content of acetoni-

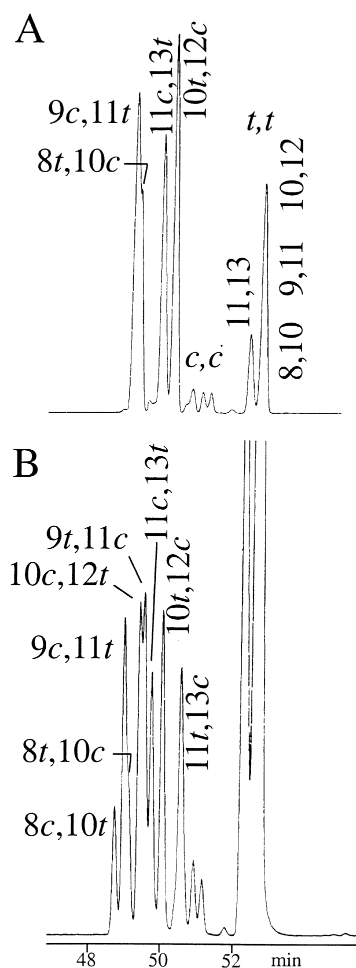

FIG. 3. Partial gas chromatographic profile of the CLA region of a commercial CLA FAME mixture (A) before and (B) after isomerization. For abbreviations see Figure 2.

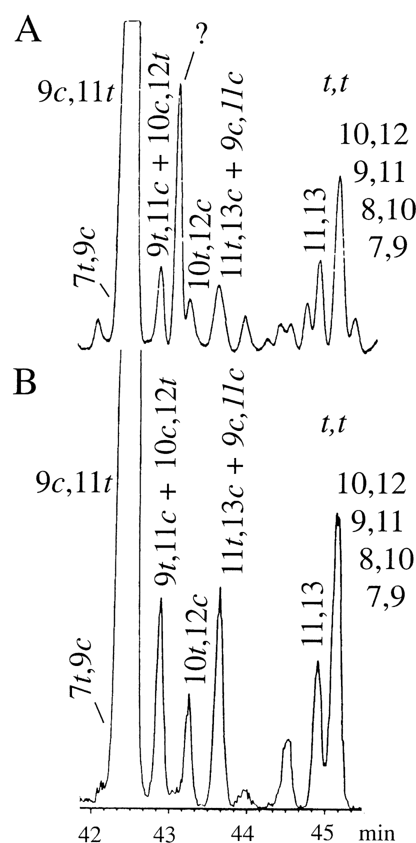

FIG. 4. Partial gas chromatographic profile of the CLA FAME region for a cheese test sample (A) obtained using flame-ionization detection, and (B) analyzed by high-resolution selected ion recording of the molecular ion of CLA FAME at *m/z* 294.2559. For abbreviations see Figure 2.

trile in the mobile phase, and as indicated previously (1–5), the 0.1% acetonitrile in the hexane solvent system was found to give the best resolution of CLA isomers.

GC separation of the isomerized CLA mixture. A comparison of the original and isomerized CLA mixture separated by GC using a 100-m CP-Sil 88 fused-silica capillary column is shown in Figures 3A and 3B, respectively. The individual CLA isomeric peaks are labeled in Figure 3. As is evident, the total *trans,trans* content was high in the isomerized mixture, and the peak attributed to 11,13-18:2 separated from that of the remaining three coeluting *trans,trans* CLA isomers (8,10-18:2, 9,11-18:2, and 10,12-18:2).

The individual CLA isomers were identified by coinjection with known CLA isomers. Coinjection of the isomerized mixture with cheese lipids identified 9*cis*,11*trans*-18:2. Isomerized 9,11-18:2 and 10,12-18:2 were coinjected separately with the total isomerized CLA mixture to confirm their identities. The identities of all the CLA positional isomers were also confirmed by GC–EIMS after derivatization to 4,4-dimethyloxazolines (DMOX). Characteristic mass spectral patterns were previously published for CLA DMOX isomers (1,3,4). The first-eluting CLA isomer was established as 8,10-18:2, while 11*trans*,13*cis*-18:2 coeluted with the minor *cis,cis* CLA isomers in the mixture (see Fig. 3B). For all geometric pairs, the *cis,trans* eluted before the *trans,cis* CLA isomer. These results confirm previous reports (8) and the prediction of the GC elution order of CLA isomers (4).

Inspection of Figure 3B indicates that the relative amounts of the *cis/trans* geometric isomers in the isomerized commercial CLA mixture separated by GC were fairly similar. By contrast, the corresponding distributions found for natural products were vastly different, as shown for cheese in Figure 4A. The large abundance of 9*cis*,11*trans*-18:2 in natural products obscured the possible presence of adjacent minor CLA isomers, such as 8*trans*,10*cis*-18:2 and 7*trans*,9*cis*-18:2 (3). Furthermore, the higher Δ CLA *cis/trans* isomers would elute in the retention time window of the minor *cis,cis* CLA isomers, as observed for 11*trans*,13*cis*-18:2 (Figs. 3 and 4). In cases where CLA isomers were obscured by the large abundance of another CLA isomer, GC–EIMS of the DMOX derivatives with reconstructed ion chromatograms was used to identify and confirm coeluting CLA isomers (3,4).

The occurrence of non-CLA FAME in the CLA methyl

ester region of the GC is a concern in the identification of CLA isomers. Generally, this region of the gas chromatogram is relatively free of other FAME, except for 21:0 (2) and an unknown peak eluting just after 9*trans*,11*trans*-18:2 (Adlof, R.O., unpublished results). We recently observed, however, a peak by FID in the GC chromatogram of cheese lipids, which we tentatively identified as 11*trans*,13*cis*-18:2 (4). We were unable to relate this large FID response to any peak in the Ag^+–HPLC chromatogram, and therefore placed a question mark next to that GC–FID peak (Fig. 4A) (4). We have subsequently developed a high resolution mass spectrometric method to distinguish between CLA and non-CLA signals in the CLA methyl ester region.

High-resolution MS. High-resolution selected-ion recording (SIR) is a useful tool to verify GC peak assignments based on nonselective FID data. The high-resolution SIR mode of GC–MS analysis increases the sensitivity of the instrument for the monitored ions more than 80-fold vs. the signals recorded for the same ions at low resolution with mass scans of 368 to 66 Da at 1 s/decade. This technique is suitable for the analysis of the same test portions examined by GC–FID. A high-resolution SIR of the FAME mixture from the same cheese test sample shown in Figure 4A is presented in Figure 4B. The molecular ion of CLA FAME at *m/z* 294.2559 was used. Comparison of Figures 4A and 4B indicates that the peak identified in the FID data with a question mark was not a CLA methyl ester. The other CLA isomers in the GC–FID trace of cheese FAME were found to have been correctly identified (4).

For the purpose of this GC–MS check on FID detection, the high-resolution SIR data record is intentionally limited to molecular ion information to maximize sensitivity. Thus, the potential for misinterpretation of the SIR data does exist. The molecular ions of 18:0, 18:1, 18:2, 18:3, and 18:4 congeners contribute, to a limited extent, to each other's signals with isotope cluster contributions and losses of H_2. The contributions of 18:1 and 18:3 to any 18:2 signal are recognized by comparing the absolute abundances of the *m/z* 292, 294, and 296 signals. A more interesting *m/z* 294 response is provided by artifacts of methylation with acid catalyst (11). The artifacts elute just beyond the CLA methyl ester region and fragment in several ways, including expulsion of the elements of methanol, to form *m/z* 294.

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Synthesis of 9*Z*,11*E*-Octadecadienoic and 10*E*,12*Z*-Octadecadienoic Acids, the Major Components of Conjugated Linoleic Acid.

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ABSTRACT: Linoleic acid was efficiently converted into the two major components of conjugated linoleic acid, 9*Z*,11*E*-octadecadienoic (**1a**) and 10*E*,12*Z*-octadecadienoic acid (**1b**) using either the superbase (*n*-butyllithium/potassium *tert*-butoxide) or by simply refluxing with KOH in 1-butanol. In turn, **1a** and **1b** were separated from each other using the lipase from *Aspergillus niger via* stereoselective esterification in 1-butanol. This enzyme has a preference for the 9*Z*,11*E* isomer, **1a**, and has excellent selectivity. This method has allowed the ready preparation of gram quantities of **1a** and **1b** in their highly purified forms, which are not readily accessible by current methods.

Paper no. L8215 in *Lipids 34,* 879–884 (August 1999).

Conjugated linoleic acid (CLA) is a general term used to describe a mixture of octadecadienoic acids (18:2) that possess a conjugated double-bond system. In 1987 Pariza and coworkers (1) at the University of Wisconsin isolated an anticarcinogenic principle from extracts of grilled ground beef and established that it consisted of a series of conjugated dienoic isomers of linoleic acid. Since that time, the mixture has been referred to as CLA. In the last decade, interest in CLA has risen with reports that dietary CLA reduced atherosclerosis (2), carcinogenesis (3–5), and body fat (6) in laboratory animals. However, commercial synthetically derived CLA samples, prepared *via* alkali isomerization of linoleic acid (7), are a complex mixture of CLA isomers, some of which still remain to be identified. Consequently, at the present time, there is no definitive evidence as to which isomers are the biologically active components and whether different isomers are responsible for different bioactivities.

Using silver ion-impregnated high-performance liquid chromatography (HPLC), Sehat *et al.* (8) reported a clear separation of CLA isomers into three groups related to their *E,E*; *Z,E* or *E,Z*; and *Z,Z* configurations of the conjugated double-bond system. Application of this method to the analysis of a commercial CLA mixture revealed the presence of 12 isomers. The four major positional isomers—11,13; 10,12; 9,11; and 8,10—with *Z,E* and/or *E,Z* double-bond configurations accounted for 92.5% of the mixture. To establish which isomers are associated with which types of biological activities, it is necessary to prepare the CLA isomers in their pure forms for in-depth biological studies. In a preliminary communication (9), we recently disclosed a chemoenzymatic method for the synthesis of two of the major components, 9*Z*,11*E*, **1a**, and 10*E*,12*Z*, **1b**, of CLA (10). We now report the experimental details which allowed the successful preparation of these two major CLA components, free of geometric isomers.

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Abbreviations: CLA, conjugated linoleic acid; HPLC, high-performance liquid chromatography; NMR, nuclear magnetic resonance; THF, tetrahydrofuran.

MATERIALS AND METHODS

Linoleic acid (99%) and an isomeric mixture of CLA were purchased from Nu-Chek-Prep, Inc. (Elysian, MN). *Candida rugosa* (MY) and *Alkaligenes* sp. (PL) lipases were products of Meito Sangyo Ltd. (Nagoya, Japan). Novozym 435 (*C. antarctica*) was obtained from Novo Nordisk (Danbury, CT). The following lipases were purchased from the Amano Co. (Troy, VA): *Aspergillus niger* (APF-12); *Candida* sp. (AY); *Geotrichum candidum* (GC4). Hexane and acetonitrile (HPLC grade) were products from Aldrich Chemical Co. (Milwaukee, WI). All other chemicals were of reagent grade. General experimental techniques and analytical measurements were applied as previously described (11).

HPLC. HPLC analysis was performed with a Hitachi L-6200 solvent delivery system (Tokyo, Japan), a Hitachi L-3000 photo array detector (233 nm) and a Hewlett-Packard integrator (Palo Alto, CA). An analytical silver-impregnated column (4.6 mm i.d. × 250 mm stainless steel; 5 µm particle size) was used (Chrompack, Bridgewater, NJ). The solvent flow rate was 1 mL/min. The mobile phase was 0.1% acetonitrile in hexane and prepared fresh daily. The flow was commenced for 0.5 h prior to sample injection. Typical injection volumes were 5–15 µL.

*Preparation of 9*Z*,11*E *and 10*E*,12*Z *CLA isomeric mixture using Schlosser base.* To a −78°C solution of *n*-butyllithium (14.2 mL of a 2.5M solution in hexane, 35.5 mmol) in tetrahydrofuran (THF) (150 mL) was added potassium *tert*-butoxide (35.4 mL of a 1 M solution in THF, 35.4 mmol). After 5 min, a solution of linoleic acid (3.1 g, 11.1 mmol) in THF (10 mL + 10 mL) was added. The resulting solution was stirred at

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–78°C for another 30 min and poured into 150 mL of 6 N HCl at 0°C. The aqueous layer was extracted with ether (150 mL × 3), and the combined organic layers were washed with a saturated sodium chloride solution, dried over magnesium sulfate, filtered, and concentrated under reduced pressure.

The crude extracts were dissolved in anhydrous diethyl ether (45 mL) and methanol (5 mL). To this solution was added diazomethane (ether solution) until the solution turned yellow, and the mixture was stirred at 24°C for 30 min to yield the methyl esters. Excess diazomethane was evaporated with a stream of nitrogen. The reaction mixture was concentrated under reduced pressure and then purified by $AgNO_3$-silica gel chromatography using hexane/ethyl acetate (60:1) as eluent to afford a mixture of 9*Z*,11*E* and 10*E*,12*Z* CLA methyl esters, **2a** and **2b** (2.5g, 77%). Analysis of this mixture using silver ion-impregnated HPLC showed the presence of only two peaks in a ratio of 1:1.5, corresponding to the retention times of **2a** and **2b** (Fig. 1), respectively (Scheme 1).

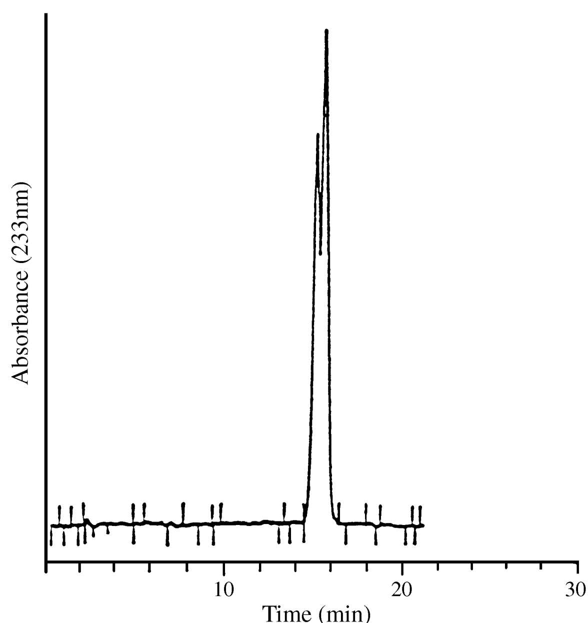
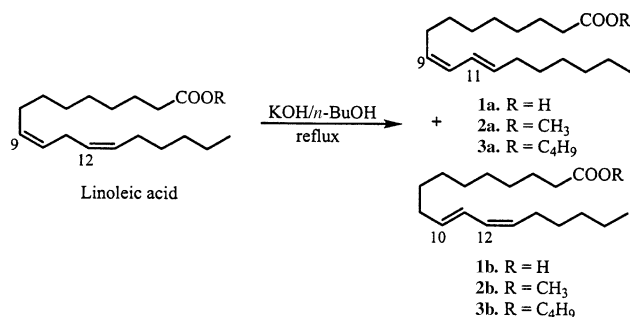


FIG. 1. Silver-ion high-performance liquid chromatography (HPLC) profile of a conjugated linoleic acid (CLA) mixture obtained from *n*-BuLi/*t*-BuOK isomerization. The conditions are described under the Materials and Methods section.



SCHEME 1

Base-catalyzed isomerization of linoleic acid. To a solution of linoleic acid (30 g, 0.107 mol) in 1-butanol (100 mL) was added KOH (30 g, 0.47 mol). The mixture was refluxed for 12 h and then was poured into water. The solution was acidified to pH 1.0 with HCl and extracted with hexane. The combined organic extract was washed with brine, dried over $MgSO_4$, and concentrated to dryness under reduced pressure to yield an isomeric mixture of CLA (29 g, 97%). The silver ion-impregnated HPLC profile is shown in Figure 2.

*Representative procedure for lipase-catalyzed selective hydrolysis of **2a** and **2b**.* To a solution of *A. niger* (APF-12) lipase (5 mg) in 2 mL of 0.1 M phosphate buffer, pH 7.0, was added 22 mg of **2a** and **2b** in 0.24 mL of acetone, and the mixture was stirred at 24°C for 3 h. After acidification of the mixture with 10% HCl, the aqueous layer was extracted with ether (10 mL × 3). The combined organic extracts were washed with a saturated sodium chloride solution and dried over magnesium sulfate. After evaporation of the solvent under reduced pressure, the residue was chromatographed over a silica gel column. The column was eluted first with ethyl acetate/hexane (30:1) followed by methanol/methylene chloride (5:95) to yield **2a** and **2b** in a ratio of 18:82 and **1a** and **1b** in a ratio of 87:13, respectively (46% conversion).

*Representative procedure for lipase-catalyzed selective esterification of **1a** and **1b**.* To a solution of **1a** and **1b** (19 mg) in 0.2 mL of 0.1 M phosphate buffer, pH 7.0, was added 0.2 mL of l-butanol and 1 mg of *A. niger* (APF-12) lipase. After stirring at 24°C for 2.5 h, the mixture was acidified with 10% HCl (1 mL) and extracted with ether (3 mL × 3). The combined organic extracts were washed with a saturated solution of sodium chloride, dried over $MgSO_4$, filtered, and concentrated to dryness under reduced pressure. The crude products were dissolved in 5 mL of diethyl ether and 1 mL of methanol. To this solution was added an ethereal solution of diazomethane until the solution turned yellow, and the mixture was left standing for 30 min. Excess diazomethane was evaporated with a stream

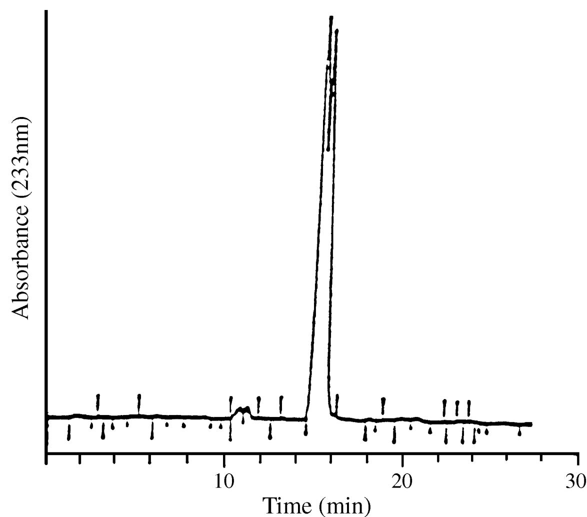
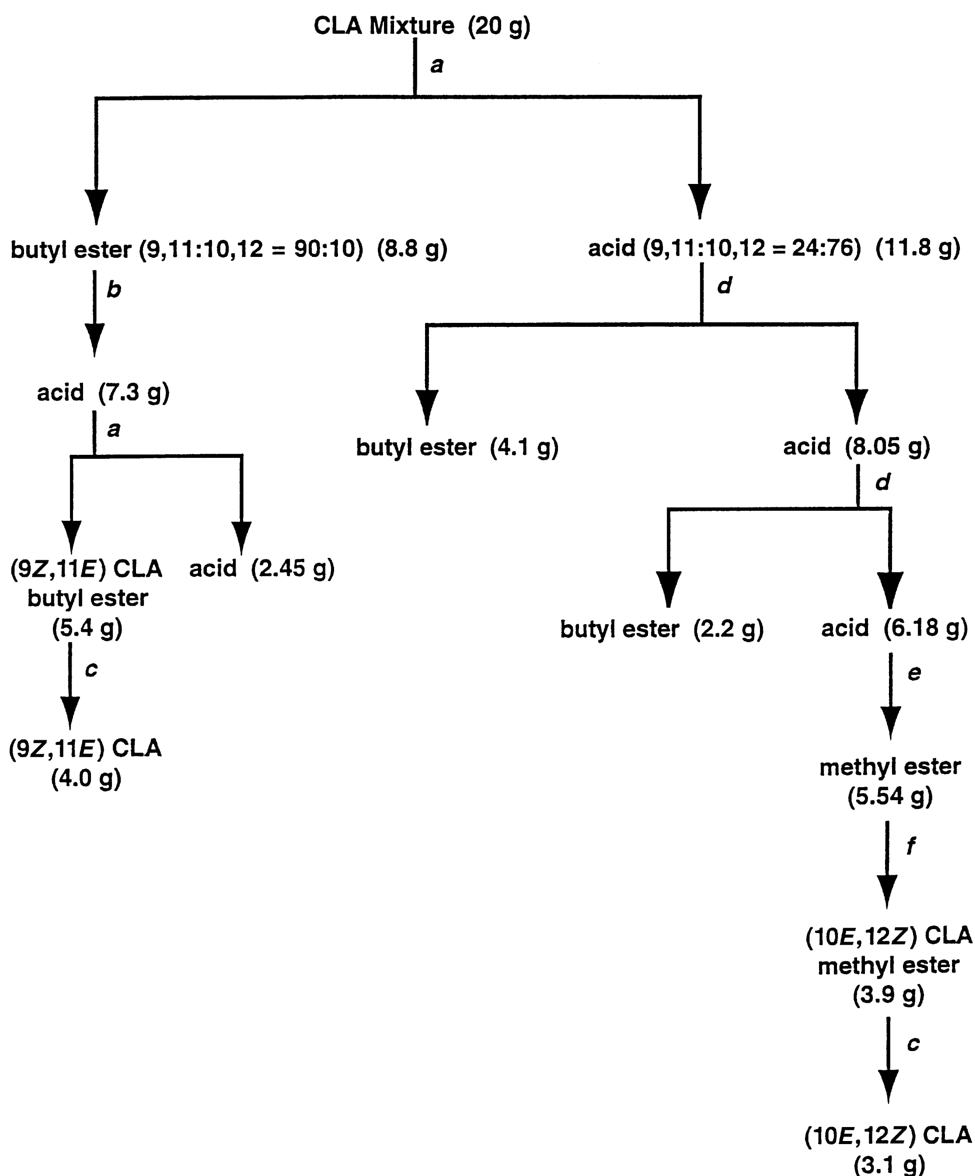


FIG. 2. Silver-ion HPLC profile of a CLA mixture derived from KOH isomerization. Conditions and abbreviations are the same as for Figure 1.

of nitrogen, and the reaction mixture was concentrated to dryness under reduced pressure. Silver ion-impregnated HPLC of this mixture showed the presence of 9*Z*,11*E* and 10*E*,12*Z* butyl esters, **3a** and **3b**, in a ratio of 95:5 and **2a** and **2b** in a ratio of 31:69, respectively (29% conversion).

General procedure for the separation of butyl esters from free acids. To a mixture (5 g) of butyl esters, **3a** and **3b**, and free acids, **1a** and **1b**, in 100 mL of hexane was added 100 mL of 1 M NaOH. After mixing, a mixture of 100 mL of ether, 100 mL of methanol, and 5 mL of *tert*-butanol was added to this solution, the mixture was shaken well, and the upper layer was decanted. To the lower layer was added 50 mL of hexane and 50 mL of methanol, and the mixture was shaken and the layers were separated again. The two upper layers were combined and washed successively with 50 mL of 1 M NaOH, 50 mL of methanol, water, and brine. After drying the solvent with $MgSO_4$, the solvent was removed under reduced pressure to give the butyl esters, **3a** and **3b**. The combined lower layers were acidified with HCl and extracted with ether. The extract was washed with brine, dried over $MgSO_4$, and concentrated to yield the free acids, **1a** and **1b**.

*Enzymatic separation of **1a** and **1b**.* To a mixture of 20 g of **1a** and **1b** in 200 mL of 1-butanol and 80 mL of water was added 4 g of *A. niger* (APF-12) lipase. The mixture was shaken on a rotary shaker (120 rpm) at 24°C for 10 h and then extracted with hexane three times. The combined organic extract was washed with brine, dried over $MgSO_4$, and concentrated to dryness under reduced pressure. The residue was subjected to the above procedure to yield 8.8 g of butyl ester (**3a** and **3b**)



SCHEME 2

and 11.8 g of free acids (**1a** and **1b**) (step *a*; Scheme 2). The butyl ester was dissolved in methanol; KOH (3.5 g) was added to the solution. After stirring at 24°C for 24 h, the reaction mixture was poured into water, acidified to pH 1.0, and extracted with ether. The ethereal extract was washed with brine and evaporated to dryness under reduced pressure to give 7.3 g of **1a** and **1b** (step *b*) which was again incubated with 1.5 g of the APF-12 lipase in 75 mL of 1-butanol and 30 mL of water. After incubation on a rotary shaker for 10 h, the butyl ester and free acid were separated to 5.4 g of **3a** and **3b** and 2.45 g of **1a** and **1b** (step *a*). This free acid fraction was reincubated with the lipase, and the resulting butyl ester was again hydrolyzed with KOH. Two crystallizations of the crude **1a** from methanol afforded 4 g of pure **1a** (step *c*).

To the acid fraction from the first enzymatic incubation, dissolved in 120 mL of 1-butanol and 48 mL of water, was added 2.4 g of the APF-12 lipase. The reaction mixture was incubated at 24°C in a rotary shaker for 18 h, and then was separated to give 8.05 g of acid and 4.1 g of butyl ester (step *d*). The acid (8.05 g) was subjected to APF-12 lipase esterification again to yield 6.18 g of the acid (**1a** and **1b**) (step *d*), which was dissolved in 100 mL of methanol and incubated with 1 g of Novozym 435 for 48 h to afford 5.54 g of a mixture of the methyl esters **2a** and **2b** (step *e*). The methyl esters were crystallized with urea (16.6 g) in methanol (110 mL) (step *f*). The mother liquor fraction was acidified with 200 mL of 2 M HCl and then extracted with hexane three times. The combined organic extract was washed with brine, dried, and concentrated to give 3.9 g of the 10*E*,12*Z* methyl ester, **2b**, which was hydrolyzed with KOH in methanol to afford crude **1b** (step *c*). Recrystallization of **1b** from methanol (65 mL) afforded 3.1 g of pure 10*E*,12*Z* acid, **1b** (Scheme 2).

RESULTS AND DISCUSSION

Since diallylic protons generally have pK values around 30 (12), it occurred to us that the superbasic mixture of *n*-butyllithium and potassium *tert*-butoxide (Schlosser base) would be a suitable reagent for the metalation of linoleic acid (13). In fact this organometallic reagent has been successfully used for the deprotonation of 1,4-pentadienyl systems with excellent selectivity (14). When linoleic acid was reacted with this superbase at −78°C in THF, only two major products were formed (Scheme 1), which accounted for more than 99% of the material recovered. After treatment of the reaction mixture with diazomethane, the resulting methyl esters were analyzed using silver ion-impregnated HPLC (8), which revealed the presence of only two peaks in a ratio of 4:6, corresponding to the retention times of methyl 10*E*,12*Z*- and 9*Z*,11*E*-octadecadienoates (**2a** and **2b**), respectively (Fig. 1).

For larger-scale preparations, we found it more convenient to use an alternative procedure by simply refluxing linoleic acid with NaOH in 1-butanol for 12 h. After treatment of the reaction mixture with diazomethane, the resulting methyl esters were again analyzed by silver ion-impregnated HPLC. The profile in Figure 2 shows the presence of two major peaks corresponding to the retention times of the 9*Z*,11*E* and 10*E*,12*Z* CLA isomers but in a ratio of 1:1, accompanied by about 2.5% of *trans* isomers (9*E*,11*E* and 10*E*,12*E*) and a trace (<1%) of the *cis* isomers (9*Z*,11*Z* and 10*Z*,12*Z*).

To our knowledge no current analytical techniques are available to separate the 9*Z*,11*E* and 9*E*,11*Z* isomers. Consequently, it was necessary to establish more definitely the position and geometric configurations of double bonds in the synthetic CLA preparation. This was carried out following a published procedure (15): (i) the double bonds of the CLA isomers were partially reduced with hydrazine; (ii) the resulting *E* monoene fraction, isolated by silver-impregnated silica gel column chromatography, was subjected to oxidative ozonolysis followed by methylation (16); and (iii) the diesters were isolated by silica gel column chromatography and their structures were verified by mass spectral analyses. When this procedure was applied to the synthetic CLA isomers, only dimethyl-1,10-decanedioate and 1,11-undecanedioate were obtained (17). These results showed the absence of geometric isomers and demonstrated the remarkable selectivity of the deprotonation–protonation procedure. This supposition was further confirmed by the ^{13}C nuclear magnetic resonance (NMR) spectra of pure **1a** and **1b**, which exhibited signals free of geometric isomers.

Because the regioisomers of CLA are difficult to separate from each other using chromatographic methods, we resorted to the use of enzymatic methods for the separation of the 9*Z*,11*E* from the 10*E*,12*Z* isomer. It is well documented that the lipase from *G. candidum* has a proclivity for unsaturated fatty acids possessing a 9*Z* double bond (18). That is, esters of 9*Z* unsaturated fatty acids are attacked more rapidly than other types of unsaturated fatty acid esters (19). However, when the synthetic CLA methyl esters **2a** and **2b** (1:1) were incubated with this lipase at pH 7.0 in 0.1 M phosphate buffer, the 9*Z*,11*E* isomer, **2a**, was preferentially hydrolyzed with a selectivity factor (*S*) (20) of only about 9. For systems containing two competing regioisomers, the selectivity factor $S = \ln[(1 - c)(1 - \mathrm{SE})]/\ln[(1 - c)(1 + \mathrm{SE})]$, where *c* denotes the extent of conversion and SE is the excess of one remaining substrate divided by the total remaining substrate at conversion *c*. See References. A 1:1 mixture of regioisomers (**1a** and **1b** or **2a** and **2b**) was used for the determination of *S*. Since the selectivity may often be improved by conducting the reverse reaction in organic solvents (21), we incubated the synthetic CLA isomers with the same lipase in 1-butanol for esterification. Indeed, the enzyme preferentially esterified the 9*Z*,11*E* isomer, **1a**, but the selectivity (factor *S*) was enhanced to only 12. This moderate improvement encouraged us to examine other lipase preparations for improved selectivities.

The results in Table 1 clearly show that the only enzyme that was more selective than the *G. candidum* lipase was the *A. niger* lipase (APF 12, Amano), which has the same stereochemical preference but the selectivity factor was enhanced to 25. In contrast, most of the other lipases exhibited low selectivities with *S* in the range of 1–3. In contrast to all the other lipases examined, immobilized lipase from *C. antarc-*

TABLE 1
Lipase-Catalyzed Selective Esterification of an Isomeric Mixture of 1a and 1b[a]

Entry	Lipase[b]	Time (h)	% Conversion[c] (*c*)	*S* (20)
1[d]	AY	4	40	6
2[d]	APF-12	8	60	25
3[d]	PL	20	40	1
4[d]	MY	1.5	58	5
5[d]	GC4	72	33	12
6[e]	Novo-435	6.5	61	4

[a]**1a** + **1b** $\xrightarrow[\text{1-Butanol}]{\text{Lipase}}$ **3a** + **3b**, where **1a** is 9*Z*,11*E*-octadecadienoic acid (18:2); **1b** is 10*E*,12*Z*-octadecadienoic acid; **2a** is the methyl ester of **1a**, and **2b** is the methyl ester of **1b**.
[b]AY, *Candida* sp. lipase (Amano Co., Troy, VA); APF-12, *Aspergillus niger* lipase (Amano Co.); PL, *Alkaligenes* sp. lipase (Meito Sangyo Ltd., Nagoya, Japan); MY, *C. rugosa* lipase (Meito Sangyo Ltd.); GC4, *Geotrichum candidum* lipase (Amano Co.); Novo-435, *C. antarctica* lipase (Novo Nordisk, Danbury, CT).
[c]$c = [\mathbf{3a} + \mathbf{3b}]/[(\mathbf{1a})_o + (\mathbf{1b})_o]$, where $(\mathbf{1a})_o$ and $(\mathbf{1b})_o$ are initial concentrations and **3a** and **3b** are butyl esters of **1a** and **1b**.
[d]1 g conjugated linoleic acid (CLA), 200 mg lipase in 10 mL of 1-butanol and 2 mL water, 24°C; 9,11-CLA was preferentially esterified.
[e]1 g CLA, 50 mg lipase in 10 mL of 1-butanol, 24°C; 10,12-CLA was preferentially esterified.

tica exhibited an opposite stereochemical preference in that it preferentially attacked the 10*E*,12*Z* CLA isomer, but the selectivity factor was low (*S* = 3.3).

To test the applicability of this methodology for the preparation of pure CLA isomers, we exposed 20 g of a 1:1 mixture of 9*Z*,11*E* and 10*E*,12*Z* isomers to the *A. niger* lipase in *n*-butanol for selective esterification. After 10 h of incubation, the ester fraction consisted of a mixture of 9*Z*,11*E* and 10*E*,12*Z* butyl esters in a ratio of 9:1 as determined by silver ion-impregnated HPLC and ^{13}C NMR. The remaining acid fraction consisted of 9*Z*,11*E* and 10*E*,12*Z* acids in a ratio of 24:76. To obtain the pure 9*Z*,11*E* isomer, the butyl ester fraction was hydrolyzed with methanolic KOH back to the free acid, which was subjected again to esterification to provide 5.4 g of the butyl ester, which upon base hydrolysis and crystallization from methanol afforded 4 g of pure 9*Z*,11*E* CLA, **1a**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.88 (3H, *t*, *J* = 6.1 Hz), 1.20–1.50 (16H, *m*), 1.63 (2H, *m*), 2.12 (4H, *m*), 2.34 (2H, *t*, *J* = 7.6 Hz), 5.28 (1H, *m*), 5.65 (1H, *m*), 5.94 (1H, *t*, *J* = 10.8 Hz), 6.29 (1H, *dd*, *J* = 15 Hz, 11 Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.5 (C1), 134.8 (C12), 129.8 (C9), 128.7 (C10), 125.6 (C11), 34.1 (C2), 32.9 (C13), 31.8 (C16), 29.7, 29.4, 29.1, 29.0, 28.9 (C4, C5, C6, C7, C14, C15), 27.6 (C8), 24.6 (C3), 22.6 (C17), 14.1 (C18). The remaining acidic fraction consisted of a mixture of **1a** and **1b** and could be recycled to obtain additional quantities of pure **2a**.

To secure the pure 10*E*,12*Z* CLA, **1b**, it was necessary to remove **1a** completely. This was achieved by subjecting the initial acid fraction to two cycles of enzymatic esterification in 1-butanol. The remaining acid fraction (6.18 g) was then subjected to selective esterification using the lipase of *C. antarctica*, which preferentially attacks the 10*E*,12*Z* isomer, to yield the methyl ester, **2b**. HPLC analysis of the methyl ester fraction revealed that **2b** was present as the main component, contaminated with small quantities of 9*Z*,11*Z*, 10*Z*,12*Z*,9*E*,11*E*, and 10*E*,12*E* CLA methyl esters. These impurities were removed by urea fractionation (7) to yield pure **2b**, which was subjected to base hydrolysis to afford **1b**; recrystallization from methanol gave the pure 10*E*,12*Z* CLA, **1b**: ^{1}H NMR ($CDCl_3$, 300 MHz) δ 0.89 (3H, *t*, *J* = 6.3 Hz), 1.20–1.50 (16H, *m*), 1.63 (2H, *m*), 2.12 (4H, *m*), 2.34 (2H, *t*, *J* = 7.5 Hz), 5.30 (1H, *m*), 5.65 (1H, *m*), 5.94 (1H, *t*, *J* = 10.9 Hz), 6.30 (1H, *dd*, *J* = 15 Hz, 11Hz); ^{13}C NMR ($CDCl_3$, 75 MHz) δ 180.5, 134.5, 130.1, 128.6, 125.7, 34.1, 32.9, 31.5, 29.4, 29.3(8), 29.3, 29.1(8), 29.1(5), 29.0, 27.7, 24.7, 22.6, 14.1. The results of this fractionation procedure are summarized in Scheme 2.

In conclusion, we have shown that linoleic acid was transformed into the two major components of CLA, **1a** and **1b**, by reaction with the Schlosser superbase (*n*-butyllithium/potassium *tert*-butoxide) or by simply refluxing with KOH in 1-butanol. In turn, **1a** and **1b** were separated from each other using the lipase from *A. niger via* selective esterification in 1-butanol. This enzyme has a preference for the 9*Z*,11*E* isomer, **1a**, and has excellent selectivity. This method has allowed the preparation of gram quantities of **1a** and **1b** in their highly purified forms, which are not readily accessible by current methods (22–25). The availability of these pure CLA isomers allows one to conduct in-depth experiments to determine which isomer is responsible for the reported biological activities.

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ARTICLE

Lipidemic Effects of an Interesterified Mixture of Butter, Medium-Chain Triacylglycerol and Safflower Oils

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ABSTRACT: The objective of this study was to determine if the positional structure of dietary triacylglycerol affected lipidemic responses. Thirty healthy adults (16 men and 14 postmenopausal women) with low-density lipoprotein cholesterol (LDL-C) concentrations >3.37 mM (130 mg/dL) enrolled in a prospective, single-blind, cross-over outpatient clinical trial that consisted of two 5-wk dietary phases. After baseline screening, subjects were instructed to follow individualized meal plans (weight maintenance diets with 36% of total energy from fat, half of which was from a test oil) and randomized to receive either butter (B) or an interesterified mixture (IM) of butter, medium-chain triacylglycerol (MCT), and safflower oils. Blood drawn during weeks 5 and 10 of feeding was analyzed for total cholesterol (TC), high density lipoprotein cholesterol (HDL-C),LDL-C, and triacylglycerols (TAG). Mean plasma levels of TC (B, 6.98 ± 1.06 mM; IM, 7.09 ± 1.20 mM), HDL-C (B,1.30 ± 0.35 mM; IM, 1.29 ± 0.34 mM), and LDL-C (B, 4.91 ± 0.95 mM; IM, 4.92 ± 1.10 mM) were not significantly different between the two dietary treatments. Mean TAG levels were higher for the interesterified B–MCT mixture (B, 1.75 ± 0.72 mM; IM, 1.96 ± 0.86 mM, $P < 0.05$). We conclude that an IM of B, MCT, and safflower oils as compared to native B has no appreciable effect on plasma cholesterol concentrations but is associated with a modest rise in plasma TAG.

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Traditionally, the cholesterolemic effect of dietary lipids has been thought of in terms of their degree of saturation (1,2). However, work with *trans* unsaturated fatty acids has suggested that geometric isomers of unsaturated fatty acids affect cholesterol metabolism differently from their *cis* counterparts (3). Over the years, data have been reported which demonstrate a differential effect of the cholesterolemic response from native or interesterified dietary triacylglycerols (TAG) (4–8). Interesterification or randomization is a process frequently used in the food industry to harden liquid oils. It alters the physical properties of a dietary fat by redistributing the fatty acids randomly, so that all three positions of the TAG molecule contain equal portions of the fatty acids (9). During interesterification of vegetable oils, the unsaturated fatty acids that commonly occupy the *sn*-2 position may move to the *sn* -1 or *sn*-3 positions that typically carry saturated fatty acids. In the small intestine, dietary fatty acids in the *sn*-2 position are preferentially absorbed as monoacylglycerols, whereas those esterified to the *sn*-1,3 positions are absorbed as free fatty acids (10). Thus, an interesterified fat may be metabolized differently from native fat. A recent intravenous study demonstrated slower clearance of a TAG that had stearic acid (as opposed to oleic or linoleic acid) in the number 2 position (11). Christophe *et al.* (8) reported that randomized butter (B) does not give the same hypercholesterolemic response as native B. Subjects fed 84 g/d of randomized or interesterified B oil for 20 d had an 11% reduction in mean serum cholesterol levels compared to when they were fed the same amount of native B.

In a series of experiments designed to determine the cholesterolemic effect of individual fatty acids, McGandy *et al.* (6) reported inconsistent effects of stearic acid. The authors hypothesized that the discrepancies were due to the position of the fatty acid on glycerol and that stearic acid esterified to the *sn*-2 position raised cholesterol concentrations more than when it was esterified to *sn*-1,3 positions. They also reported that mean serum cholesterol concentrations fell by 13.6 ± 5.1 mg/dL when subjects consumed a transesterified mixture of 50% medium-chain triacylglycerols (MCT)/myristic (60% MCT/40% trimyristin) and 50% safflower oil. When the amount of MCT/myristic was lowered to 20% and safflower was increased to 80%, mean serum cholesterol levels rose by 31.6 ± 7.0 mg/dL. Other studies have reported a neutral effect of MCT on blood cholesterol concentrations (12–14). Medium-chain fatty acids have been effective in treating individuals with fat malabsorption. Although most medium-chain fatty acids bypass the lymphatics and enter directly into the portal circulation where they are readily oxidized in the liver for energy, recent work in animals (15,16) has shown that medium-chain fatty acids in the *sn*-2 position can be absorbed into the lymphatics, and human studies have demonstrated they can be incorporated into chylomicron TAG (17).

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Abbreviations: B, butter; HDL-C, high density lipoprotein cholesterol; IM, interesterified mixture; LDL-C, low density lipoprotein cholesterol; MCT, medium-chain triacylglycerol; TAG, triacylglycerol; TC, total cholesterol.

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If the positional arrangement of fatty acids on the glycerol molecule does indeed influence cholesterol metabolism, interesterification would conceivably be favorable if it moved cholesterol-raising fatty acids out of the *sn*-2 position and neutral fatty acids in. It would also be unfavorable, as in some vegetable oils, if the concentration of saturated fatty acids on the middle carbon increased. Palmitic acid is the most abundant saturated fatty acid in the diet. In animal fats such as pork and dairy fat, palmitic acid is largely in the *sn*-2 position, unlike plant oils such as palm oil where it is most often esterified to the first and third carbons (18). One recent study addressed the question of what effect interesterification of palm oil would have on blood lipoprotein concentrations in humans and reported little effect (19). Another study investigated whether interesterification of a high palm oil margarine would have harmful effects on plasma lipids and also demostrated no significant effect from interesterification (20).

The present study was designed to investigate whether an IM of B (60%), MCT (35%), and safflower oils (5%), when incorporated into baked goods and then fed to healthy hypercholesterolemic adults, would show a differential lipidemic response when compared to butter.

EXPERIMENTAL PROCEDURES

Subjects. Adults were recruited by advertising in local Boston newspapers. All subjects underwent a medical history evaluation and physical examination. They were eligible for the study if their plasma low-density lipoprotein cholesterol (LDL-C) was >3.37 mM and they were free of any diseases (cardiac, renal, thyroid, or hepatic) or medications (hypolipidemic drugs, beta-blockers, diuretics, estrogens, progestins, or thyroxine) capable of influencing plasma lipid levels. They were required to maintain their body weight within a 2-kg range and to avoid extremes in dietary or exercise habits throughout the study period. Two subjects smoked cigarettes (each <5 per day). The study was approved by the Institutional Review Board on Human Studies at the Deaconess Hospital. Written informed consent was obtained from all subjects at the time of enrollment.

Of the 226 people who were screened *via* a telephone interview, 106 were eligible to receive written medical history questionnaires. Of these mailed questionnaires, 53 were returned from which 49 individuals were selected for baseline clinical screening. Of these 49 people who came to the clinic, six were ineligible as their plasma LDL-C was below the cut-off point and 10 were unable or unwilling to comply with the study protocol. Three subjects were dropped during the study: one completed phase 1, but due to a change in work schedule was unable to continue into phase 2; one developed a cardiac arrhythmia during phase 2 and was started on atenolol; and one violated the weight criterion.

Study design. The study followed a single-blind, randomized, two-phase, cross-over design. Baseline plasma lipid levels were determined after a 12-h fast on the first clinic visit. After a 2–3 wk screening period to confirm eligibility, establish the test diet, teach food recording and fat gram counting techniques and to document stable weight, subjects were randomized to receive either regular B oil or an interesterified B–MCT oil mixture. Subjects were fed one of the fats for 5 wk and immediately crossed over to the alternate fat after fasting-blood samples were taken on three separate days during week 5. Plasma lipids have been reported to stabilize by week 4 of fat-modified diet consumption (21). In each treatment phase of this study, all subjects followed the test diets for 5 wk in order to allow for triplicate fasting-blood sampling during week 5. They were required to report to the clinic each week in order to monitor their health status, weight, and food records, and to pick up their supply of baked goods for the subsequent week.

Diet. The test diet was designed to simulate a typical American diet with an energy distribution of 15–49–36% of each subject's total weight maintenance energy from protein, carbohydrates, and fats, respectively, and a cholesterol concentration of 35 mg/kJ (22). Energy requirements were estimated by the Harris–Benedict equation and from energy intakes reported in the 3-d food records. In each dietary phase, the test oil was designed to provide 18% of total daily energy and was baked into muffins (18 g of fat each) and cookies (8 g of fat each). Stepan Company Inc. (Maywood, NJ) provided the test oils; their fatty acid composition and positional TAG distribution are listed in Tables 1 and 2, respectively. The muffins and cookies were made by the Dietary Department of Deaconess Hospital and frozen until ready for use.

Subjects were instructed by the research coordinator (CEM, a registered dietitian) to follow a low-fat, exchange-style meal plan (18% fat energy) supplemented with high-fat baked goods in amounts that provided 18% of the subjects' energy from the test oil. Three-day food records were completed each week. In an attempt to minimize the error and variability associated with dietary-assessment protocols, food intake was recorded on two nonconsecutive weekdays and one weekend day (23). Subjects also were taught to count and record the grams of fat in the foods they reported in their records. As there was variability in food preferences and subsequently in food choices among the subjects, it was impossible to precisely control the specific fatty acid profile of the diet. However, as each person served

TABLE 1
Fatty Acid Composition of Test Oils[a]

Fatty acid mixture	Butter	Interesterified butter-MC
4:0, butyric	14.9	6.9
6:0, caproic	4.8	2.8
8:0, caprylic	2.1	32.4
10:0, capric	3.8	17.5
12:0, lauric	3.6	2.1
14:0, myristic	10.2	5.3
16:0, palmitic	24.5	12.6
18:0, stearic	10.5	5.0
16:1n-7, palmitoleic	1.0	0.5
18:1n-9, oleic, *cis*	10.5	5.0
18:1n-9, elaidic, *trans*	1.7	0.9
18:2n-6, linoleic	2.3	3.5
18:3n-3, α-linolenic	0.4	0.0

[a]wt% of total fatty acids; MCT, medium-chain triacylglycerol.

TABLE 2
Fatty Acid Composition and *sn*-2 Positional Distribution in Test Fats[a]

	Butter		Interesterified butter-MCT mixture	
Fatty acid	*sn*-2	*sn*-1,3	*sn*-2	*sn*-1,3
6:0	0.0	2.0	0.0	1.2
8:0	1.1	3.3	32.7	36.7
10:0	4.4	5.4	20.9	20.2
12:0	5.6	4.2	2.4	2.2
14:0	19.2	9.7	6.2	5.7
16:0	34.4	28.1	14.7	13.8
18:0	7.1	15.3	5.7	5.3
20:0	0.4	0.7	0.0	0.0
16:1n-7	1.8	2.5	0.6	0.6
18:1n-9	17.0	22.2	10.9	9.1
18:1n-9,t	1.8	2.5	0.9	0.9
18:2n-6	3.0	2.4	4.0	3.5
18:3n-3	0.4	0.5	0.0	0.0

[a]Percentage of triacylglycerol in *sn*-2 position determined by Grignard procedure; see Table 1 for abbreviations.

as his/her own control, the test fat was the only significantly different dietary component between each phase.

The 3-d food records from baseline and from weeks 2 and 4 of each dietary phase were analyzed for mean daily totals of energy and nutrients using Minnesota Nutrition Data System© (24).

Anthropometric data. Height was measured to the nearest 0.5 cm and weight to the nearest 0.1 kg using a Seca™ scale (8920A RT. 108; Columbia, MD). Body fat was measured by bioelectrical impedance using a Quantum BIA-101Q™ (RJL Systems Inc., Clinton Township, MI) as previously described by Lukaski (25).

Test oils. The B used in the study was clarified B oil which contained 99.48% (by weight) fat and 0.24% water. The interesterified B-MCT mixture was made by blending clarified B oil (60% by weight), MCT oil (35%), and safflower oil (5%) in a stainless steel batch reactor and heating the mixture to 75–80°C under nitrogen as per the method described by Sreenivasan (9). Sodium methoxide was used as a catalyst. After 30–60 min, the resulting product was washed, dried, deodorized, and filtered to give the final interesterified product.

Test oil TAG structure (Grignard degradation). The dietary oils (10 mL) were dissolved in 1 mL of hexane and methylated using KOH in methanol (26). The fatty acid methyl esters were analyzed with a Hewlett-Packard 5890 series II gas chromatograph equipped with a fused-silica column (SP2380, 60 m, 0.25 mm i.d., Supelco Inc., Bellefonte, PA). Helium was the carrier gas with a split ratio of 1:20. The mixture was heated to 75°C for 0.5 min, and the temperature was then increased as follows: 15°C/min until 160°C was reached, followed by 30°C/min until 225°C was reached. This was maintained for 5 min. The fatty acid methyl esters were identified and quantified by internal standards added prior to the methylation (Nu-Chek-Prep Inc., Elysian, MN).

The distribution of the fatty acids within the TAG was determined using Grignard degradation (27). The *sn*-2 monoacylglycerol fractions were then isolated and the fatty acid composition was determined in the same manner as the total TAG.

Blood collection, preparation, and analyses. Fasting (14-h) blood samples were collected in tubes containing EDTA (0.1%). Plasma was separated by centrifugation at 3000 rpm at 4°C. Total cholesterol (TC) and TAG were analyzed with an Abbott Diagnostics Spectrum CCX bichromatic analyzer (Abbott Park, IL) using enzymatic reagents. High-density lipoprotein cholesterol (HDL-C) was measured in the supernatant fraction after precipitation of other lipoproteins by a dextran sulfate-magnesium procedure as described elsewhere (28). LDL-C was separated directly using a reagent kit from Genzyme Corp.© (Cambridge, MA) which precipitates very low-density lipoprotein and high-density lipoprotein with antibodies to apolipoproteins A-I and E, as previously described (29).

Plasma TAG structure (Grignard degradation). The total lipid fraction from the plasma samples was extracted according to the method of Folch *et al.* (30). Methanol (1.5 mL) was added to 200 mL of plasma. Then, 3 mL of chloroform and 0.2× the volume of saline (0.73 wt% NaCl) were added and the mixture was shaken vigorously. The mixture was left to separate into two phases and the aqueous phase was discarded. The organic phase was filtered through a column of anhydrous Na_2SO_4 and the column was washed twice with 1 mL of chloroform. The solvent was evaporated and the lipid fraction redissolved in 2 mL of chloroform/methanol (95:5, vol/vol) containing 0.005% butylated hydroxytoluene (Kock-Light Laboratories, England). One milliliter of the lipid extract was applied on thin-layer chromatographic plates and developed using heptane/propanol-1/acetic acid (95:5:1, by vol). The plates were sprayed with 2,7-dichlorofluorescein and the lipid classes visualized under an ultraviolet lamp. The TAG fractions were scraped off for fatty acid analysis. The TAG fraction was extracted from the plate material using 3 × 1 mL of ether which was evaporated and the TAG fraction was redissolved in 0.5 mL of hexane and methylated using KOH in methanol. The phospholipid fraction was saponified and methylated using BF_3 (31). The 2-monoglyceride band was scraped off the plate, since 2-monoglyceride yields the best results in a regiospecific analysis (27). The 2-monoglyceride was extracted from the silica gel with 3 × 1 mL diethyl ether, the ether was evaporated under nitrogen, and the glycerides were dissolved in hexane.

Statistical analysis. All statistical analyses were carried out using Systat® (32). Paired *t*-tests were used to compare the averaged plasma lipid and lipoprotein values between the B and interesterified B-MCT phases. An α level of 0.05 was used. Dietary variables were expressed as a percentage of total energy, and paired *t*-testing was used to determine if any differences existed. Carryover effects of the previous diet were determined by comparing the differences between the plasma lipid values averaged over the entire study with those subjects who received B first and those who received the interesterified B-MCT mixture first.

RESULTS

The sample consisted of 16 men and 14 postmenopausal women whose mean age was 59 ± 11 yr (women, 64.3 ± 7.4 yr; men, 54.3 ± 12.3 yr) and body-mass index (kg/m^2) was 27.2

± 5.3 (women, 27.9; men, 26.7). The women weighed 69.2 ± 16.1 kg and men 80.4 ± 12.7 kg. The mean blood pressure (mm Hg) was 129/80 (women, 137/80; men, 122/80). The mean percent body fat was 26.7 ± 9.7 (women, 34.0; men, 20.4). As such, the patient population was, on average, both hypercholesterolemic and obese, and less responsive to dietary changes in unsaturated fatty acids. Food records from weeks 2 and 4 of each dietary phase were averaged together and analyzed for mean energy and selected nutrient concentrations. Nutrients were expressed as a percentage of total energy and, as *t*-tests revealed no significant differences for any of the nutrients between men and women, the data were combined. There were no significant differences seen in any mean nutrient intakes from diet alone between the two dietary phases. However, when the test foods were added to the nutrient data file, paired *t*-tests revealed the expected differences that are reported in Table 3. During the B phase, significantly more butyric (4:0), myristic (14:0), palmitic (16:0), stearic (18:0), and oleic (18:1n-9) acids were consumed, while in the interesterified phase more caprylic (8:0) and capric (10:0) acids and less cholesterol were consumed. The overall energy distribution was slightly higher in carbohydrates (4%) and lower in protein (2%) and fats (4%) than the intended goal.

Mean intake of alcohol (per 1000 kJ) was 0.7 ± 1.3 g during baseline, and dropped to 0.4 ± 1.0 g and 0.4 ± 0.8 g during the B and IM phases, respectively. Subjects were instructed to abstain from alcohol intake during the weeks of blood sampling. Mean fiber intakes were stable throughout the study.

Table 4 shows the plasma lipid concentrations at baseline and during the two treatment phases of the study. The subjects were moderately hypercholesterolemic and hypertriglyceridemic upon entry. There were no significant differences in plasma cholesterol concentrations between the two dietary treatments. The interesterified B-MCT mixture resulted in a significantly ($P < 0.05$) higher fasting plasma TAG concentration. Statistical comparisons were not made with baseline measurements given unknown dietary intakes prior to screening. Analysis showed that there was no effect due to the order of treatment.

TAG molecular structure. As shown in Table 2, the more common fatty acids in the *sn*-2 position in the B were myristic and palmitic acids, accounting for 66 and 55%, respectively, of these fatty acids relative to the *sn*-1,3 position. In the IM, the most common fatty acids in the *sn*-2 position were caprylic and capric acids, accounting for 49 and 50%, respectively, of these fatty acids relative to the *sn*-1,3 position. The latter distribution is essentially what would be predicted from the random distribution of the fatty acids in the oils used in the manufacturing of the interesterified oil. Unfortunately, during the Grignard procedure, the shorter-chain fatty acids were volatilized, accounting for the lower values, as compared to those in Table 1.

Table 5 shows the fatty acid composition seen in the subjects' plasma TAG. Subjects had significantly more capric, stearic, and dihomo-γ-linolenic acid, and less oleic acid when they consumed the IM as compared to B.

TABLE 3
Mean Nutrient Intakes and Percent Energy Distribution (diet plus test foods) for Baseline and Dietary Phases[a]

Nutrient	Baseline	Butter	Interesterified butter + MCT
Carbohydrate (% of energy)	55.5 ± 8.5	53.9 ± 5.2	54.2 ± 5.2
Protein (% of energy)	16.2 ± 3.3	13.3 ± 2.0	12.7 ± 1.8
Fat (% of energy)	29.6 ± 7.3	32.1 ± 4.2	32.4 ± 4.5
Saturates (% of energy)	9.5 ± 3.1	18.2 ± 2.3	18.6 ± 2.2
4:0	0.2 ± 0.1	2.6 ± 0.4	1.2 ± 0.2[b]
6:0	0.1 ± 0.1	0.9 ± 0.1	0.5 ± 0.1
8:0	0.1 ± 0.1	0.4 ± 0.1	5.3 ± 0.7[b]
10:0	0.2 ± 0.1	0.7 ± 0.0	2.9 ± 0.4[b]
12:0	0.3 ± 0.2	0.7 ± 0.1	0.5 ± 0.1
14:0	0.8 ± 0.4	2.1 ± 0.3	1.3 ± 0.2[b]
16:0	5.1 ± 1.5	6.7 ± 0.9	4.8 ± 0.9[b]
18:0	2.5 ± 0.9	2.9 ± 0.4	2.0 ± 0.4[b]
Monounsaturates (% of energy)	11.8 ± 3.6	8.8 ± 1.7	7.8 ± 2.1[b]
16:1n-7	0.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.1
18:1n-9	10.9 ± 3.3	8.2 ± 1.6	7.2 ± 2.0[b]
Polyunsaturates (% of energy)	6.0 ± 2.0	3.9 ± 1.2	4.2 ± 1.2
18:2n-6	5.2 ± 2.0	3.3 ± 1.1	3.5 ± 1.0
18:3n-3	0.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.2
Cholesterol (mg/1000 kJ)	26 ± 13	31 ± 6	29 ± 6[c]
Ethanol (g/1000 kJ)	0.7 ± 1.3	0.4 ± 1.0	0.4 ± 0.8
Fiber (g/1000 kJ)	2.4 ± 0.9	2.1 ± 0.4	2.0 ± 0.4
Soluble fiber	0.8 ± 0.3	0.8 ± 0.2	0.7 ± 0.2
Insoluble fiber	1.5 ± 0.6	1.3 ± 0.3	1.2 ± 0.3

[a]Averaged values from weeks 2 and 4 of each treatment phase reported as mean + SD; see Table 1 for abbreviations.
[b]Butter value different from interesterified mixture value ($P < 0.0001$).
[c]Butter value different from interesterified mixture value ($P < 0.05$).

TABLE 4
Plasma Lipid Concentrations at Baseline and During Each Diet Phase[a]

Lipid	Baseline (mM/L)	Butter (mM/L)	Interesterified butter + MCT (mM/L)
Total cholesterol	6.60 ± 1.04	6.98 ± 1.06	7.09 ± 1.20
LDL-cholesterol	4.50 ± 1.22	4.91 ± 0.95	4.92 ± 1.10
HDL-cholesterol	1.30 ± 0.37	1.30 ± 0.35	1.29 ± 0.34
Triacylglycerols	1.46 ± 0.82	1.75 ± 0.72	1.96 ± 0.86[b]

[a]Mean ± S.D; LDL, low-density lipoprotein; HDL, high-density lipoprotein; MCT, medium-chain triacylglycerol.
[b]Significantly different from butter value, $P < 0.05$.

TABLE 5
Fatty Acid Composition of Subjects' Plasma Triacylglycerols[a]

Fatty acid	Butter	Interesterified butter-MCT
10:0	0.03 ± 0.06	0.11 ± 0.12[b]
12:0	0.26 ± 0.17	0.21 ± 0.14
14:0	2.74 ± 0.77	2.56 ± 0.75
16:0	25.95 ± 2.67	26.13 ± 3.38
18:0	3.40 ± 0.61	3.80 ± 0.67[c]
16:1n-7	3.83 ± 1.10	3.71 ± 0.93
18:1n-9	33.94 ± 3.19	32.28 ± 3.0[d]
18:2n-6	17.45 ± 3.21	18.66 ± 3.79
18:3n-3	1.30 ± 0.51	1.34 ± 0.58
20:3n-6	0.23 ± 0.20	0.34 ± 0.15[e]
20:4n-6	1.55 ± 0.31	1.58 ± 0.35

[a]Mean ± SD expressed as percentage by weight of total fatty acids; see Table 1 for abbreviations.
[b]Significantly different from butter value, $P < 0.01$.
[c]Significantly different from butter value, $P < 0.02$.
[d]Significantly different from butter value, $P < 0.007$.
[e]Significantly different from butter value, $P < 0.009$.

DISCUSSION

The results of this study demonstrate that the mean plasma cholesterol concentrations did not differ significantly when subjects consumed the interesterified oil vs. the B oil. This finding is interesting in view of the composition and molecular structure of the test oil. Medium-chain fatty acids accounted for 50% of the fatty acids in the test oil as compared with 6% in the B oil. Caprylic and capric acids in the *sn*-2 position of the IM appear to have the same potential to increase plasma cholesterol concentrations as the longer-chain saturated fatty acids, such as myristic and palmitic acids, which were of greater concentrations in the B oil. We cannot, however, definitely conclude this since interesterification of the oil mixture was performed, rather than merely feeding the oil mixture.

After a 20-yr hiatus, there has been a renewed interest in the effect of interesterified fat on serum lipid levels. Unlike results from earlier studies that reported interesterified fats had different effects on serum lipids, the present study and studies by Zock *et al.* (19) and Nestel *et al.* (20) have found little or no difference with interesterified fats.

The majority of research on MCT and cholesterol metabolism was conducted in the 1960s. Beveridge *et al.* (12) reported no significant change in serum cholesterol levels in healthy students when an MCT-containing formula replaced a fat-free regime. When coconut oil formula was substituted for MCT, slight increases in serum cholesterol levels occurred, whereas a B-containing formula produced a marked elevation of serum cholesterol levels.

Roels and Hashim (14) compared liquid formulae containing MCT, corn oil, or B as the sole source of dietary fat (50% of total energy). In comparison with corn oil, MCT induced a slight rise in serum cholesterol levels, whereas serum cholesterol increased when B followed MCT and fell when MCT replaced B.

McGandy *et al.* (6) reported that mean serum cholesterol concentrations decreased by 3.2 ± 8.4 mg/100 mL in a group of 10 men and by 11.0 ± 4.3 mg/100 mL in a group of nine men when these groups consumed a diet that provided 38% of total energy as fat of which 5.7 total energy % was added from MCT.

In the present study, there was a significant rise in fasting TAG levels when subjects were consuming the test oil. Uzawa *et al.* (33) substituted MCT for long-chain saturated or unsaturated fatty acids in a diet (45% of energy from fat) and reported an increase of serum TAG levels of 100% or more. Resumption of long-chain fatty acid intake resulted in an immediate drop of serum TAG concentrations to previous levels. The study is limited, however, as two of the three subjects were diabetic.

McGandy *et al.* (6) reported a significant ($P < 0.01$) increase in mean serum TAG concentrations of 61.1 ± 17.8 mg/100 mL when MCT provided 38% of the total dietary energy in a group of 10 institutionalized men. In another group of nine given the same diet, the mean increase in TAG was 20.1 ± 3.0 mg/100 mL. This effect was diminished in both groups when the MCT was mixed with trimyristate and safflower oil.

Kuo and Huang (34) also reported a significant increase in serum TAG concentrations in a group of patients with cystic fibrosis when they were given a formula supplemented with MCT. However, the ranges of variation were large and the level of significance was marginal ($P < 0.10$).

In summary, there appears to be consistency in the reporting of increased TAG concentrations when subjects consume MCT. Even though cholesterol levels were unaffected, interesterified TAG can influence the atherosclerotic process as shown by Kritchevsky *et al.* (4). Other studies emphasize alterations in lipoprotein composition after different types of dietary fats (35,36).

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Mechanisms Mediating Lipoprotein Responses to Diets with Medium-Chain Triglyceride and Lauric Acid

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ABSTRACT: Medium-chain triglycerides (MCT) are often used in specialized formula diets or designer fats because of their special properties. Yet their influence on lipid metabolism is not completely understood. In this two-period cross-over study, the effects of MCT (8:0 + 10:0) in contrast to a similar saturated fatty acid (12:0) were compared. Eighteen healthy women ate a baseline diet [polyunsaturated (PUFA)/saturated fat = 0.9] for 1 wk. Then, they consumed test diets (PUFA/saturated fat = 0.2) for 4 wk. Monounsaturated fat and cholesterol were constant in baseline and treatment diets. MCT and 12:0, substituted for part of the PUFA, provided 14 energy (en)% of the test diets. In comparison to the PUFA baseline diet, a 16% increase in mean serum low density lipoprotein (LDL)-cholesterol (C) on the 12:0 diet was accompanied by a 21% decrease in mean receptor-mediated degradation of LDL by freshly isolated mononuclear cells (MNC) *in vitro*. The MNC assay theoretically gives an indication of receptor-mediated degradation of LDL. In contrast, the MCT diet raised mean receptor-mediated degradation of LDL by 42%, a finding out of line with the mean 11% increase in serum LDL-C. Perhaps MCT, by increasing the rate of LDL-C production, overcame the rate of LDL-C clearance. The 12:0 diet enhanced some factors involved in reverse cholesterol transport (e.g., high density lipoprotein fractions) while MCT had a different or less pronounced effect. The overall effects of MCT on cholesterol metabolism may or may not be desirable, whereas those of 12:0 appear largely undesirable as previously reported.

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Medium-chain triglycerides (MCT) are composed of fatty acids (FA) with 6–12 carbons (1). Although they are found in several natural fats and oils, FA with 8 and 10 carbons, henceforth referred to as MCT, are not present in high amounts in fats and oils produced in the United States. Yet they play an important role in certain American diets. For example, because they are easily digested and absorbed, they have been incorporated into nutritional products developed for patients with diseases of fat malabsorption (2). Very low birth-weight infants are often given formulas with MCT to improve absorption of fat and calcium (3). MCT are on a list of supplements frequently used by body builders (4), sometimes in large amounts (5). Gene manipulation can be employed to modify the MCT content of oils such as canola (6) and so might be used to enhance the MCT content of food products if this should prove desirable.

MCT, in addition to being more digestible, are metabolized somewhat differently from long-chain triglycerides (LCT) (7). For example, [^{13}C]trioctanoate, in contrast to [^{13}C]trioleate, is oxidized more rapidly after both oral and parenteral administration. This rapid oxidation may amount to 50% of the dietary energy use in very low birth-weight infants (3,7). There is evidence of incorporation of label from orally administered [^{13}C]octanoate into myristic and palmitic acids in plasma triglycerides (TG), a result suggestive of chain elongation of a portion of the medium-chain FA (3). In addition, MCT are metabolized to other, less usual compounds compared to LCT. More ketones (3-hydroxybutyrate) are recovered in plasma and more dicarboxylic acid is found in urine of low birth-weight infants when MCT constitute 50% rather than 0% of formula fat (8). Not all dietary MCT are metabolized immediately. Infants may store up to 12% of the medium-chain FA they consume in adipose tissue (9). These metabolic data suggest that MCT can have a somewhat different effect on energy and lipid metabolism from that of LCT. In fact, excess dietary energy in the form of MCT, in contrast to LCT, seems to increase postprandial thermogenesis, as assessed by indirect calorimetry, possibly because of enhanced lipogenesis in the liver and reduced efficiency of energy storage (10). As regards effects on lipid metabolism, the multiple regression coefficients compiled by Hegsted *et al*. (11) suggest that 10:0 has a slightly elevating effect on serum cholesterol concentrations in contrast to oleic, linoleic, and stearic acids, which have different degrees of negative effect. In a two-study series conducted by Wardlaw *et al*. (12), a structured TG made of randomly distributed 8:0, 10:0, and 22:0 had the same cholesterolemic properties as the comparison diets that were high in 12:0, 14:0, and 16:0, as provided by butter or a mixture of palm oil/palm kernel oil. Even though the structured TG had reduced metabolizable energy

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Abbreviations: apo, apolipoprotein; CE, cholesterol ester; CETP, cholesterol ester transport protein; FA, fatty acid; FC, free cholesterol; HDL-C, high density lipoprotein-cholesterol; LCAT, lecithin cholesterol acyl transferase; LCT, long-chain triglyceride; LDL-C, low density lipoprotein-cholesterol; MCT, medium-chain triglyceride; MNC, mononuclear cell; PL, phospholipid; PRO, protein mass; PUFA, polyunsaturated fatty acid; RCT, reverse cholesterol transport; SFA, saturated fatty acid; TC, total cholesterol; TG, triglyceride; VLDL-C, very low density lipoprotein-cholesterol.

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content compared to the natural products, there was no evidence in this study that it could be used as a heart-healthy substitute for animal fats or tropical oils.

Since MCT have the potential for extensive use in specialized formulas and designer TG, it is important to know more about their metabolic effects. In this study, the effects of diets high in MCT were compared to the changes in lipid metabolism produced by a diet high in lauric acid (12:0), which has consistently been demonstrated to raise serum cholesterol concentrations compared to a neutral diet, whether fed in a natural oil (palm kernel oil) or as a synthetic TG (11,13,14).

METHODS AND MATERIALS

Subjects. Eighteen healthy, premenopausal women were randomized into this two-period cross-over study, the minimal number needed to detect a 10% mean difference with an 8% standard deviation by a power of 0.9. Since one subject left the study after period one, 17 subjects consumed the MCT (8:0 + 10:0) diet and 18 subjects finished the lauric acid (12:0) diet. The average age of all subjects was 24 ± 4 yr (mean ± SEM), range 19 to 33 yr; the average weight was 62.2 ± 11.7 kg, range 40.5 to 91.8 kg; and the average height was 161.9 ± 7.7 cm, range 147.9 to 176.5 cm. The fasting serum cholesterol concentration at screening was 167 ± 22 mg/dL (mean ± SEM) with a range between 143 and 237 mg/dL. The health of subjects was verified by blood chemistry and a health questionnaire. Anyone taking medications influencing lipid metabolism was not recruited into this study. The ethnic background of subjects was 10 Caucasians, seven Asians, and one African American. Apolipoprotein E (apoE) phenotype of subjects was 12 apoE 3/3, three apoE 4/3, and three apoE 3/2. Each subject signed a consent form approved by The Ohio State University Biomedical Human Subjects Committee.

Design. In this two-period and two-sequence design involving 18 subjects, nine subjects ate the MCT diet in the first period, and the other nine received the lauric acid diet. Then, in the second period, all subjects consumed the alternate diet. In order to standardize nutrient intake prior to the experimental treatments and provide a comparison base, the subjects were given a baseline diet designed to be higher in polyunsaturated fatty acids (PUFA) and lower in saturated fatty acids (SFA) than the treatment diets for the initial week of each period. The experimental diets were given during the following 4 wk. A 7-wk washout period was held between periods. The study was designed so that blood samples were obtained at the same phase of each subject's menstrual cycle. Blood samples were collected twice during the last 3 d of baseline and twice during the last 3 d of experimental feeding, and the two data points were averaged. The blood samples were drawn into vacutainers with or without anticoagulant depending on the tests to be run. For lipoprotein analyses, serum was obtained by centrifuging at 500 × *g* for 20 min. Some serum samples were used immediately for procedures requiring fresh blood. The remaining samples were frozen at –80°C for later analysis.

Diets. All diets were prepared in a metabolic kitchen in the Department of Human Nutrition and Food Management. The subjects ate breakfast and dinner in the metabolic dining room and carried out lunch during weekdays. Except for Saturday night dinner, they took out weekend meals. According to the nutrient analysis by Food Processor II software (ESHA Research, Salem, OR), the baseline diet contained 40 en% total fat, 11.9 en% saturated fat, 14.9 en% monounsaturated fat, and 10.5 en% polyunsaturated fat, and 323 mg cholesterol

TABLE 1
Daily Energy and Nutrient Intake on Two Saturated Fat Diets and Baseline Diet[a] (mean ± SEM)

Dietary variable	Baseline (*n* = 35)	MCT (*n* = 17)	Lauric acid (*n* = 18)
Energy (MJ)[b]	8.1 ± 0.1	8.3 ± 0.1	8.4 ± 0.1
Protein (g)[b]	72 ± 0 (15)	73 ± 0 (15)	73 ± 0 (15)
Carbohydrate (g)[b]	225 ± 2 (46)	230 ± 3 (46)	231 ± 3 (46)
Total fat (g)[b]	86 ± 1 (40)	89 ± 1 (40)	89 ± 1 (40)
Fatty acids (g)[c]			
8:0 + 10:0 (MCT)	1.8 ± 0.0 (0.8)	30.1 ± 0.5 (13.6)	1.0 ± 0.0 (0.5)
12:0	3.6 ± 0.0 (1.7)	2.1 ± 0.0 (0.9)	31.4 ± 0.5 (14.1)
14:0	2.3 ± 0.0 (1.0)	1.4 ± 0.0 (0.6)	1.4 ± 0.0 (0.6)
16:0	8.2 ± 0.1 (3.8)	5.9 ± 0.1 (2.7)	5.9 ± 0.1 (2.7)
18:0	7.9 ± 0.1 (3.7)	2.9 ± 0.1 (1.3)	2.9 ± 0.1 (1.3)
SFA	25.7 ± 0.5 (11.9)	43.9 ± 0.6 (19.8)	44.1 ± 0.7 (19.8)
MUFA	32.0 ± 0.4 (14.9)	32.8 ± 0.4 (14.8)	32.9 ± 0.5 (14.8)
PUFA	22.6 ± 0.3 (10.5)	7.5 ± 0.1 (3.4)	7.5 ± 0.1 (3.4)
P/S ratio	0.89 ± 0.00	0.17 ± 0.00	0.17 ± 0.00
Cholesterol (mg)[b]	323 ± 1	316 ± 1	316 ± 1

[a]Values in parentheses are calculated values for percentage of food energy provided by the nutrient.
[b]Analyzed from daily dietary records using Food Processor II Analyses Software (ESHA Research, Salem, OR).
[c]As determined by gas–liquid chromatography. MCT, medium-chain triglyceride; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; P/S, ratio of PUFA to SFA.

(Table 1). The MCT and lauric diets provided 40 en% total fat, 19.8 en% saturated fat, 14.8 en% monounsaturated fat, and 3.4 en% polyunsaturated fat, and 316 mg cholesterol (Table 1). The test fats furnished over two-thirds of the saturated fat. Dietary fat was present in lean beef (56 g), turkey breast (56 g), and an experimental fat/oil blend. In the baseline diet, the fat/oil blend was 32.7% high-oleic safflower oil, 30.9% safflower oil, 10.9% coconut oil, 12.7% sheanut oil, and 12.7% butter. Commercial miglyol (a 8:0 + 10:0 TG) or trilaurin (42% of the fat blend), obtained from HULS America Inc. (Piscataway, NJ), was the main fat in the experimental fat blends, which also contained 46% high-oleic safflower oil, 6% butter, and 6% coconut oil. The fat blends were used as spread on bread and added to the main dishes at dinner. They were also incorporated into cakes and cookies. The basal menu initially provided 8.1 MJ. The subjects, who were weighed daily, were given extra food energy with the same fat composition to maintain their initial body weight. Food Processor II software was used to calculate the components of the diets by adding data on FA composition in each menu to the software database. (Table 1). The specific FA composition of 1-d composites of each menu was analyzed by gas–liquid chromatography (Table 1).

Biochemical procedures. (i) Serum lipoprotein cholesterol. Total cholesterol (TC) and free cholesterol (FC) in previously frozen serum samples were analyzed by enzymatic assays (Sigma procedure No. 352, St. Louis, MO) (15). Total high density lipoprotein-cholesterol (HDL-C), HDL_2-C (by difference) and HDL_3-C concentrations were determined by the same enzymatic method after precipitation of very low density lipoprotein (VLDL) and low density lipoprotein (LDL) portions with dextran sulfate-Mg^{2+} solution followed by removal of HDL_2-C with a stronger dextran sulfate-Mg^{2+} solution (16). Serum TG concentration was assayed enzymatically (Sigma procedure No. 339). LDL-cholesterol (in mg/dL, LDL-C) was calculated as TC minus [HDL-C and 0.2 (TG)] (17). Data for cholesterol and TG concentrations from two pre- and two postperiod measurements were averaged. The coefficients of variation, evaluated for the assay using previously frozen pool samples, were 0.9% for TC, 1.2% for total HDL-C, 3.2% for HDL_3-C, and 1.6% for TG levels.

(ii) Apolipoprotein (apo) concentration. ApoA-1 and apoB were assayed by a turbidimetric procedure (Raichem, San Diego, CA) in one pre- and one postperiod blood sample which was frozen at −80°C immediately after the blood draw. The coefficient of variation of two control sera provided in the kits was 2.9 and 1.9% for apoA-I and 2.5 and 2.5% for apoB.

(iii) LDL receptor activity. LDL binding and degradation mediated by LDL receptors in mononuclear cells (MNC) were determined as in previous studies (18,19). Briefly, LDL was iodinated 3 d before the blood drawing day using pooled blood obtained from a constant group of donors consuming self-selected diets. The composition of LDL was assumed to be constant throughout the whole study. Pooled serum treated with 0.1% disodium EDTA was used to isolate the LDL fraction from density 1.019 to 1.063 by sequential ultracentrifugation after KBr layering. Then, LDL was radioiodinated after dialysis against phosphate buffer saline to produce a specific activity of 100–600 cpm/ng protein. Free iodine content was less than 3%.

MNC were isolated by centrifugation (800 × *g* for 20 min at 25°C) from blood anticoagulated with sodium citrate and diluted 1:1 with NaCl (0.9%), and the dilute blood was layered over Nycoprep™ (Mediatech, Inc., Herndon, VA). Freshly isolated MNC were then counted and incubated with ^{125}I-LDL (20 μg/mL) in the presence and absence of a 20-fold excess of unlabeled LDL at 37°C for 5 h, a period determined to be suitable for demonstrating differences in ^{125}I-LDL degradation if they existed among treatments (18). After incubation, trichloroacetic acid was added to the samples to achieve a final concentration of 20% in the presence of bovine serum albumin to precipitate undegraded, protein-bound iodotyrosine. The precipitate then was sedimented by centrifugation to obtain supernatant, which was counted in a gamma counter to determine the amount of LDL degraded. Specific degradation was expressed as the difference in degradation in the presence and absence of 20-fold excess unlabeled LDL.

The specific and nonspecific binding activity of LDL receptors was determined by incubating MNC at 4°C with 5, 10, 20, 40, and 80 μg/mL ^{125}I-LDL in the presence and absence of excess unlabeled LDL. After incubation, cells were washed three times with RPMI 1640 medium, collected by centrifugation at 12,000 × *g*, and counted in a gamma counter.

(iv) Lecithin cholesterol acyl transferase (LCAT) and cholesterol ester transport protein (CETP) activity. Endogenous cholesterol esterification (LCAT) and transfer (CETP) were measured by an isotopic method (20). In order to determine esterification rate, fresh serum was preincubated at 4°C for 1 h with ^{3}H-cholesterol-albumin emulsion, and the mixture was incubated at 37°C for 3 h after equilibration of the added cholesterol and endogenous cholesterol. Then lipids were extracted, separated by thin-layer chromatography, and cholesterol ester (CE) and FC spots were scraped and counted by liquid scintillation. To determine CETP, transfer of radiolabeled CE into apoB-containing lipoproteins was measured first by precipitating these lipoproteins from the incubation media with dextran sulfate followed by counting total radioactivity in the incubate, in the HDL-containing supernatant, and in the apoB-containing lipids by liquid scintillation. From these data the rate of CE transfer was calculated.

(v) ApoE phenotype. ApoE phenotype was determined by an isoelectric focusing (IEF) method (21,22) using whole serum samples. After delipidating serum with a dithiothreitol solution overnight, the apoE isoforms were separated in gels of 7.5% polyacrylamide/3 M urea, containing 1:2 ampholine to pharmalyte on an IEF machine (Bio-Rad Bio-Phoresis Horizontal electrophoresis cell, Hercules, CA) with NaOH (1 M) and H_3PO_4 (1 M) as cathode and anode solutions, respectively. After isoelectrofocusing, each lane of apoE phenotype was identified by Western blot using goat anti-human apoE as first antibody, rabbit anti-goat as second antibody, and visualization

with color-developing solution containing β-naphthyl phosphate and Fast Blue BB and $MgSO_4$ in boric acid developer.

(vi) Lipoprotein isolation. A single-spin density gradient ultracentrifugation method (23,24) was used to separate VLDL, LDL, HDL_2, and HDL_3 fractions from whole serum. After blood drawing, 2 mL fresh serum was pipetted into the bottom of a cellulose-nitrate centrifuge tube containing 0.77 g KBr to make a density of 1.25 g/mL, followed by sequentially overlaying with equal amounts of density 1.225 g/mL KBr solution and density 1.1 g/mL KBr solution and, finally, distilled water. Then the tubes were placed in a SW41 rotor (L7-65 Beckman) and centrifuged at 39,000 rpm at 10°C. After 24 h, samples were taken out carefully without shaking and each fraction of lipoprotein was withdrawn by Pasteur pipet.

(vii) LDL size and composition. The composition of LDL isolated as described above was analyzed to estimate the average diameter of LDL obtained from analysis on the two experimental diets. LDL particle diameters were calculated from the core to surface volume ratio using the following formula (25):

$$r = \{[1.093(TG) + 1.044(CE)]/[0.968(FC) + 0.97(PL) + 0.705(PRO)]\} \times 3 \times 21.5 \text{ Å} \quad [1]$$

where $d = 2r + 2 \times 21.5$ Å and r = radius of the particle, d = diameter, PL = phospholipid mass, TG = triglyceride mass, CE = cholesterol ester mass, FC = free cholesterol mass, and PRO = protein mass; 21.5 Å is the assumed thickness of the lipoprotein surface layer; 1.093, 1.044, 0.968, 0.97, and 0.705 are the corresponding specific volumes (26).

LDL-C, TC, and FC concentrations were analyzed by an enzymatic assay (15). LDL-CE value was calculated by difference. LDL-TG concentration was determined by the same assay as the serum TG level. LDL-PL concentration was assayed by an enzymatic colorimetric method (Biochemical Diagnostics reagent, Edgewood, NY). The concentration of LDL-PRO was quantified by total protein reagent (Sigma). The core to surface ratio was calculated as:

$$\text{core/surface} = (4/3\pi r^3)/(4\pi r^2 \times 21.5) = \{[1.093(TG) + 1.044(CE)]/[0.968(FC) + 0.97(PL) + 0.705(PRO)]\} \quad [2]$$

(viii) Fatty acid analysis. Fat was extracted from the blended menu composites with hexane and isopropanol (3:2), and the extracted fat was saponified and methylated with 10% methanolic HCl (27). After heating at 90°C for 2 h, the samples were cooled on ice, and hexane and K_2CO_3 were added. Then a small amount of sodium sulfate and charcoal was added to the extracted hexane fraction followed by centrifugation. The clean hexane layer was analyzed for fatty acyl contents by gas–liquid chromatography (GLC).

Statistical analysis. All data from the beginning and the end of each period and the changes from the baseline produced by the experimental diets were analyzed statistically by MINITAB (Minitab Inc., State College, PA). Differences between the baseline and experimental values were analyzed by paired t-test. Differences between the two diets were analyzed by two-sample t-test. Significance level was set at $\alpha = 0.05$. Relationships among relevant variables were determined by Pearson correlation analysis.

RESULTS

Diet composition. According to computer (Food Processor II) analysis, the experimental diets provided almost the same amount of monounsaturated fatty acids and cholesterol as the baseline diet. There were no significant differences in energy intake between the two preperiods and between the two postperiods. The protein, carbohydrate, and fat intakes as percentages of total energy intake were the same among baseline and the two experimental diets (Table 1). According to the gas–liquid chromatographic analysis, the baseline diet provided three times more PUFA than the experimental diets and only about three-fifths as much SFA (Table 1). Therefore, the PUFA/SFA ratio of the baseline diet was five times higher than the experimental diets. Except for MCT and lauric acid, the contents of all other SFA in the experimental diets were lower than in the baseline diet. Because the carbon chain length of the experimental FA in this study was 12 or less, it was assumed that the absorption of major dietary fats was the same. Therefore, fecal excretion of FA was not measured.

Serum lipoprotein-cholesterol and apo concentrations. The mean ± SEM serum cholesterol concentration of the subjects at screening and representing their usual diet was 167 ± 22 mg/dL. This value was reduced by 10 ± 3 mg/dL after the subjects had consumed the baseline diet for 1 wk during the first period. In comparison to the baseline diet, both MCT (P = 0.032) and lauric acid (P = 0.0001) diets raised mean serum TC concentrations. The 12:0 diet produced higher postperiod values for mean serum TC than the MCT diet (P = 0.021) (Table 2), which tended to return serum cholesterol to levels observed on the subjects' usual, self-selected diet. Among the 18 subjects who consumed the 12:0 diet, TC concentrations were raised in 16 women compared to baseline (Fig. 1), whereas on the MCT diet TC rose in 12 out of 17 subjects (Fig. 2). There was no correlation between changes produced by the two diets (r = −0.058), meaning that individual subjects did not have uniform responses to the two treatments. There was no difference (P > 0.05) in changes in TC among the three different apoE phenotypes represented in this study on either diet.

Both diets increased LDL-C values (P = 0.05 for MCT, P = 0.0016 for 12:0) from baseline value (Table 2). Fifteen out of 18 subjects had increased LDL-C levels after eating the 12:0 diet, in agreement with the changes in TC except for one subject (Fig. 3), and 12 out of 17 subjects had LDL-C increases after consuming the MCT diet, which was also in agreement with the changes in TC except for one subject (Fig. 4). The correlation between changes in LDL-C pro-

TABLE 2
Serum Lipoprotein Concentrations (mean ± SEM) on Two Saturated Fatty Acid Mixtures

Serum parameter	MCT (n = 17)	Lauric acid (n = 18)	Difference between diets
Total cholesterol (mg/dL)			
Preperiod	157 ± 4	162 ± 4	NS
Postperiod	168 ± 4	182 ± 4	S (P = 0.021)
Difference	10 ± 4[a]	20 ± 4[a]	NS (P = 0.14)
LDL-cholesterol (mg/dL)			
Preperiod	88 ± 5	92 ± 4	NS
Postperiod	98 ± 5	107 ± 4	NS (P = 0.17)
Difference	10 ± 5[a]	15 ± 4[a]	NS
HDL-cholesterol (mg/dL)			
Preperiod	56 ± 2	57 ± 2	NS
Postperiod	56 ± 3	62 ± 2	NS (P = 0.089)
Difference	0 ± 1	5 ± 1[a]	S (P = 0.0073)
HDL_2-cholesterol (mg/dL)			
Preperiod	19 ± 2	19 ± 2	NS
Postperiod	16 ± 2	22 ± 2	S (P = 0.044)
Difference	−4 ± 1[a]	2 ± 1[a]	S (P = 0.0002)
HDL_3-cholesterol (mg/dL)			
Preperiod	36 ± 1	37 ± 1	NS
Postperiod	40 ± 1	40 ± 1	NS
Difference	4 ± 1[a]	3 ± 1[a]	NS
Triglycerides (mg/dL)			
Preperiod	66 ± 5	68 ± 5	NS
Postperiod	69 ± 5	66 ± 5	NS
Difference	3 ± 4	−3 ± 5	NS
Apolipoprotein A-1 (mg/dL)			
Preperiod	166 ± 5	167 ± 6	NS
Postperiod	159 ± 6	171 ± 7	NS (P = 0.17)
Difference	−7 ± 8	4 ± 7	NS
Apolipoprotein B (mg/dL)			
Preperiod	69 ± 5	67 ± 4	NS
Postperiod	74 ± 4	73 ± 3	NS
Difference	4 ± 2	6 ± 4	NS

[a]Significant difference between pre- and postperiod values (P < 0.05). NS, nonsignificant; S, significant; LDL, low density lipoprotein; HDL, high density lipoprotein. For other abbreviation see Table 1.

duced by two experimental diets was small (r = −0.267). There was no significant difference in LDL-C changes among the three apoE phenotypes on either diet, even though LDL-C was increased in all three subjects with apoE 4/3 phenotype compared to values on the baseline diet.

Total HDL-C (P = 0.0017), HDL_2-C (P = 0.026), and HDL_3-C (P = 0.0019) all increased on the 12:0 diet, whereas on the MCT diet, HDL_2-C decreased (P = 0.0031), HDL_3-C increased (P = 0.0002), but total HDL-C did not change (Table 2). At the end of treatment, values for serum HDL_2-C differed (P = 0.04) between MCT and 12:0, and values for total HDL-C concentrations appeared lower on MCT than 12:0, suggesting that MCT and 12:0 had different effects on certain parameters of reverse cholesterol transport (RCT). There were no significant differences in TG concentration either on the MCT or on the 12:0 diet or in changes from baseline (Table 2). ApoA-I and apoB concentrations did not change significantly on either diet (Table 2).

Changes in variables associated with RCT. Both serum FC (P = 0.002) and CE (P = 0.003) concentrations increased on the 12:0 diet, but not on the MCT diet (P > 0.05) (Table 3). At the end of the period serum CE concentration was higher on 12:0 than MCT (P = 0.023). The rate of cholesterol esterification (LCAT) rose on the 12:0 diet (P = 0.0003) but was

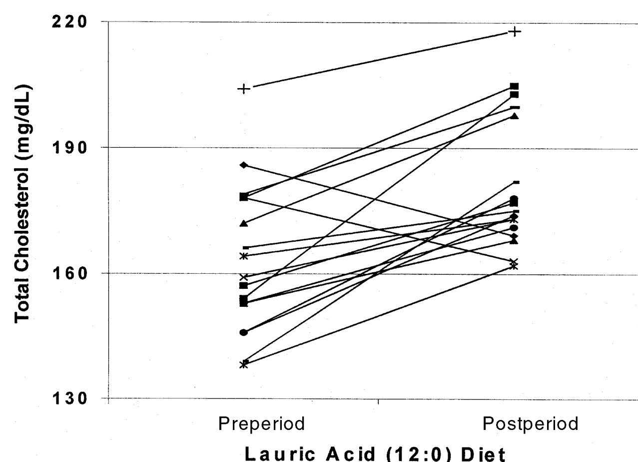

FIG. 2. Individual total cholesterol change on the 12:0 diet.

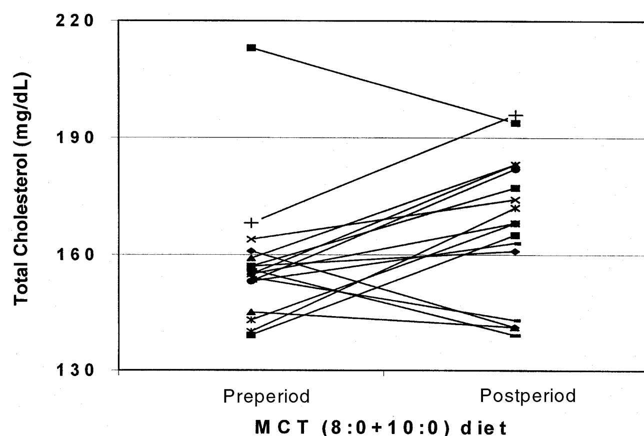

FIG. 1. Individual total cholesterol change on the medium-chain triglyceride (MCT) diet.

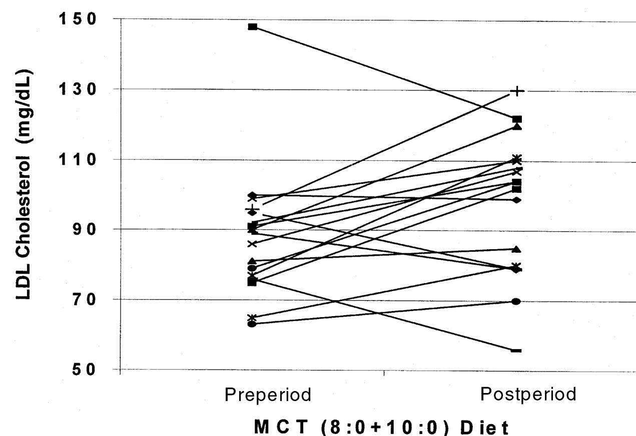

FIG. 3. Individual low density lipoprotein (LDL)-cholesterol change on the MCT diet. For abbreviation see Figure 1.

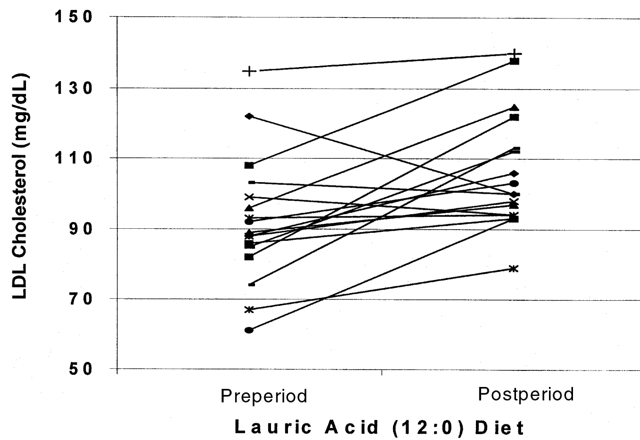

FIG. 4. Individual LDL-cholesterol change on the 12:0 diet. For abbreviation see Figure 3.

TABLE 4
Saturated Fat and LDL Receptor Activity in MNC (mean ± SEM)

MNC variable	MCT (n = 17)	Lauric acid (n = 18)	Difference between diets
Receptor-mediated degradation of ^{125}I-LDL (ng/10^6 MNC/h)			
Preperiod	12 ± 1	14 ± 1	NS (P = 0.19)
Postperiod	17 ± 2	12 ± 1	S (P = 0.033)
Difference	5 ± 1[a]	−3 ± 1[a]	S (P = 0.0001)
Nonspecific degradation of ^{125}I-LDL (ng/10^6 MNC/h)			
Preperiod	7.3 ± 1.1	5.2 ± 0.9	NS (P = 0.14)
Postperiod	7.9 ± 1.3	9.0 ± 1.7	NS
Difference	0.6 ± 1.5	3.7 ± 2.0	NS
Receptor-mediated ^{125}I-LDL binding (ng/10^6 MNC)			
Preperiod	16 ± 2	18 ± 1	NS
Postperiod	21 ± 2	16 ± 1	NS (P = 0.056)
Difference	4 ± 1[a]	−2 ± 1	S (P = 0.0023)

[a]Significant difference between pre- and postperiod values (P < 0.05). MNC, mononuclear cell; for other abbreviations see Tables 1 and 2.

not altered significantly on the MCT diet. However, differences in cholesterol esterification at postperiod were not significant between the two diets. The rate of cholesterol ester transfer (CETP) from HDL to apoB-containing lipoproteins also increased on the 12:0 diet (P = 0.0002). In addition, differences in cholesterol transfer rates at postperiod were significant between the two diets (P = 0.0041). The correlation between changes in CETP and LCAT was moderate on MCT (r = 0.667) and was small on 12:0 (r = 0.452).

Changes in LDL receptor activity in MNC. The rate of receptor-mediated degradation of ^{125}I-LDL increased on the MCT diet (P = 0.0018) (Table 4) although the increase was inconsistent with the rise in serum LDL-C. However, the reduced rate of specific LDL degradation on the 12:0 diet (P = 0.0088) was consistent with the increased serum LDL-C. Postperiod values for specific LDL degradation by MNC were higher on MCT than 12:0 (P = 0.033). The rate or changes in the rate of nonspecific degradation of ^{125}I-LDL (degradation not involving the LDL-receptor) was not significant between the two diets (Table 4). The relationship between ^{125}I-LDL concentration in the incubation media and ^{125}I-LDL binding by MNC is shown in Figure 5 for the baseline and the two experimental diets. Differences among treatments were apparent when the incubation media contained 20 μg ^{125}I-LDL/mL, a concentration at which MCT increased receptor-mediated LDL binding (P = 0.0069). This result was in line with the increased receptor-mediated LDL degradation but not with the increased serum LDL-C levels. Receptor-mediated LDL binding decreased but not significantly (P = 0.14) on the 12:0 diet, a tendency consistent with the decreased receptor-mediated LDL degradation and increased serum LDL-C values. Changes in specific LDL binding induced by the two dietary treatments differed (P = 0.0023), and the lower value for specific binding of LDL by MNC on 12:0 compared to MCT approached significance (P = 0.056). In

TABLE 3
Saturated Fat and Rates of Cholesterol Esterification and Transfer in Serum (mean ± SEM)

Serum variable	MCT (n = 17)	Lauric acid (n = 18)	Difference between diets
Free cholesterol (mg/dL)			
Preperiod	51 ± 2	52 ± 2	NS
Postperiod	55 ± 2	58 ± 2	NS
Difference	3 ± 2	6 ± 2[a]	NS
Cholesterol ester (mg/dL)			
Preperiod	106 ± 3	111 ± 3	NS
Postperiod	113 ± 3	124 ± 3	S (P = 0.023)
Difference	7 ± 4	14 ± 4[a]	NS
Rate of cholesterol esterification (μmol/L/h)			
Preperiod	50 ± 3	50 ± 3	NS
Postperiod	53 ± 4	59 ± 3	NS
Difference	3 ± 4	8 ± 2[a]	NS
Rate of cholesterol ester transfer (μmol/L/h)			
Preperiod	27 ± 2	25 ± 2	NS
Postperiod	27 ± 2	30 ± 2	NS (P = 0.12)
Difference	−1 ± 1	5 ± 1[a]	S (P = 0.0041)

[a]Significant difference between pre- and postperiod values (P < 0.05). For abbreviations see Tables 1 and 2.

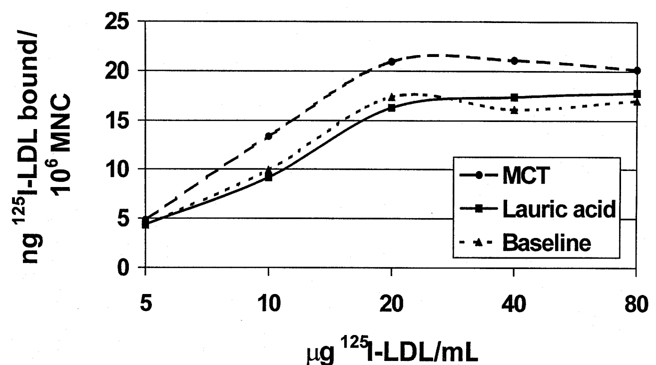

FIG. 5. Binding of ^{125}I-LDL to LDL receptors when freshly isolated mononuclear cells (MNC) obtained from subjects eating two saturated fat mixtures were incubated with different amounts of ^{125}I-LDL. (Baseline n = 35, MCT n = 17, lauric acid n = 18). For abbreviations see Figures 1 and 3.

addition, the correlation between changes in receptor-mediated LDL degradation and binding was small on 12:0 (r = 0.422) and was not demonstrated on MCT (r = –0.153).

The associations of changes in LDL-C with mechanisms of LDL removal were various. Except for the moderate correlations between changes in LDL-C concentration and specific LDL (r = 0.528) and nonspecific LDL (r = 0.499) degradation on MCT, the changes in serum LDL-C concentration were not correlated with changes in receptor-mediated binding on either diet.

Effect of different SFA on LDL size and composition. Although no significant LDL diameter changes were observed after either dietary treatment, and the postperiod LDL diameters were not different, there was a slight tendency for LDL diameter to increase in subjects fed MCT (P = 0.069) and 12:0 (P = 0.11) (Table 5). There were some compositional changes in LDL that occurred during the treatment periods, but the changes effected by the two diets were not different (P > 0.05). LDL-FC concentration increased on 12:0 (P = 0.028) but was not significantly different on MCT. LDL-TG (P = 0.0008) and PL (P = 0.0092) concentrations increased but LDL-PRO (P = 0.028) decreased on the MCT diet. Furthermore, although the core to surface ratio was not altered significantly on either diet, the ratio tended to increase on MCT (P = 0.069) and 12:0 (P = 0.11) compared to the PUFA baseline diet.

TABLE 5
LDL Composition (mean ± SEM) from Subjects Fed Two Saturated Fat Diets

Parameter	MCT (n = 17)	Lauric acid (n = 18)	Difference between diets
LDL diameter (Å)			
Preperiod	172 ± 8	175 ± 6	NS
Postperiod	188 ± 4	187 ± 3	NS
Difference	15 ± 8	12 ± 7	NS
LDL-free cholesterol (mg/dL)			
Preperiod	19 ± 1	18 ± 1	NS
Postperiod	18 ± 1	19 ± 1	NS
Difference	–1 ± 1	2 ± 1[a]	NS (P = 0.054)
LDL-cholesterol ester (mg/dL)			
Preperiod	61 ± 3	61 ± 3	NS
Postperiod	65 ± 4	68 ± 2	NS
Difference	4 ± 2	7 ± 4	NS
LDL-triglyceride (mg/dL)			
Preperiod	26 ± 2	29 ± 3	NS
Postperiod	32 ± 2	32 ± 2	NS
Difference	6 ± 1[a]	3 ± 2	NS
LDL-phospholipid (mg/dL)			
Preperiod	47 ± 3	49 ± 3	NS
Postperiod	51 ± 2	53 ± 1	NS
Difference	5 ± 2[a]	4 ± 2	NS
LDL-protein (mg/dL)			
Preperiod	47 ± 6	42 ± 3	NS
Postperiod	35 ± 2	35 ± 2	NS
Difference	–13 ± 5[a]	–7 ± 5	NS
Core/surface			
Preperiod	1.00 ± 0.06	1.02 ± 0.05	NS
Postperiod	1.12 ± 0.03	1.12 ± 0.03	NS
Difference	0.12 ± 0.06	0.09 ± 0.06	NS

[a]Significant difference between pre- and postperiod values (P < 0.05). For abbreviations see Tables 1 and 2.

DISCUSSION

This is the most thorough study to date of the effects of MCT on lipoprotein metabolism in humans. The objective was to compare the effects of diets providing MCT (8:0 + 10:0) or lauric acid (12:0) with each other and with a baseline diet having half as much saturated fat, three times as much polyunsaturated fat, and the same amount of total fat, monounsaturated fat, and cholesterol. Because MCT and lauric acid are normally obtained from imported tropical oils, they were fed in greater amounts (14 en%) in this study than are typically consumed in the United States, except possibly by persons consuming certain formula diets or taking supplements. The major findings were (i) that both MCT and 12:0 raised serum LDL-C concentrations compared to the more polyunsaturated baseline diet. The effect of MCT was about two-thirds that of 12:0. (ii) Lauric acid produced higher levels of serum TC than MCT partly because of its effect on HDL_2. (iii) The MCT diet suppressed or did not affect some measures of RCT in many subjects whereas 12:0 consistently enhanced the same parameters compared to the PUFA diet. (iv) Increases in serum LDL-C in subjects consuming MCT were positively associated with higher rates of specific LDL degradation, not lower rates as might be expected. These results suggest that MCT and 12:0 have somewhat different effects on lipoprotein metabolism despite their closeness in chain length and the fact that MCT and at least some 12:0 enter the blood *via* the portal circulation (28).

The influence of MCT and lauric acid on LDL-C concentration. The effect of MCT on blood TC and LDL-C concentrations is somewhat controversial. Several human feeding trials with MCT, dating back to 1959, are summarized in Table 6. These studies differed considerably in the amount of MCT fed (5–40 en%), in the type of fat fed along with the MCT (none or a blend of different fats), in the type of diet fed (conventional foods vs. liquid formula diets), in the comparison fat (linoleic acid, oleic acid, other SFA, or a fat-free diet), in the length of the feeding period (5–28 d), and in the type of subject used (healthy vs. hospital patients, men vs. women).

In early studies (29,30), in which MCT or comparison fats were usually fed alone and in high amounts, MCT appeared to be considerably less cholesterolemic than butter or coconut oil. Total blood cholesterol levels were measured in these studies, not HDL-C or LDL-C. In more recent studies (12,31, 32, and this one), diets providing MCT in varying amounts (5–43 en%) had similar effects on blood cholesterol and LDL-C levels as the subjects' usual (presumably not heart-healthy) diets or a baseline diet high in butter or tropical oils. Either the latter studies were short in duration (31) or the MCT diets contained the minimum recommended levels of 18:2 in addition to MCT and other FA (12,32, and this study). These more recent studies consistently indicated that in con-

TABLE 6
Summary of Studies on the Effect of Diets with MCT on Blood Total and LDL-Cholesterol Levels[a]

Investigator, date (ref. no.)	Subjects	Baseline diet	Treatment or comparison diets	Results	Comments
Hashim *et al.*, 1960 (29)	8 middle-aged male and female hospital patients	Regular hospital diet	40 en% corn oil, butter, or MCT diets for 2 wk (butter) or 4 wk (MCT, corn oil). Patients consumed 2 of the 3 test fats.	In corn oil–MCT comparisons, MCT produced 8–9% higher TC; in butter–MCT comparisons, butter produced 20–50% higher TC.	MCT diet was devoid of POLY; it took up to 4 wk to establish a steady-state response to the MCT diet.
Beveridge *et al.*, 1959 (30)	83 college males and females	Fat-free diet was fed for 8 d to all subjects	One of 8 treatment diets was fed for 8 d and replaced 30 en% of carbohydrate in the baseline diet. Treatments were MCT, coconut oil, and distilled fractions of butter with or without added cholesterol.	MCT diets raised TC 5–7% compared to the fat-free diet. Coconut oil raised TC by 17%. Butter diets raised TC up to 60%.	MCT was not neutral contrasted with the fat-free diet, but it was considerably less cholesterolemic than coconut oil or the butter fractions. Treatment diets were low in or devoid of 18:2. Periods were short.
Snook *et al.*, 1985 (33)	12 college-age males and females	Conventional food, butter-based diet providing 42 en% fat was fed for 1 wk.	Liquid formula diets, with 32 en% fat, were fed for 1 wk in a cross-over design. Corn oil was the sole fat in Diet 1. MCT replaced some POLY in Diet 2.	Compared to the butter-based diet, Diet 1 lowered TC by 24% in 1 wk. Diet 2 lowered TC by 12% in 1 wk.	Replacement of some of the POLY in the formula diet with MCT reduced the cholesterol-lowering properties of the formula. Study periods were short.
Hill *et al.*, 1990 (31)	10 healthy males	Usual diet	Liquid formula diets with 40 en% fat were fed for 5 d in a cross-over design. Fat was soybean oil or MCT.	Soybean oil lowered TC by 15% in 5 d while MCT produced no change compared to usual diet.	POLY diet lowered TC compared to usual diet while MCT did not. The study was short. The MCT diet was devoid of 18:2.
Cater *et al.*, 1997 (32)	9 males with documented coronary artery disease	Outpatient *ad libitum* diet	Diets with 53 en% fat, 43 en% of which was palm oil, high-oleic sunflower oil or MCT, were given for 3 wk periods in cross-over design. All diets had at least 3 en% PUFA. Palm oil diet provided 20 en% 16:0.	TC and LDL-C were 12 and 15% higher, respectively, on MCT than MONO diet. Effects of MCT and palm oil did not differ.	MCT raised TC and LDL-C compared to MONO diet. According to authors, cholesterol-raising potency of MCT appeared to be half that of 16:0. All diets provided at least 3 en% 18:2. Periods were of adequate length.
Wardlaw *et al.*, 1995 (12)	34 males (study 1) and 14 males (study 2) with mildly elevated cholesterol	Diets had 38–41 en% total fat and 15–17 en% 12:0–16:0 provided by palm oil/palm kernel oil (study 1) or butter (study 2) and were fed for 3 wk.	MCT + 22:0 replaced about 10 en% of the 12:0–16:0 for 6 wk in diets of half the men. The other half continued on the baseline diet.	TC and LDL-C were not altered; HDL was higher on palm kernel oil than on the MCT + 22:0 diet.	MCT, butter, and palm oil/palm kernel oil had equivalent effects on TC when about 5 en% 18:2 was in all diets. Cholesterolemic effect of 22:0 is not known.
Tsai *et al.*, 1999 (this paper)	17–18 healthy, premenopausal females	40 en% fat diet with 10 en% PUFA and 10 en% saturated fat was fed for 1 wk.	40 en% fat diets with 3.4 en% PUFA and 14 en% MCT or 12:0 were fed for 4 wk in cross-over design.	MCT raised TC and LDL-C by 7 and 11%, respectively. 12:0 raised TC and LDL-C by 12 and 16%, respectively.	When substituted for the POLY baseline diet, MCT had about 2/3 the cholesterol-raising potency of 12:0.

[a]TC, total cholesterol; POLY, dietary polyunsaturated fat; MONO, dietary monounsaturated fat; LDL-C, LDL-cholesterol; 12:0–16:0, dietary content of lauric, myristic, and palmitic acids, the saturated fatty acids considered to be the most cholesterolemic; for other abbreviations see Tables 1 and 2.

trast to diets high in PUFA, MCT diets produced 8–15% higher levels of TC in serum or plasma (29,31,33, and this study). Furthermore, an MCT diet resulted in 12 or 8–9% higher levels of TC than a high monounsaturated FA diet (32) or a fat-free diet (29), respectively. One of the multiple regression equations developed by Hegsted *et al*. (11) predicted the effects of seven FA and dietary cholesterol on serum concentrations of TC. The positive partial regression coefficient describing the effect of 10:0 was about two-thirds that of 12:0 and was positive in contrast to the negative coefficients for 18:0, 18:1, and especially 18:2. In a companion study to the one reported herein (34), mean serum concentrations of TC and LDL-C were the same on the PUFA baseline diet as on a diet with 14 en% 18:0, suggesting, as did the work of Hegsted *et al*. (11), that 18:0 is less cholesterolemic than MCT. In summary, in this study, MCT was more cholesterolemic than 18:2 and less so than 12:0, which has been shown to raise blood cholesterol concentrations in previous studies (11,13, 14). In addition, reports in the literature suggest MCT is more cholesterolemic than 18:1 and 18:0 and may not lower blood cholesterol levels in some cases when substituted for self-selected diets or for 12:0–16:0 SFA. Thus, when an effect on serum cholesterol or LDL-C concentrations is the criterion, MCT is not a convincing heart-healthy substitute for other saturated fats. However, MCT do have some unique effects on lipoprotein metabolism that merit further attention.

The influence of MCT and lauric acid on intravascular lipoprotein metabolism. Despite the fact that both MCT and 12:0 raised blood cholesterol levels when substituted for PUFA, their influence on cholesterol metabolism was not identical. For example, the lauric acid diet enhanced some factors involved in RCT, but MCT sometimes had a different or less pronounced effect. In comparison to corresponding baseline values, the mean rates of cholesterol esterification (LCAT) and transfer (CETP) increased significantly on the lauric acid diet but not on the MCT diet, although there was less conformity in the response to MCT. The concentrations of both HDL_2-C and HDL_3-C increased when lauric acid replaced PUFA whereas the concentration of HDL_2-C declined and HDL_3-C increased in serum when subjects consumed MCT instead. This finding suggests the MCT diet reduced conversion of HDL_3-C to HDL_2-C, which involves TG-rich lipoproteins, LCAT, CETP, and lipoprotein lipase, or increased reconversion of HDL_2 to HDL_3, which involves removal of lipid components by the liver (35). Previous studies (12,36–38) indicated that MCT decrease HDL-C levels. One explanation is that MCT reduces chylomicron production, and chylomicron metabolites are used in part to produce the HDL fraction (39). In this study, total HDL-C concentrations were not changed by the MCT diet. Cater *et al*. (32) also found that MCT did not influence total HDL-C levels. Furthermore, serum TG level was increased on MCT in many studies (31,32,37) possibly because MCT may enhance hepatic *de novo* FA synthesis. In this study, serum TG levels were not changed by MCT feeding although LDL-TG levels were higher on the MCT diet than on the baseline ($P < 0.05$). In summary, in this study and in a companion study (34), SFA ranging in chain length from 8:0 to 18:0 had subtly different effects on intravascular lipoprotein remodeling. The physiological implications of these differences are unclear at this point.

The influence of MCT and lauric acid on LDL receptor activity. MCT, but not lauric acid, had an unusual effect on LDL receptor activity. In a previous study (18), we noted that changes in serum LDL cholesterol concentration and specific LDL degradation were moderately and negatively associated ($r = -0.4$ to -0.6) when diets high (>20 en%) in either saturated fat (butter) or polyunsaturated fat (corn oil) were compared, an effect that might be expected. Curiously, in this study, receptor-mediated degradation of LDL in MNC was positively associated ($r = 0.53$) with an increase in LDL-C concentration in serum on the MCT diet despite the fact that polyunsaturated fat, which is presumed to upregulate the LDL receptor, was lower on the MCT diet than on the baseline comparison diet. In agreement with our observation, Woollett *et al*. (40) noted in hamsters that addition of MCT and cholesterol to a chow diet increased receptor-mediated LDL transport and LDL-cholesterol production but did not alter plasma LDL-C concentrations.

When the intake of either cholesterol or SFA increases, a marked increase of LDL-C delivery into the plasma and a reduction of the receptor-dependent LDL uptake by the liver occur in hamsters (41). With both the decreased hepatic clearance and increased LDL production, the plasma LDL-C level elevates progressively until the velocity of the remaining receptor-dependent and -independent activities in the various tissues achieve a rate of LDL-C clearance equaling the rate of LDL-C introduction into the plasma (42). Another determinant of the plasma LDL-C is the rate of LDL-C entry into plasma. According to Woollett *et al*. (41), both MCT and longer-chain FA (mainly 12:0) had similar effects on the rate of delivery of LDL-C into the plasma pool. As reported in one baboon study (43), this increased LDL-C delivery rate might result from the increased production rate of cholesterol-rich particles. The increased rate of LDL-C delivery may be associated with an elevated rate of hepatic apoB synthesis, an elevated rate of conversion of VLDL to LDL, or an elevated transfer of cholesterol from other lipoproteins (44). In addition, although MCT and longer-chain SFA are delivered to the liver differently (45,46), both of them could expand the hepatic lipid pool either by *de novo* FA synthesis from acetyl CoA or *via* chylomicron remnants, which may increase the secretion of hepatic VLDL and then increase LDL-C production (47,48). Since in this study both plasma apoB level and CETP activity on the MCT diet did not change, a high rate of VLDL conversion to LDL may cause LDL-C level to increase. Meanwhile, as suggested by Cater *et al*. (32), the newly synthesized elongated FA from MCT in the liver could act like dietary longer-chain SFA. Therefore, a single parameter such as LDL receptor activity, may not predict the change in plasma LDL-C level. In agreement with the hamster study by Woollett *et al*. (41), the feeding of 12:0 suppressed LDL

receptor activity and increased LDL-C levels in the plasma in this study.

The composition of LDL as well as receptor activity influences LDL turnover (49). When we assayed LDL receptor activity in MNC, we used a constant donor pool as the source of LDL in order to eliminate the effect of dietary-induced changes in LDL composition. Thus, there is a possibility that MCT-induced changes in the composition of LDL of the subjects may influence the uptake of this particle by LDL receptors. There was an indication in this study that LDL particle size (diameter) increased although not significantly, and LDL number (protein content) declined when MCT or 12:0 replaced the baseline diet (Table 5). However, the two diets did not differ significantly in their effect on LDL composition.

Although receptor-mediated LDL degradation and serum LDL-C concentrations were positively associated with each other when the subjects consumed MCT, there was a significant negative correlation between LDL degradation activity and serum TG concentrations ($r = -0.54$, $P < 0.05$). Since VLDL remnants are normally cleared from the plasma by hepatic LDL receptors without conversion to LDL (50–52), LDL receptor activity is not only associated with LDL-C clearance. Possibly, the output by liver of TG-rich particles increased on MCT. Since serum TG concentrations did not increase significantly, the TG-rich lipoproteins may have been cleared from serum relatively rapidly at the expense of uptake of cholesterol-rich particles *via* a receptor-mediated mechanism. This may be one of the factors to promote upregulation of receptor-mediated activity.

In summary, the results of this study indicate that lipoproteins produced during MCT and lauric acid feeding are metabolized somewhat differently. An enhancement of LDL receptor activity in MNC, which presumably is coordinated with an enhancement in liver LDL receptors, did not guarantee a reduction in LDL-C concentration in serum. Not only receptor-mediated uptake of LDL but also rate of LDL-C production, RCT, rates of efflux and clearance of individual lipoproteins, and gene expression of various apo may be involved in mediating the lipoprotein response to the fats fed in this study. These variables need further investigation before MCT can be promoted for use in therapeutic diets and designer fats.

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Hepatic Zonation of the Formation and Hydrolysis of Cholesteryl Esters in Periportal and Perivenous Parenchymal Cells

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ABSTRACT: The periportal (PP) and perivenous (PV) zones of the liver acinus differ in enzyme complements and capacities for cholesterol and bile acid synthesis and other metabolic processes. The aim of this investigation was to determine the acinar distribution of the catalytic activity of the enzymes governing the formation and hydrolysis of cholesteryl esters using PP and PV hepatocytes from normal or cholestyramine-fed rats. The hepatocyte subpopulations were isolated by centrifugal elutriation, characterized according to the distribution pattern of a number of cell parameters and marker enzymes, and assayed for acyl-CoA:cholesterol acyltransferase (ACAT) and lysosomal, cytosolic and microsomal cholesteryl ester hydrolase (CEH). In normally fed rats, no zonation was found in the activity of lysosomal CEH and ACAT, and the activity of both cytosolic and microsomal CEH zonated toward the PV zone of the acinus. Concentrations of free and esterified cholesterol in homogenates, cytosol, and microsomes of PP and PV cells were, however, similar. Cholestyramine raised significantly the PV/PP ratio of ACAT because of an exclusive PP reduction of activity and abolished the heterogeneity in microsomal CEH because of a greater inhibitory PV response, whereas the PV dominance of cytosolic CEH and the homogeneous distribution of lysosomal CEH were unaffected. These results demonstrated homogeneity within the liver acinus for the enzymatic degradation of endocyted lipoprotein-derived cholesteryl esters, a structural zonation of the cytosolic CEH and a dynamic zonation of ACAT and the microsomal CEH, with a PV dominance of the enzymatic capacity for the degradation of stored cholesteryl esters in normal livers.

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That the microcirculation-related heterogeneity of parenchymal liver cells is an important factor in the regulation of many liver functions is firmly established (see 1). Cells in the periportal (PP) afferent zone are exposed to blood richer in oxygen, substrates, hormones, and mediators than cells in the perivenous (PV) efferent zone, and the two subpopulations have been shown to differ in their subcellular structures and enzyme complements and therefore to elicit different functional capacities (1). Different types of zonation may, however, exist. Zonation of nitrogen metabolism seems to be static and structural, whereas that of carbohydrate and lipid metabolism seems dynamic and functional.

The liver is the central organ in maintaining whole body cholesterol homeostasis. It is responsible for the uptake of cholesterol from the circulating lipoproteins and the subsequent excretion of cholesterol from the body into bile, and it is also a major site for the synthesis of free and esterified cholesterol and for their secretion into plasma in new lipoproteins. These multiple functions are mainly carried out by the parenchymal cells. A number of processes involved in cholesterol metabolism have been found to be heterogeneously distributed in the PP and PV liver parenchyma. Most of the available data result from studies on the enzymes 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) reductase and cholesterol 7α-hydroxylase (C7αH), rate-limiting cholesterol and bile acid synthesis (2,3), and on bile formation itself. In intact livers of normally fed rats, HMG-CoA reductase was found to be located mainly in the PP zone (4,5), whereas under simultaneous administration of the HMG-CoA reductase inhibitor, mevinolin, and of the bile acid sequestrant, cholestyramine, HMG-CoA reductase was induced in all hepatocytes (5,6). The capacity for bile formation predominates in hepatocytes that are localized periportally (7), although C7αH activity has been reported to be approximately eightfold higher in PV as compared to PP hepatocytes, and mass production of bile acids by cultured hepatocytes showed a PV/PP ratio of 4.4 (8). These apparently contradictory observations can be explained by greater feedback inhibition of bile acid synthesis by bile acids returning to the liver in the enterohepatic circulation in the PP zone (9). Hence, cholestyramine, which releases this feedback inhibition (10), decreases the PV/PP ratio of C7αH activity (8). In view of the separated dominance of the enzymes governing cholesterol and bile acid synthesis, and to fulfill cellular and body cholesterol homeostasis, we hypothesize that the cholesterol-cholesteryl ester (CE) cycle would also exhibit heterogeneity within the liver acinus. To date, however, no information is available about such zonal regulation.

The metabolism of CE in the liver is primarily controlled

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Abbreviations: ACAT, acyl-CoA:cholesterol acyltransferase; C7αH, cholesterol 7α-hydroxylase; cCEH, cytosolic cholesteryl ester hydrolase; CE, cholesteryl ester; CEH; cholesteryl ester hydrolase; HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; lCEH, lysosomal CEH; mCEH, microsomal CEH; PP, periportal; PV, perivenous; SEM, standard error of the mean; TG, triacylglycerol.

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at four levels. Esterification of cholesterol with long-chain fatty acyl-CoA to form CE is accomplished by the action of acyl-CoA:cholesterol acyltransferase (ACAT), which is an integral membrane protein located in the endoplasmic reticulum and allosterically regulated by cholesterol (recently reviewed in 11). CE and triacylglycerols (TG) entering hepatocytes *via* receptor-mediated endocytosis of plasma lipoproteins are broken down by the lysosomal cholesteryl ester hydrolase (lCEH) or lysosomal acid lipase (12). The resulting cholesterol is released into the cytosol and plays a key role in cellular sterol homeostasis (13,14). The hydrolysis of CE from intracellular lipid is achieved by the two neutral cholesteryl ester hydrolase (CEH) isoforms located in cytosol (cCEH) and microsomes (mCEH) (15). Although the contribution of the latter to the mobilization of metabolically active free cholesterol is greater in the female than in the male rat liver, the cCEH is the dominant activity in both sexes (16,17). The aim of this work was to investigate the acinar distribution of the specific activity of the enzymes governing the formation and hydrolysis of CE using PP and PV liver parenchymal cells. The flexibility of zonation was evaluated under conditions that are known to perturb the cholesterol flux across the liver, namely, in hepatocytes isolated from rats with a portal bile acid load decreased by cholestyramine feeding, which upregulates C7αH and HMG-CoA reductase (10). Separation of liver parenchymal cells into subpopulations originating from the PP and PV regions has been used to study zonal differences in a wide variety of metabolic liver functions (i.e., see 1). The main limitation of this approach is that the conditions used *ex vivo* do not necessarily reflect the situation that occurs *in vivo*. Notwithstanding, this approach allows the quantitative assessment of functional parameters and rates of metabolic processes under defined conditions. Our findings demonstrate homogeneity within the liver acinus for the acid lysosomal hydrolysis of CE and complex zonation of the formation and neutral hydrolysis of CE, with an acinar location-dependent response of each individual enzyme activity to cholestyramine.

EXPERIMENTAL PROCEDURES

Animals and chemicals. Female Sprague-Dawley rats weighing 200 g were housed under constant day length (12 h, lights off 0300–1500) and temperature (25°C), and allowed access to food and tap water *ad libitum*. Rats were fed either a normal low-fat pellet diet or the same diet supplemented with 5% (w/w) cholestyramine (Laboratorios Rubió, Barcelona, Spain). After 7 d, the rats were used at the sixth hour of the dark period. 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 Amersham International plc (Buckinghamshire, United Kingdom). Cholesteryl [^{3}H]oleate (47 Ci/mmol) was from DuPont New England Nuclear (Boston, MA). Collagenase was purchased from Boehringer Mannheim GmbH (Mannheim, Germany). All other chemicals were from Sigma Chemical Co. (St. Louis, MO).

Preparation and characterization of PP and PV hepatocytes. Liver parenchymal cells were isolated by the two-step collagenase perfusion technique of Seglen (18) and fractionated by centrifugal elutriation as described in detail previously (19). Briefly, cells were washed twice in Krebs-Henseleit buffer containing 20 mM glucose and 2.5 mM $CaCl_2$ and once in the so-called incubation medium consisting of Dulbecco's modified Eagle's medium supplemented with 20 mM glucose, 1% bovine serum albumin, 10 mM pyruvate, 10 mM HEPES, and 40 mM sodium bicarbonate. Hepatocytes were fractionated using a Beckman JE6-B elutriator rotor on a Beckman J2-21 centrifuge adapted for operation at low speed. Routinely, 120×10^6 cells suspended in 6 mL of the incubation medium were submitted to centrifugal elutriation. PP and PV cells were eluted from the rotor using Krebs-Henseleit buffer with 1 mM $CaCl_2$, 5 mM glucose, 10 mM pyruvate, 6.5 mM HEPES, and 0.05% gelatin (elutriation buffer) by changes in rotor speed and buffer flow rate. Four populations of cells were recovered at 950 rpm–18 mL/min, 950 rpm–21 mL/min (PP cells), 950 rpm–24 mL/min, and 880 rpm–24 mL/min (PV cells). Cells were counted and sized using a Coulter counter (Multisizer II) with a 70-µm diameter aperture. The final hepatocyte suspension contained cells from two animals with a viability, as determined by Trypan Blue exclusion, exceeding 85%. The enrichment of PP and PV parenchymal cells was monitored by the distribution pattern of a number of marker enzymes and cell parameters that have been shown to reflect the asymmetry. Alanine and tyrosine aminotransferase activities were measured with a commercial assay kit (Sigma Chemical Co.) and according to Granner and Tomkins (20), respectively. The size, cytoplasmic complexity, mitochondria content, and ploidy of the PP and PV cells were determined in a flow cytometer (EPICS, model 752; Coulter, Hialeh, FL) equipped with an argon laser, using conventional fluorescent dyes (21,22). The estimated PP/PV ratio of alanine aminotransferase activity (1.5) and tyrosine aminotransferase activity (1.2) was in good agreement with published values (22–24), as was the size (PP, 18.69 ± 0.13 µm; PV, 23.13 ± 0.15 µm; means ± SEM for 20 preparations), cytoplasmic complexity (PP/PV ratio of 0.6), mitochondria content (PP/PV ratio of 1.4), and ploidy (higher in PV than in PP) (21–24). According to a dual parameter statistical analysis performed by the EPICS flow cytometer that considers the data of cytoplasmic complexity and size, the purity of the preparations in PP and PV hepatocytes averaged 93 and 72%, respectively, and cross-contamination of the populations was not significant.

Determination of ACAT, and lysosomal, microsomal, and cytosolic cholesterol esterase activities. The cell pellets were homogenized and submitted to differential centrifugation as described earlier (25). The lysosome-enriched fraction was resuspended in 250 mM sucrose solution at a 1 mg/mL protein concentration, and the microsome fraction in 20 mM Tris-HCl buffer pH 7.4 containing 250 mM sucrose at a protein concentration of 5 mg/mL. ACAT activity was assayed in microsomes under saturation cholesterol conditions by

measuring the rate of transfer of labeling from [1-^{14}C]oleoyl-CoA to cholesterol as described previously (26). CEH activity was estimated in lysosomes, cytosol, and microsomes, in terms of the release of labeled oleic acid from a cholesteryl [1-^{14}C]oleate micellar substrate as described previously (26).

Other analytical methods and statistical analysis. Protein was determined by the dye-binding method of Bradford (27) using bovine serum albumin as standard. TG and free and esterified cholesterol in cell homogenates, cytosol, and microsomes were quantified by the method of Ruiz and Ochoa (28) using a Bio Image image analysis system equipped with a Kodak Videk Megaplus digital camera and Whole Band Sun View commercial software from Bio Image Corporation (Ann Arbor, MI). Data were analyzed by Student's *t*-test, with $P \leq 0.05$ taken as indicating a statistically significant difference.

RESULTS

The present study was aimed at investigating the functional heterogeneity of liver PP and PV parenchymal cells for the metabolism of CE. To this end, specific activities of the enzymes catalyzing the formation and hydrolysis of CE were measured in PP and PV hepatocytes isolated from rats fed a normal diet or the same diet supplemented with cholestyramine. Table 1 shows that, in normally fed rats, ACAT activity and lysosomal acid lipase activity in PP cells did not differ from those in PV cells, while both neutral cCEH and mCEH activities zonated toward the PV zone of the acinus. It seems that the ratio between the enzymatic capacities for the esterification of cholesterol and for the hydrolysis of CE from lipoproteins taken up by receptor-mediated endocytosis does not depend on the acinar location of the hepatocyte, while the ratio between the enzymatic capacities for cholesterol esterification and for the mobilization of stored CE by cCEH and mCEH is moderate, though significantly higher in PP than in PV hepatocytes. This relative PP dominance of the esterification side of the cholesterol–CE cycle was not, however, accompanied by a higher net content of CE. Table 2 shows that the levels of TG, and free and esterified cholesterol in cell homogenates, cytosolic fractions, and microsomal fractions did not differ substantially in PP and PV cells. Nevertheless, the ratios of free to esterified cholesterol and of TG to esterified cholesterol were about 35-40% higher in PP cell microsomes. This suggests a superior relative availability of the former lipids in PP hepatocytes and of CE in PV hepatocytes for those metabolic events involving the endoplasmic reticulum.

Adding cholestyramine to the diet is well known to drain cell cholesterol to bile acids. This upregulates cholesterogenesis and low density lipoprotein receptor-mediated lipoprotein uptake, this latter leading to a depressed portal load of plasma lipoproteins (10). As shown in Table 1, PP and PV hepatocytes responded to cholestyramine with specific changes of activity of the enzymes controlling the cholesterol-CE cycle. Esterification of cholesterol decreased selectively in hepatocytes from the PP zone, which elevated the PV/PP ratio of ACAT activity from 1 to near 1.5. The specific activity of the acid CEH dropped by about 50% in both types of hepatocyte subpopulations, hence the homogeneous distribution of the lCEH activity within the liver acinus was not altered significantly by cholestyramine. The PV dominance of the cCEH activity was also maintained, since no significant change in activity was found in the two cell subpopulations. And finally, the initial asymmetry of the mCEH was abolished because of an inhibition of activity which was more prominent in PV than in PP hepatocytes. In consequence, the ratio between the activities of ACAT and lCEH doubled in PV cells, and the ratio between ACAT activity and neutral CEH activity, considered as the addition of cCEH and mCEH, increased also exclusively in hepatocytes from the PV zone. Therefore, the two ratios zonated toward the PV zone of the acinus by cholestyramine. It is also of interest to note that both the formation and the hydrolysis of CE occur at lower rates in hepatocytes isolated from cholestyramine-fed rats.

The contribution of non-PP-non-PV hepatocytes to CE metabolism and confirmation of cholestyramine effects were assessed in aliquots of the whole hepatocyte suspensions used for Table 1 taken prior to elutriation. Cells were homogenized and subfractionated, and enzyme activities were determined in the corresponding subcellular fraction simultaneously with those performed in fractions from PP and PV hepatocytes.

TABLE 1
Specific Activities of the Enzymes Catalyzing the Formation and Hydrolysis of Cholesteryl Esters in Periportal (PP) and Perivenous (PV) Hepatocytes from Rats Fed a Normal or a Cholestyramine Diet[a]

	Normal diet			Cholestyramine diet		
Enzyme	PP cells	PV cells	PV/PP ratio	PP cells	PV cells	PV/PP ratio
ACAT (pmol/min/mg protein)	2315 ± 435 (8)	2409 ± 410 (8)	1.04	1667 ± 336 (6)[d]	2497 ± 303 (6)[a]	1.50
lCEH (pmol/min/mg protein)	3575 ± 505 (8)	3300 ± 304 (8)	0.92	1719 ± 179 (6)[d]	1591 ± 153 (6)[d]	0.93
cCEH (pmol/min/mg protein)	24.4 ± 3.3 (10)	32.4 ± 3.0 (10)[c]	1.33	23.9 ± 3.6 (6)	34.8 ± 4.4 (6)[b]	1.46
mCEH (pmol/min/mg protein)	67.9 ± 10.1 (10)	83.4 ± 12.3 (10)[c]	1.23	50.6 ± 4.9 (7)[d]	52.9 ± 5.1 (7)[d]	1.05
Ratio ACAT/lCEH	0.648 ± 0.093	0.730 ± 0.108	1.13	0.969 ± 0.152	1.563 ± 0.144[a,d]	1.61
Ratio ACAT/(cCEH + mCEH)	40.76 ± 5.11	20.80 ± 2.60[c]	0.51	22.37 ± 3.11[d]	28.34 ± 2.96	1.27

[a]The specific activity of ACAT and lysosomal, cytosolic, and microsomal CEH was determined in the corresponding subcellular fraction of PP and PV hepatocytes isolated from normal or cholestyramine-fed rats. All values are means ± SEM for the number of preparations indicated in parentheses. Significant differences from the PP zone in the same diet group are indicated by [a]$P \leq 0.05$, [b]$P \leq 0.01$, and [c]$P \leq 0.001$ (Student's paired *t*-test). Significant differences from the same parameter in normally fed rats are indicated by [d]$P \leq 0.05$ minimum (Student's unpaired *t*-test). ACAT, acyl-CoA:cholesterol acyltransferase; lCEH, lysosomal cholesteryl ester hydrolase; cCEH, cytosolic CEH; mCEH, microsomal CEH.

TABLE 2
Lipid Composition of the Cell Homogenate and the Cytosolic and Microsomal Subcellular Fractions of PP and PV Hepatocytes Isolated from Normal Fed Rats[a]

Cell fraction	Lipid (nmol lipid/mg cell protein)	PP cells	PV cells
Homogenate	Triacyglycerides	58.4 ± 12.8	56.8 ± 14.8
	Free cholesterol	23.3 ± 3.6	23.2 ± 0.6
	Cholesterol ester	5.69 ± 0.50	4.93 ± 0.57
	Ratio FC/CE	4.38 ± 1.13	4.94 ± 0.67
Cytosol	Triacylglycerides	15.7 ± 2.5	18.2 ± 6.6
	Free cholesterol	1.35 ± 0.17	1.19 ± 0.12
	Cholesterol ester	2.36 ± 0.49	2.06 ± 0.22
	Ratio FC/CE	0.63 ± 0.12	0.62 ± 0.15
Microsomes	Triacylglycerides	32.9 ± 6.1	28.0 ± 3.9
	Free cholesterol	19.4 ± 1.9	14.7 ± 2.3
	Cholesterol ester	3.45 ± 0.29	3.94 ± 0.55
	Ratio FC/CE	5.84 ± 0.98	4.18 ± 0.92[a]
	Ratio TG/CE	9.54 ± 1.21	7.10 ± 0.42[a]

[a]Levels of triacylglycerol (TG), free (FC) and esterified cholesterol (CE) were determined in the cell homogenate and the cytosolic and microsomal fractions of PP and PV hepatocytes isolated from normally fed rats. All values are means ± SEM for the number of preparations indicated in parentheses. Significant differences from the PP zone were analyzed by the Student's paired *t*-test and are indicated by $^{a}P \leq 0.05$ minimum. For other abbreviations see Table 1.

Comparison of data in Tables 1 and 3 concerning normally fed animals indicates that all enzyme activities were slightly higher in the total cell population than in PP or PV hepatocytes. This suggests that the formation and hydrolysis of CE might function actively in hepatocytes located in any acinar zone. Irrespective of the various models proposed to date to explain the microstructure of the liver acinus, PP hepatocytes seem to account for the bulk of the parenchymal cells within the acinus (i.e., see 1,31). In line with this, a good relationship was observed between the changes caused by cholestyramine on ACAT and lysosomal, cytosolic, and microsomal CEH in total hepatocytes and those caused in the PP hepatocyte subpopulation. Even the response of the PP cells, which are in a minority, may be masked by that of PP cells, as it is for the high capacity for cholesterol esterification through ACAT maintained by PV hepatocytes after cholestyramine feeding. In general, qualitative changes in total hepatocytes with cholestyramine were as expected, although the increase in cCEH activity, which was consistent for Ghosh *et al*. using whole livers harvested during the light period from male rats fed cholestyramine for 10 d (32), did not reach statistical significance in this study using hepatocytes isolated at midnight from female rats fed cholestyramine for 7 d. Diurnal rhythms were identified for the acid and neutral cholesterol esterases (25) and the above differences in cCEH activity are likely related, at least in part, with the time of the day at which the animals were used. As a matter of fact, a significant 30% increase in cCEH activity was observed in livers of cholestyramine fed rats over the control fed group when rats were used at the beginning of the light period (experiments not shown).

DISCUSSION

Our results demonstrate for the first time symmetry within the liver acinus of the lCEH activity, responsible for the degradation of endocyted lipoprotein-derived CE, a structural zonation of the cCEH, and a dynamic zonation of the microsomal enzymes mCEH and ACAT, with a PV dominance of the enzymatic capacity for the degradation of stored CE in normally fed rats and an exclusive PP reduction of the cholesterol esterifying ACAT activity after cholestyramine feeding. The difficulty of extrapolating these observations to the whole animal is evident: the experiments show a potential but do not reveal how it would be modified by whole body hormonal influences. We are also aware that the time lag between the handling of the intact liver and the performance of experimental determinations might produce changes in metabolic fluxes. This problem could be important in the case of ACAT activity, which is quite dependent on the conditions of hepatocyte isolation and incubation (11). Disturbance of hepatic zonation by cholestyramine offers an interesting model for the study of the significance of zonation in cholesterol metabolism. Our current view of the metabolism of CE in PP and PV hepatocytes is illustrated in Figure 1; this should not be interpreted to mean that PP and PV cells are located adjacent in a hepatic lobule. The scenario is based on results and ideas from this and other laboratories (4–9,19,24,29,30) and involves the following assumptions: (i) there is a gradient of bile acids and

TABLE 3
Specific Activities of the Enzymes Catalyzing the Formation and Hydrolysis of Cholesteryl Esters in Hepatocytes from Rats Fed a Normal or a Cholestyramine Diet[a]

Enzyme	Normal diet	Cholestyramine diet	Decrease (%)
ACAT (pmol/min/mg protein)	2752 ± 407 (8)	1496 ± 371 (6)[d]	45.6
lCEH (pmol/min/mg protein)	4074 ± 469 (8)	2463 ± 319 (6)[d]	39.5
cCEH (pmol/min/mg protein)	38.3 ± 5.1 (10)	41.1 ± 3.7 (6)	– 7.3
mCEH (pmol/min/mg protein)	91.9 ± 10.6 (10)	72.5 ± 6.9 (7)[d]	21.1
Ratio ACAT/lCEH	0.675 ± 0.077	0.607 ± 0.054	10.0
Ratio ACAT/(cCEH + mCEH)	21.14 ± 3.04	13.17 ± 2.11[d]	37.7

[a]One aliquot of each preparation of hepatocytes from normal or cholestyramine fed rats used in the experiments reported in Table 1 was homogenized before submission to centrifugal elutriation, and the specific activities of ACAT, lCEH, cCEH, and mCEH were determined in the corresponding subcellular fraction. All values are means ± SEM for the number of preparations indicated in parentheses. Significant differences from normal fed rats were analyzed by the Student's unpaired *t*-test and are indicated by $^{d}P \leq 0.05$, minimum. For abbreviations see Table 1.

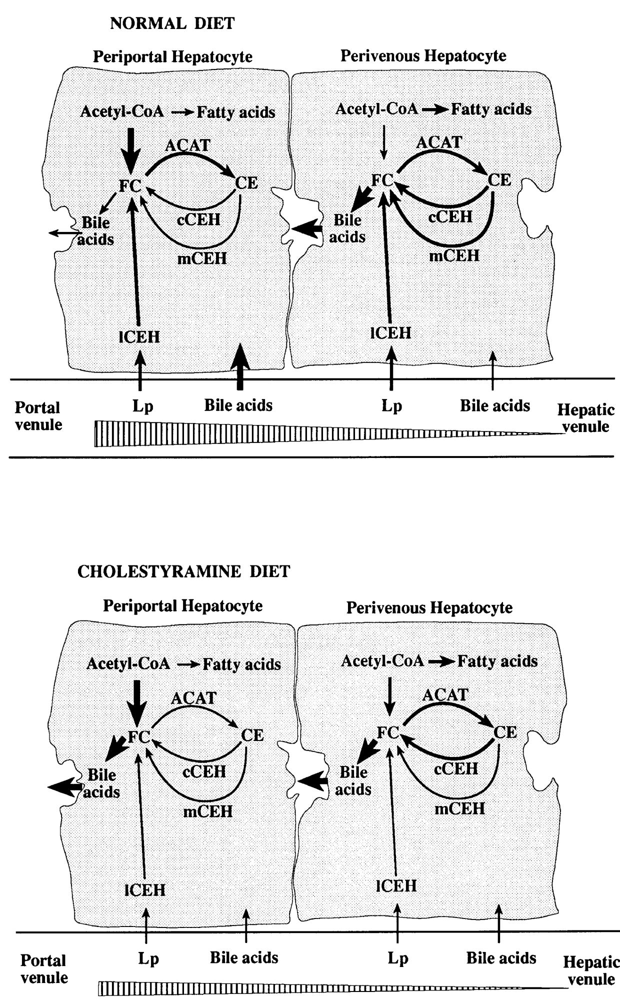

FIG. 1. Hypothetical scheme of cholesteryl ester metabolism in PP and PV hepatocytes from rats fed a normal or a cholestyramine diet. This model involves three key assumptions. (i) There is a gradient of bile acids and lipoproteins (Lp) from the portal to the hepatic venule that is attenuated by cholestyramine, with higher PP uptake of bile acids and symmetrical uptake of lipoproteins. The concentration gradients are represented by the elongated triangles shown at the bottom of each panel. (ii) There is no zonation of lCEH and ACAT activity, and PV dominance of cCEH and mCEH activity exists in normally fed rats. (iii) There is a predominance in the increase in bile acid synthesis and the decrease in ACAT activity in PP hepatocytes, and of the increase in cholesterol synthesis and the decrease in mCEH in PV cells in response to cholestyramine. The close proximity of the PP and PV cell types in the scheme does not indicate that these cells are located adjacent in a hepatic lobule. Abbreviations: FC, free cholesterol; ACAT, acyl-CoA:cholesterol acyltransferase; CE, cholesteryl ester; cCEH, cytosolic cholesteryl ester hydrolase; lCEH, lysosomal CEH; mCEH, microsomal CEH; PP, periportal; PV, perivenous.

lipoproteins from the portal to the hepatic venule that is attenuated by cholestyramine, with a higher PP uptake of bile acids and a homogeneous uptake of lipoproteins, (ii) there is no zonation of lCEH and ACAT activity, and PV dominance of both neutral cCEH and mCEH activities exists in livers of normally fed rats, and (iii) in response to cholestyramine feeding, the increase in bile acid synthesis and the decrease in ACAT activity dominate in PP hepatocytes, and the increase in cholesterol synthesis and the decrease in mCEH dominate in PV cells.

Comparable lCEH activity in PP and PV hepatocytes from normal or cholestyramine-fed rats was observed in this work. PP hepatocytes, *in vivo*, are likely to be exposed to higher concentrations of lipoproteins than PV cells, and such an acinar lipoprotein gradient might be expected to lead to zonal differences of the processes involved in lipoprotein uptake and processing. Unfortunately, this is as yet hardly explored. Results, though indeed not conclusive, suggest little zonation. For example, Fong *et al.* (29) found that low density lipoprotein receptors in the normally fed rat liver were present on the sinusoidal membranes without an obvious portal to central gradient. Vooschur *et al.* (24) reported that the lipoprotein-remnant receptor density and the binding of β-migrating very low density lipoproteins to cell membranes was similar in PP and PV hepatocytes, and recent research has revealed that, although PP hepatocytes have a greater capacity for the uptake of ^{125}I-chylomicron remnants than PV cells, both PP and PV cells bind and internalize similar amounts of remnants up to a certain remnant protein (19). Moreover, little zonation of the lysosomal degradation of proteins was reported (30). All these findings are in apparent harmony with the observed lack of zonation for the lysosomal acid lipase activity in normally fed rats and the identical decrease of lCEH activity in PP and PV cells promoted by cholestyramine. A minor activity in the putative regulatory enzyme of the hydrolysis of lipoprotein-derived CE and TG may underlie a minor uptake concomitant to the well-described lipoprotein-lowering effect of cholestyramine. It might thus be hypothesized that, although there is a concentration gradient in blood levels of lipoproteins between the PP and PV parenchyma, the uptake and internalization of lipoproteins and further degradation of components by the two subpopulations of hepatocytes are similar. This would guarantee effective nutrient extraction even when the circulating lipoprotein load is low. The question is open as to whether there are zonal differences for the selective uptake of lipoproteins where intracellular nonlysosomal lipolytic enzymes, such as the cCEH, may be involved.

ACAT plays a crucial role in the maintenance of cellular cholesterol homeostasis. The enzyme activity is highly activated by cholesterol substrate, which also acts as an allosteric activator of the enzyme (11). Here ACAT activity was determined in microsomes with saturated cholesterol as the substrate. ACAT showed a symmetrical distribution in control livers. However, zonation toward the PV region was induced by cholestyramine because of an exclusive depression of activity in cells from the PP zone. Hence, if the cellular demand of unesterified cholesterol increases, cholesterol esterification *via* ACAT might not occur at its maximal velocity in PP hepatocytes, although it might in PV hepatocytes. On the contrary, PP upstream hepatocytes adapt more actively than PV downstream cells to cholestyramine when C7αH is considered. The fact that opposite changes in ACAT and C7αH activity occur only in PP cells suggests that restriction of the esterification in favor of the 7α-hydroxylation of cholesterol

may not be a common hepatocyte response to cholestyramine but may be reserved to the PP hepatocytes. The balance between cholesterol esterification and hydrolysis of CE by cCEH and mCEH was also affected by cholestyramine only in PP cells, in which provision of metabolically active cholesterol dominates over cholesterol esterification as compared to normal PP cells. On the contrary, adaptation of cholesterogenesis and hydrolysis of CE by mCEH to cholestyramine seem to be more pronounced in hepatocytes from the PV area of the acinus. HMG-CoA reductase is widely accepted to be localized mainly in the PP zone of normal rat livers (4–6), though with a questionable degree of zonation, and it has been shown to be induced more efficiently in PV than in PP hepatocytes by increased cholesterol demand underlying dietary manipulations (5,6). Here we report that the activity of mCEH declined more markedly in PV than in PP hepatocytes, thus leading to the loss of the initial PV dominance of this activity. Taken together, the observed changes in the hepatic enzyme activities would stabilize free cholesterol in cholestyramine-fed rats by decreasing ACAT and mCEH, as well as by enhancing *de novo* cholesterol synthesis and the conversion of cholesterol to bile acids. This occurs with prominent contributions of PP cells to bile acid and CE formation and of PV cells to cholesterol synthesis and the microsomal hydrolysis of CE.

The metabolic scenario illustrated in Figure 1 restricts a direct linkage between all these pathways and points to a more complex integrated regulation than originally stated. Further, the purpose of this compartmentation is not entirely clear. With regard to this and the microsomal CEH isoform, whose function is not still ascribed, it is noteworthy that the observed qualitative changes indicate that mCEH does not seem to mediate the provision of a pool of cholesterol that is particularly committed to esterification or to hydroxylation. In contrast with the dynamic zonation displayed by the mCEH, the cCEH showed a structural type zonation. Not only the specific but also the total cCEH activity was always predominant in PV over PP hepatocytes, irrespective of the perturbation in the hepatic flux of cholesterol. Complex regulation of rat liver cCEH by hormones, signal transduction, and changes in cholesterol flux, which takes place by changes both in mRNA levels and postranscriptional mechanisms, has been reported recently (32). The identity of the factors involved in creating and maintaining the acinar heterogeneity of the hepatic cCEH activity deserves further investigation. At least part of the difference between the PP and PV zones involves long-term mechanisms that survive hepatocyte isolation. The physiological significance of an elevated neutral enzymatic capacity for the mobilization of CE in PV cells is unclear, since PV hepatocytes apparently constitute only a minor fraction of the parenchymal cells within the liver acinus (1,31). Nevertheless, PV hepatocytes are localized in the less aerobic, efferent side of the acinus, and an intriguing possibility is that the secretion of CE as components of plasma lipoproteins is zonated. The question of whether there is acinar heterogeneity of hepatocytes for very low density lipoprotein assembly and secretion is a matter of ongoing research.

The hepatic zonation of some pathways of lipid metabolism has been recently reviewed (31). These pathways appear to be less zonated than those of carbohydrate or amino acid metabolism, and the zonation of a certain step or pathway by biochemical studies appears to be less marked than by immunohistochemical approaches, in which information about the amount of total enzyme protein but not of enzyme activity is acquired. A general conclusion that may be inferred from our investigation is that the acinar distribution of the formation and the neutral hydrolysis of CE is complex and changes according to the requirements of the organ itself, whereas the acid lysosomal hydrolysis of CE shows a symmetrical distribution. Overall, this indicates that acinar heterogeneity may be an additional factor in the regulation of CE metabolism.

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Eicosapentaenoic Acid and Docosahexaenoic Acid Selectively Attenuate U46619-Induced Smooth Muscle Cell Proliferation

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ABSTRACT: It is well known that vascular smooth muscle cell (SMC) proliferation is a key step in atheromatous plaque formation. Thromboxane A_2 (TxA_2), released from aggregating platelets and an injured vessel wall, may play an important role in the development of atheromatous plaque. Many animal studies have suggested that n-3 polyunsaturated fatty acids eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) present in the fish oils have antiatherosclerotic effects. In the present study, we investigated the effect of EPA and DHA on TxA_2-induced SMC proliferation. To determine the functional selectivity of n-3 fatty acids, we also tested the effect of arachidonic acid (AA, 20:4n-6), γ-linolenic acid) (LNA, 18:3n-6), and oleic acid (OA, 18:1n-9) on TxA_2-induced SMC proliferation. Only EPA and DHA prevented the SMC proliferation induced by the TxA_2 mimetic U46619. When EPA and DHA were added together in the ratio in which they are present in menhaden oil, EPA and DHA acted synergistically to block the SMC proliferation induced by the TXA_2-mimetic. These findings suggest that the n-3 polyunsaturated fatty acids in fish oils may exert antiatherosclerotic effects by blocking the mitogen-stimulated proliferation of SMC.

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The amount and source of dietary lipids can affect a variety of disease processes in humans (1,2). In particular, dietary long-chain polyunsaturated fatty acids (PUFA) have been shown to modulate a number of cellular responses to environmental stimuli (3,4). Among PUFA, a clear distinction of properties has emerged between the n-3 and n-6 families with some notable exceptions (5). Epidemiological studies have suggested an inverse relationship between intake of n-3 PUFA and incidence of cardiovascular disease (6–8). Consumption of n-3 PUFA diminishes atherogenesis in some non-human primate models (9), as well as in other animal species (3,4). In addition to atherothrombotic disease, intimal hyperplasia in autologous vein grafts (10,11) has been inversely associated with dietary intake of n-3 PUFA. Efforts to understand the mechanism of action of n-3 PUFA have largely focused upon their effects on eicosanoid production. However, the hypothesis that n-3 PUFA act through alterations of eicosanoid metabolism cannot explain many aspects of their biological activity. These include decreased production of tissue factor (12), cytokines from monocyte-derived macrophages (13), platelet-derived growth factor-like mitogens from endothelial cells (14), and monocyte macrophages (15,16). Thus, there is a need to understand the noneicosanoid effect that may play a role in antiatherogenic effects of n-3 PUFA.

Production of thromboxane A_2 (TxA_2) at sites of vascular injury contributes to lesion formation characteristic of atherosclerosis and restenosis (17,18). Although well known as a potent vasoconstrictor and inducer of platelet aggregation (19,20), TxA_2 also has been shown to induce smooth muscle cell (SMC) migration and proliferation (21–29). In this study, we demonstrate that when the TxA_2 mimetic U46619 was added together with n-3 fatty acids eicosapentaenoic acid (EPA) or docosahexaenoic acid (DHA), U46619 failed to induce vascular SMC proliferation as measured by ^{3}[H]-thymidine incorporation. We also show that this inhibitory effect is specific for n-3 fatty acids because the n-6 fatty acids arachidonic (AA) and γ-linolenic acid (LNA) or the n-9 fatty acid oleic acid (OA) failed to inhibit TxA_2-induced ^{3}[H]-thymidine incorporation.

MATERIALS AND METHODS

Serotonin (as the creatine sulfate), EDTA, pargyline, AA (20:4n-6), LNA (18:4n-6), OA (18:1n-9), and Hank's balanced salts were obtained from Sigma Chemical Co. (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) were obtained from Whittaker Bioproducts (Walkersville, MD), and ^{3}[H]-thymidine (20 Ci/mole) came from New England Nuclear Corp. (Boston, MA). Other reagents were purchased from local vendors. EPA and DHA were provided by the FOTM program, United States Department of Commerce, National Oceanic and Atmospheric Administration (Charleston, SC).

Isolation, culture, and characterization of canine aortic SMC. Primary aortic SMC were isolated using the explant method previously described (29). The intima was peeled off from the aorta and the media was carefully stripped away

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FBS, fetal bovine serum; IP_3, inositol triphosphate; LNA, γ-linolenic acid; OA, oleic acid; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acids; SMC, smooth muscle cell; TxA_2, thromboxane A_2.

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from the adventia and placed in a petri dish containing warmed DMEM (37°C). The media layer was cut into approximately 1-mm squares, which were transferred to a 25-cm^2 tissue culture flask and barely covered with DMEM supplemented with 10% FBS. The blocks of tissue were cultured in a humidified atmosphere of 95% air and 5% CO_2 (vol/vol) at 37°C. After 1–2 wk, the tissue blocks were removed and the migrated SMC were cultured. Following isolation, the identity of the SMC was confirmed by morphological examination and by staining for β-actin.

Subcultures of SMC were done once they became confluent; media from the plates was aspirated and the cells were washed with 10 mL of phosphate-buffered saline (PBS). Then, 2–3 mL of trypsin EDTA solution (0.05% trypsin, 0.53 mM EDTA in Ca^{2+}-, Mg^{2+}-free Hank's balanced salts) was added to the cells and incubated at room temperature for 2–3 min. The action of trypsin on cells was blocked by the addition of 7–8 mL of DMEM containing 10% FBS. The cells were collected by centrifugation at 150 × *g* for 10 min. After removing the supernatant, the pelleted cells were dispersed in 10 mL of DMEM containing 10% FBS and fresh cultures were initiated from these cells.

^{3}[H]-thymidine incorporation. Aortic SMC from passages 2 and 3 were seeded into 35-mm diameter plates at a density of 65,000 to 75,000 cells/plate in DMEM containing 10% FBS and allowed to proliferate for approximately 72 h. After 72 h, the growth medium was replaced with 2 mL of DMEM containing 0.1% FBS and incubated for approximately 72 h to arrest cell growth and cause synchronization. After growth arrest, the medium was replaced with 2 mL of DMEM containing 1% FBS and 100 mM pargyline. Where indicated, U46619 (a TxA_2 mimetic) alone or the indicated fatty acid (EPA, DHA, AA, OA, LNA) alone or both together were added to this medium. After 20 h of incubation with U46619 and/or indicated fatty acid, 1 μCi of [^{3}H]-thymidine was added to each plate and then incubated for an additional 4 h. The medium was removed and the plates were washed three times with ice-cold PBS. Then, 6% trichloroacetic acid was added to the cells and the acid-insoluble material containing [^{3}H]-thymidine was collected on a glass fiber filter. The filters were washed with 100% ethanol, air dried, and [^{3}H]-thymidine was quantified using a liquid scintillation counter. In a selected number of dishes, the cells were counted (Coulter counter) on the day of seeding (before cells were attached) and before changing to 0.1% serum medium (on day 3) to ensure that they were growing, and finally on day 6 to determine that the cells were growth-arrested. To determine the cell number on days 3 and 6, dishes were first washed with PBS, followed by the addition of 0.2 mL of 2% (wt/vol) crude pancreatic trypsin in PBS, containing 0.152 M EDTA. The dishes were incubated for 2–3 min at room temperature before addition of 0.4 mL FBS. The contents of each dish were diluted to 10 mL with isoton II (Coulter Electronics, Luton Beds, England) and cell numbers determined. All experiments were performed in triplicate.

Statistical analyses. Data were analyzed by one-way analysis of variance. When a statistically significant difference was obtained, further analysis was conducted using Scheffe's Post-hoc tests. In each figure, mean ± SD are shown. For all comparisons, statistical significance was assumed as $P < 0.05$.

RESULTS

Effect of TxA_2 on ^{3}[H]-thymidine incorporation by SMC. The effect of increasing concentrations of U46619 on the [^{3}H]-thymidine incorporation by growth-arrested SMC is shown in Figure 1. When U46619, in 1% FBS-containing medium was added to growth-arrested SMC at concentrations higher than 0.33 μM, it significantly induced ^{3}[H]-thymidine incorporation. The maximal increase in ^{3}[H]-thymidine incorporation was observed at a concentration of 3.33 μM of U46619 where there was approximately a fivefold increase in [^{3}H]-thymidine incorporation, as compared to the untreated control. However, with further increases in concentrations of U46619, there was a gradual, but significant, decrease in the amount of ^{3}[H]-thymidine incorporated.

Effect of n-3, n-6, and n-9 fatty acids on ^{3}[H]-thymidine incorporation by SMC. The effect of increasing concentrations of n-3 fatty acids EPA and DHA, n-6 fatty acids AA and LNA, and n-9 fatty acid OA (in 1% FBS-containing medium) on ^{3}[H]-thymidine incorporation into growth-arrested SMC was examined. Incubation of growth-arrested SMC with LNA or OA at the concentrations tested showed no significant effect on ^{3}[H]-thymidine incorporation (Fig. 2). When growth-arrested SMC were incubated with AA up to an added concentration of 1.67 μM, AA did not have a significant effect on ^{3}[H]-thymidine incorporation. However, at higher concentrations, AA induced a significant increase in the extent of ^{3}[H]-thymidine in-

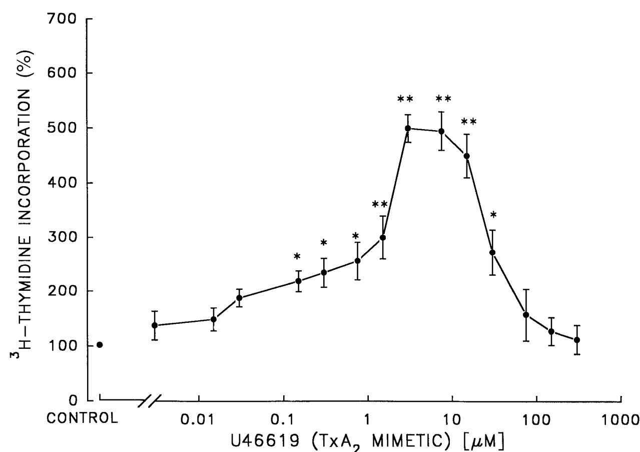

FIG. 1. Concentration-dependent stimulation of smooth muscle cells (SMC) by thromboxane A_2 (TxA_2). ^{3}[H]-thymidine incorporation into DNA was measured in growth-arrested SMC stimulated by various concentrations of U46619 in 1% fetal bovine serum (FBS), as described in the Materials and Methods section. One hundred percent equals the baseline value of ^{3}[H]-thymidine uptake (100% = 15856 ± 650 counts per minute (cpm)/10^6 cells). The experiments were performed with two different batches of cells and each batch was tested in triplicate. *$P < 0.05$, **$P < 0.01$ as compared to the control.

corporation, reaching a fourfold increase at 16.7 μM. (Fig. 2). When growth-arrested SMC were incubated with EPA or DHA, EPA up to 3.3 μM and DHA up to 7.5 μM did not have any significant effect on ^{3}[H]-thymidine incorporation. In contrast, with concentrations of EPA higher than 3.3 μM or DHA higher than 7.5 μM, there was a gradual decrease in the amount of ^{3}[H]-thymidine incorporated. At concentrations greater than 75 μM, both EPA and DHA completely inhibited ^{3}[H]-thymidine incorporation and the cells failed to respond when transferred to normal growth medium, indicating that these concentrations of EPA and DHA are cytotoxic to SMC (Fig. 2).

Effect of n-3, n-6, and n-9 fatty acids on TxA_2-induced ^{3}[H]-thymidine incorporation by SMC. To determine whether U46619-induced ^{3}[H]-thymidine incorporation into SMC can be inhibited by n-3 fatty acids, growth-arrested SMC were incubated with different concentrations of U46619 (0.05–5 μM), along with EPA or DHA in concentrations at which they did not have any significant growth inhibitory effect (0.165–0.83 μM). In addition, SMC also were incubated with the same concentrations of U46619 and varying concentrations of AA, LNA, or OA (0.165–0.83 μM). When SMC were incubated with 0.33 μM EPA or DHA, they partially blocked (≈ 25%) the U46619-induced ^{3}[H]-thymidine incorporation. However, when SMC were incubated with 0.83 μM EPA or DHA, even 5 μM of U46619 failed to stimulate any ^{3}[H]-thymidine incorporation (Fig. 3). In contrast, when SMC were incubated with AA, OA, or LNA, even at a concentrations as high as 0.83 μM, they did not inhibit U46619-induced ^{3}[H]-thymidine incorporation (Fig. 4).

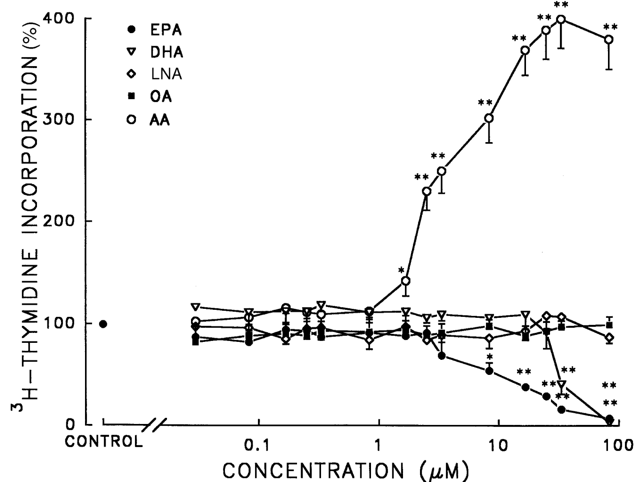

FIG. 2. Effect of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), oleic acid (OA), γ-linolenic acid (LNA), and arachidonic acid (AA) on SMC growth. Various concentrations of EPA, DHA, LNA, OA, or AA in 1% FBS containing Dulbecco's modified Eagle's medium (DMEM) were added to growth-arrested SMC and the amount of ^{3}[H]-thymidine incorporated into the DNA was measured as described in the Materials and Methods section. One hundred percent equals the baseline value of ^{3}[H]-thymidine uptake (100% = 16,580 ± 760 cpm/10^6 cells). The experiments were performed with two different batches of cells and each batch was tested in triplicate. *$P < 0.05$, **$P < 0.01$ as compared to the control. See Figure 1 for other abbreviations.

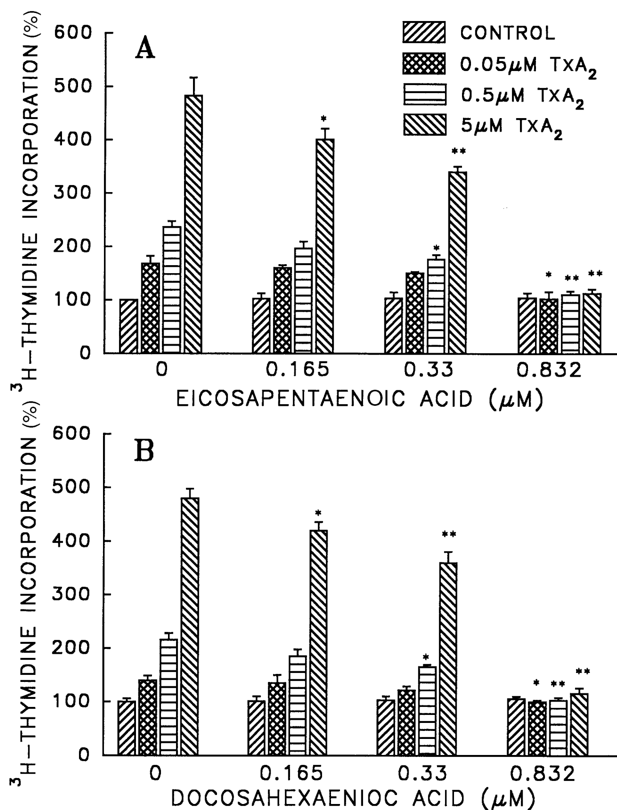

FIG. 3. Interaction between U46619 and (A) EPA or (B) DHA in modulating SMC growth. Growth-arrested canine aortic SMC were incubated with indicated concentrations of U46619 and EPA or DHA in 1% FBS containing DMEM, and the amount of ^{3}[H]-thymidine incorporated was determined as in the Materials and Methods section. One hundred percent equals the baseline value of ^{3}[H]-thymidine uptake [100% = (A) 16,120 ± 740 cpm/10^6 cells, (B) 100% = 16,470 ± 810 cpm/10^6 cells]. The experiments were performed with two different batches of cells and each batch was tested in triplicate. *$P < 0.05$, **$P < 0.01$ as compared to the corresponding control. See Figures 1 and 2 for abbreviations.

In menhaden oil, widely used in human and animal studies, EPA and DHA are present in approximately a 2:1 ratio. Therefore, we also tested the combined effects of EPA and DHA in this ratio on U46619-induced ^{3}[H]-thymidine incorporation. Growth-arrested SMC were incubated with 0.11 and 0.22 μM EPA or 0.055 and 0.11 μM DHA or 0.165 μM total n-3 fatty acids (0.11 μM EPA + 0.055 μM DHA) or 0.33 μM n-3 fatty acids (0.22 μM EPA + 0.11 μM DHA) as a mixture along with mitogenic concentrations of U46619. Although at a concentration of 0.165 μM or 0.33 μM EPA or DHA partially reversed (≈ 10 and 20%, respectively) the mitogenic effect of U46619 (Fig. 3), the same concentration of total n-3 fatty acids (0.11 μM EPA + 0.055 μM DHA and 0.22 μM EPA + 0.11 μM DHA) almost completely reversed (≈ 60 and 90%, respectively) U46619-induced ^{3}[H]-thymidine incorporation (Fig. 5). These results suggest that when present in a 2:1 ratio, EPA and DHA act synergistically in reversing U46619-induced mitogenic effects.

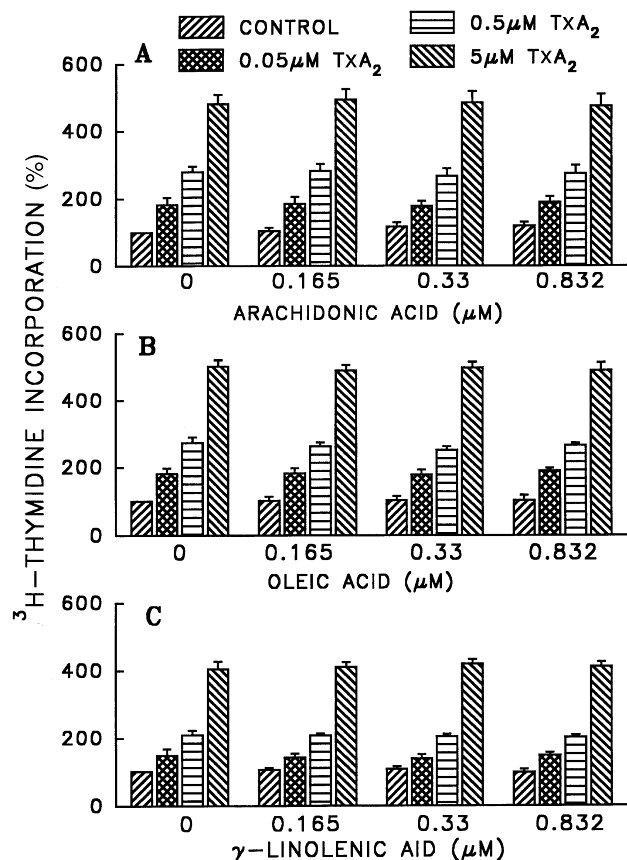

FIG. 4. Interaction between U46619 and (A) AA, (B) OA, or (C) LNA in modulating SMC growth. Growth-arrested SMC were incubated with indicated concentrations of U46619 and AA, OA, or LNA in 1% FBS containing DMEM, and the amount of ^{3}[H]-thymidine incorporated was determined as described in the Materials and Methods section. One hundred percent equals the baseline value of ^{3}[H]-thymidine uptake (100% = 16970 ± 820 cpm/10^6 cell for A; 100% = 16820 ± 640 cpm/10^6 cells for B; 100% = 17128 ± 860 cpm/10^6 cells for C). The experiments were performed with two different batches of cells and each batch was tested in triplicate. See Figures 1 and 2 for abbreviations.

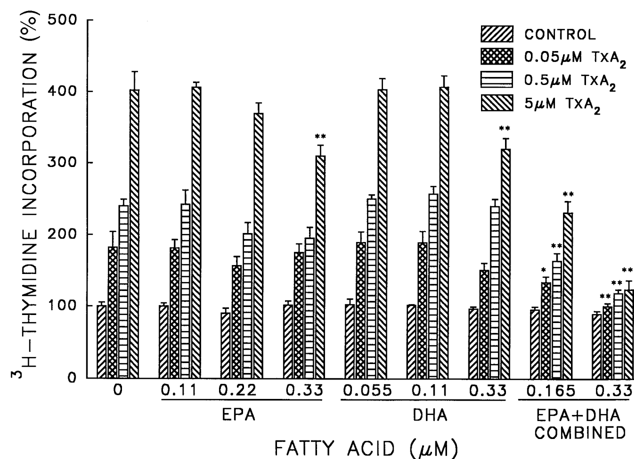

FIG. 5. Synergistic interaction between EPA and DHA in modulating U46619-induced SMC growth. Growth-arrested SMC were incubated with indicated concentrations of U46619, EPA, and DHA in 1% FBS containing DMEM and the amount of ^{3}[H]-thymidine incorporated was determined as described in the Materials and Methods section. One hundred percent equals the baseline value of ^{3}[H]-thymidine uptake (100% = 16820 ± 790 cpm/10^6 cells). The experiments were performed with two different batches of cells and each batch was tested in triplicate. *$P < 0.05$, **$P < 0.01$ as compared to the corresponding control. For abbreviations see Figures 1 and 2.

DISCUSSION

The present study demonstrates that the major n-3 PUFA present in fish oils, EPA and DHA, can attenuate the vascular SMC proliferation induced by the TxA_2 mimetic U46619. TxA_2 is A major metabolite of AA released during platelet aggregation (19,20). It also has been suggested that the vascular wall produces significant amounts of TxA_2, especially in some genetic models of hypertension (21,22). Although well known as a potent inducer of platelet aggregation and as a vasoconstrictor, TxA_2 also has been shown to induce SMC migration and hyperplasia of the cells (21,29) of the sort that is typically encountered in many occlusive vascular lesions. In studies involving cultured vascular SMC, several investigators have reported that TxA_2/prostaglandin endoperoxide (TxA_2/PGH_2) receptor agonists can induce hypertrophy or hyperplasia of the cells (24,29). Antagonism of TxA_2/PGH_2 receptors with specific receptor antagonists has been shown to reduce atheromatous plaque formation in coronary arteries of hypercholesterolemic rabbits (17), neointima formation in mechanically injured canine coronary arteries (18), and proliferation of vascular SMC in culture (28,29). Based on these studies, it seems possible that TxA_2 released from aggregating platelets and/or vascular walls may play a role in the development of atherosclerosis and/or restenosis following angioplasty.

In this study, EPA up to an added concentration of 3 μM and DHA up to an added concentration of 7.5 μM had no significant effect on ^{3}[H]-thymidine incorporation by SMC. At concentrations higher than these, both EPA and DHA were growth inhibitory. This inhibitory effect is not due to cytotoxicity because we could revive cell growth after transferring the cells to normal-growth medium (data not presented). However, at concentrations greater than 75 μM, both EPA and DHA were cytotoxic. Several investigators have reported that PUFA like EPA and DHA inhibit the proliferation of cells in culture mainly through the formation of free radicals (30–32) and this inhibition could be reversed by incubating the cells with antioxidants (32). This may explain the inhibitory and cytotoxic effects of EPA and DHA at higher concentrations. In this study, neither OA nor LNA had a significant effect on ^{3}[H]-thymidine incorporation by SMC. However, AA at concentrations greater than 3 μM significantly increased ^{3}[H]-thymidine incorporated into SMC. AA, as well as its metabolites, has been shown to modulate the proliferation of SMC (33). When added to cell cultures, AA itself has been shown to stimulate mitogen-activated protein kinases (34), which also results in increased ^{3}[H]-thymidine incorporation (35). When tested in concentrations at which they do not have any

significant effect by themselve, EPA and DHA prevented TxA_2-stimulated ^{3}[H]-thymidine incorporation, whereas similar concentrations of OA, LNA, and AA failed to prevent TxA_2 stimulation. When present together, EPA and DHA appear to exert an additive effect to prevent the TxA_2-induced stimulation of SMC.

Conflicting reports exist about the effect of TxA_2 mimetics on vascular SMC. Dorn *et al.* (27) reported that U46619 stimulates only protein synthesis, indicating that TxA_2 mimetics induce only vascular SMC hypertrophy, but not hyperplasia. In contrast, Sachinidis *et al.* (28) demonstrated that TxA_2 mimetics can stimulate DNA synthesis. However, both groups have shown that TxA_2 analogs stimulated phospholipase C, with subsequent formation of IP_3 (inositol triphosphate) and diacylglycerol and an increase in intracellular free calcium. A common response of many G protein-coupled vasoactive agents known to be mitogens is elevation of phosphoinositide turnover and elevation of intracellular free calcium. The calcium channel blocker verapamil has been shown to block U46619-induced ^{3}[H]-thymidine incorporation by rat aortic SMC (36) and I-BOP [1*S*-(1α,2β(5*Z*), 3α(1*E*,3*R**), 4α)]-7-[3-(3-hydroxy-4-(4′-iodophenoxy)-1-butenyl)-7-oxabicyclo-[2.2.1]heptan-2-y1]-5-heptenoic acid]-induced ^{3}[H]-thymidine incorporation by guinea pig coronary artery SMC (24). This indicates that influx of extracellular calcium is required for the mitogenic response of TxA_2 analogs U46619 and I-BOP. Little information is available about the influence of n-3 fatty acids on calcium transport systems. Hallaq *et al.* (37) have shown that enriching cardiac myocyte membranes with fish oil fatty acids reduced the ouabain-stimulated increase in intracellular calcium. Locher *et al.* (38,39) have shown that fish oils and EPA can attenuate the elevation of intracellular calcium in rat aortic SMC stimulated by angiotensin II, low density lipoprotein, and platelet-derived growth factor. In another report, Locher *et al.* (40) have also shown that fish oils and EPA can attenuate IP_3 formation in rat aortic SMC stimulated by low-density lipoprotein and angiotensin II. Similarly, Chetty *et al.* (41) have demonstrated that EPA interferes with the formation of inositol phosphates in U46619-stimulated rabbit platelets.

Since it has been shown that TxA_2 analogs can exert their mitogenic effects by increasing IP_3 formation and elevating intracellular calcium, a mechanism by which EPA and DHA may inhibit SMC proliferation could be by inhibiting the U46619-induced increase in IP_3 formation and preventing the increase in intracellular calcium. Another possible mechanism by which EPA and DHA might inhibit U46619-induced SMC proliferation may be by directly competing for the TxA_2 receptor. Swann *et al.* (42,43) have demonstrated that when platelets were incubated with either esterified or nonesterified EPA or DHA, there was a significant decrease in the specific binding of ^{3}[H]-U46619 to TxA_2/prostaglandin H_2 receptor. They have also shown that this effect was specific for EPA and was not shared by LNA.

In summary, we have demonstrated that EPA and DHA at very low concentrations selectively inhibit TxA_2-stimulated vascular SMC proliferation. Since it is well established that fish oils inhibit platelet aggregation resulting in a decreased production of TxA_2, fish oils may have several beneficial effects to inhibit platelet aggregation and decrease TxA_2 production, and also to directly inhibit the effect of TxA_2 on vascular SMC proliferation.

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Induction of Apoptosis and Apoptotic Mediators in Balb/C Splenic Lymphocytes by Dietary n-3 and n-6 Fatty Acids

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ABSTRACT: The present study was designed to investigate the effect of dietary n-6 and n-3 polyunsaturated fatty acids (PUFA) on anti-CD3 and anti-Fas antibody-induced apoptosis and its mediators in mouse spleen cells. Nutritionally adequate semipurified diets containing either 5% w/w corn oil (n-6 PUFA) or fish oil (n-3 PUFA) were fed to weanling female Balb/C mice, and 24 wk later mice were sacrificed. In n-3 PUFA-fed mice, serum and splenocyte lipid peroxides were increased by 20 and 28.3% respectively, compared to n-6 PUFA-fed mice. Further, serum vitamin E levels were decreased by 50% in the n-3 PUFA-fed group, whereas higher anti-Fas- and anti-CD3-induced apoptosis (65 and 66%) and necrosis (17 and 25%), compared to the n-6 PUFA-fed group, were found when measured with Annexin V and propidium iodide staining, respectively. In addition, decreased Bcl-2 and increased Fas-ligand (Fas-L) also were observed in the n-3 PUFA-fed group compared to the n-6 PUFA-fed group. No difference in the ratio of splenocyte subsets nor their Fas expression was observed between the n-3 PUFA-fed and n-6 PUFA-fed groups, whereas decreased proliferation of splenocytes was found in n-3 PUFA-fed mice compared to n-6 PUFA-fed mice. In conclusion, our results indicate that dietary n-3 PUFA induces higher apoptosis by increasing the generation of lipid peroxides and elevating Fas-L expression along with decreasing Bcl-2 expression. A reduced proliferative response of immune cells also was observed in n-3 PUFA-fed mice.

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In recent years, it has been increasingly apparent that long-chain polyunsaturated fatty acids (PUFA) are involved in maintaining a variety of physiological processes and therefore currently are viewed to play a key role in human nutrition, both in normal health and disease states (1,2). Further epidemiological evidence suggests that the low incidence of breast and colon cancer seen among traditional Mediterranean and Japanese populations may be related to diets with higher fish consumption, which can enrich tissue n-3 PUFA levels (3,4). Recently, several studies, including our own investigations, have shown that feeding a diet rich in fish oil (FO) inhibits autoimmune renal disease (5–9) and growth of human breast cancer cells in nude mice (10–12). Other studies have shown that n-3 PUFA decrease cell proliferation, induce apoptosis in *in vitro* systems (13,14), and are found more susceptible to lipid peroxidation (LPO) than n-6 PUFA (15–17). A positive correlation was established between the number of double bonds in fatty acids, their increased susceptibility to LPO, and their potential for inducing apoptosis (18). Metabolites of lipid peroxides have been implicated in many pathological conditions due to the increased oxidative stress they cause (19,20). Indeed oxidative stress is found to induce higher apoptosis in a variety of cultured cell lines and is thought to play a major role in the regulation of apoptosis (21).

Apoptosis is an essential process for normal development and for the maintenance of tissue homeostasis in multicellular organisms (22,23). In lymphocytes, apoptosis plays an important role in maintaining the T cell repertoire and deletion of autoreactive T and B lymphocytes, thus limiting autoimmune responses (24). Apoptosis can be triggered by various stimuli, such as glucocorticoids, oxidative stress, activation of Fas-ligand (Fas-L), and deprivation of cytokines and growth factors (25,26).

Apoptosis is also regulated by a number of gene products that could either promote cell death or extend cell survival (26,27). For instance, both Fas/apolipoprotein-1 (apo-1, CD95) and apolipoprotein-2L (TRAIL), members of a cell surface tumor necrosis factor-receptor family (28), mediate both T and B lymphocyte apoptosis (29). Fas-L, which is expressed predominantly in activated T cells (30,31), is a type 2 membrane protein belonging to the tumor necrosis factor receptor family. Whereas TRAIL-mediated pathways involve activation of the transcription factor, nuclear factor (NF)-κB (32), Fas-L mediates cell death by cross-linking Fas-receptor (33,34). Other gene products that modulate lymphocyte apoptosis are those of the Bcl-2 or Bcl-x1 family. Bcl-2, which is primarily located on the mitochondrial outer membrane, acts as an effective inhibitor of apoptosis in many target tissues (35).

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Abbreviations: CO, corn oil; Con A, concanavalin A; DCFH-DA, dichlorofluorescein diacetate; Fas-L, Fas-ligand; FITC, fluorescein isothiocyanate; FO, fish oil; LPO, lipid peroxidation; LPS lipopolysaccharide; PBS, phosphate-buffered solution; PE, phycoerythrin; PI, propidium iodide; PUFA, polyunsaturated fatty acids; TBARS, thiobarbituric acid reactive substances; Vit E, vitamin E.

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Recently, we showed that dietary n-3 fatty acids are able to enhance the spontaneous as well as dexamethazone-induced apoptosis in murine splenocytes when compared to n-6 fatty acids (6,36). The present study was undertaken to determine the influence of dietary n-3 and n-6 fatty acids on mediators of apoptosis and the generation of lipid peroxides in immune cells obtained from a non-autoimmune-prone strain (Balb/C) of mice.

METHODS AND MATERIALS

Materials. Thiobarbituric acid, 1,1,3,3-tetraethoxy propane, butyl hydroxyanisole, brefeldin A, ionomycin, phytohemagglutinin, and lipopolysaccharide (LPS) were obtained from Sigma Chemical Co. (St. Louis, MO). Dichlorofluorescein diacetate (DCFH-DA), dexamethasone, Annexin V, propidium iodide (PI), and all fluorescein isothiocyanate (FITC)- and phycoerythrin (PE)-labeled antibodies and antibody isotype controls were purchased from Pharmingen (San Diego, CA). Concanavalin A (Con A) was obtained from Pharmacia. All other chemicals (analytical grade) were obtained from Sigma Chemical Co.

Animals. Two-month old Balb/C female mice, five per cage, were housed in a temperature-controlled room at 24°C and were maintained on a 12/12-h dark–light cycle. The mice were provided an AIN-76 formula (37) diet containing either 5% (w/w) corn oil (CO, n-6 PUFA) or 4% FO + 1% CO (n-3 PUFA) and water on an *ad libitum* basis. FO was obtained from the National Marine Fisheries Service (Charleston, SC). The composition of the diet was 20% casein, 50% dextrose, 15% starch, 5% cellulose, 5% oil, 3.5% AIN salt mixture, 1% AIN vitamin mixture, 0.3% DL-methionine, and 0.2% choline chloride. The oils had equal levels of antioxidants, i.e., 1.3 g (1300 IU) α-tocopherol per kg oil, 1.2 g (13.2 IU) γ-tocopherol/kg, and 1 g/kg *tert*-butylhydroxyquinone, as per the guidelines of the National Institutes of Health. Food intake and weight gain were monitored at regular intervals. Fresh, semipurified moist diet prepared weekly was provided to mice daily. Leftover food was discarded to prevent lipid rancidity.

After 24 wk of feeding, mice were fasted overnight and given a mild anesthesia. Peripheral blood was then collected by retro-orbital bleeding, and mice were killed by cervical dislocation. Spleens were aseptically removed and placed in RPM1 complete medium (5% heat-inactivated fetal calf serum, 2 mM l-glutamine, and 100 U/mL each of penicillin and streptomycin). Serum was separated by centrifuging blood at 1000 × *g* for 15 min and analyzed immediately. Serum lipid peroxides (malondialdehyde) were determined using the fluorescence method as previously described (36).

Vitamin E (Vit E, α-tocopherol) assay. Vit E was quantitated by high-performance liquid chromatography as described by Rushing *et al*. (38).

Splenocyte preparation. Single-cell suspensions were prepared by disrupting the spleen between frosted-glass slides in RPMI 1640 media with 2% heat-inactivated fetal calf serum. After a 5-min centrifugation at 100 × *g* to separate cells from debris, the cells were washed twice in RPMI medium (8). Spleen lymphocytes were purified by layering over lympholyte (Cedarlane Labs. Lit., Hornby, Ontario, Canada), centrifuging at 1000 rpm for 15 min at 22°C, then washing twice in the RPMI 1640 medium.. Cells were counted and viability was determined by trypan blue exclusion.

Measurement of intracellular peroxides by flow cytometry. DCFH-DA is a lipophilic fluorophore that is converted intracellularly when exposed to peroxides into a fluorescent molecule and retained by viable cells. It is a well-established indicator of cellular peroxides. Cellular peroxide levels were measured using the method of Hockenberry *et al.* (39). In brief, 1×10^6 lymphocytes were washed with 1× PBS (phosphate-buffered saline), suspended in 1 mL of PBS, then incubated for 30 min with 10 μM DCFH-DA at 37°C. After incubation cells were washed by centrifuging at 200 × *g*, resuspended in PBS, and kept on ice in the dark until analysis. Cellular fluorescence was analyzed by flow cytometry with a Becton Dickinson FACScan flow cytometer following excitation and emission at 488 and 530 nm, respectively. For visual comparison of lymphocyte population histograms, each analysis was recorded at a single amplification setting.

Quantification of apoptosis and necrosis by flow cytometry. Lymphocytes, 1×10^6, were incubated for 8 h in RPMI complete medium with or without 1 μg anti-Fas or anti-CD3 monoclonal antibody (mAb) in a final volume of 1 mL in a 5% CO_2 humidified incubator at 37°C. After incubation, cells were washed twice with binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), resuspended in 200 μL binding buffer in the presence of 0.2 μg FITC-Annexin V and 2 μg PI, then incubated in the dark for 10 min (40). Annexin V and PI staining were used as indications of apoptosis and necrosis, respectively, and were measured with a FACScan (Becton Dickinson) using Cell Quest software. The percentage of apoptotic (stained with Annexin V alone) and necrotic (stained with Annexin and PI) cells was calculated *via* measurements of forward and side light scatter, which are proportional to cell diameter and internal density, respectively.

Flow cytometric analysis of Fas, Fas-L, Bcl-2, and splenocyte subsets. Cells were washed with isotonic NaCl/phosphate buffer containing 0.5% bovine serum albumin and 0.2% NaN_3 (wash buffer). Cells (1×10^6) were incubated with 0.1 μg of Fc block (anti mouse CD16/CD32 antibody to block the Fc receptors) for 10 min at room temperature. After two washes, 1×10^5 cells in 200 μL wash buffer were incubated at 4°C in the dark for 30 min with 2 μL of one of the following monoclonal antibodies: anti-CD4-PE, CD8-PE, or CD19-PE and anti-Fas-FITC. Isotype control for anti-Fas-FITC was used in all experiments. Cellular fluorescence was analyzed by flow cytometry with a Becton Dickinson (Mountain View, CA) FACScan flow cytometer using the Cell Quest program.

Because Fas-L is expressed on activated T cells, splenocytes (1×10^6 cells) were activated in the presence of soluble anti-CD3 (2 μg/mL) for 24 h after washing, the cells were further stimulated with Brefeldin A, phytohemagglutinin, and

ionomycin (2 µg, 10 ng, and 1 µg/mL respectively) for 4 h. For Fas-L (activated cells) and Bcl-2 staining, cells were washed with wash buffer and fixed with 200 µL of 1% paraformaldehyde in wash buffer for 15 min with continuous shaking at room temperature. Lymphocytes were washed with wash buffer, then permeabilized by suspending in 200 µL of 0.33% saponin at 4°C for 45 min. Permeabilized cells were washed and stained with anti-Fas-L-PE and Bcl-2-FITC in 200 µL of wash buffer at 4°C for 45 min. Proper isotype controls for anti-Fas-L PE and anti-Bcl-2-FITC were used in all experiments.

Cell proliferation. Splenocytes were cultured at 5×10^5 cells/well in 0.2 mL of RPMI with 10% FCS at 37°C for 48 h with or without Con A, LPS, or soluble anti-CD3 at 0.5 µg/mL, 2 µg/mL, and 1 µg/mL, respectively, in 96-well microtiter plates (8). The cells were pulsed with 1 µCi[^{3}H] thymidine ([^{3}H]TdR; sp. act. 6.7 Ci/mmol; NEN, Boston, MA) for 16 h, harvested onto glass fiber filters, and counted with a liquid scintillation counter (Becton Dickinson). [^{3}H]TdR incorporation was determined after subtracting background incorporation. A control (medium alone) was also included.

Statistical analysis. The data are expressed as means ± SEM. Data were statistically analyzed using a Student's *t*-test and $P < 0.05$ was considered significant.

RESULTS

The amount of moist semipurified AIN diet provided and consumed by mice in both groups was found similar over the 24 wk of the experimental period (5 g d^{-1} $mouse^{-1}$). There were no significant differences in weight gain among the mice fed n-6 and n-3 dietary lipids (15.5 ± 2.0 g $mouse^{-1}$, mean ± SEM, n = 10 mice/treatment). The fatty acid composition of dietary lipids (Table 1) shows that the CO (5%) diet contained 59.7% 18:2n-6, whereas the FO diet contained 9%. The FO (5%) diet contained 15.5% of 20:5n-3 and 13.7% of 22:6n-3 fatty acids which are not present in the CO diet. The FO diet had a 57.6% higher unsaturation index than the CO diet.

TABLE 1
Fatty Acid Composition of Experimental Diets

Fatty acid	Corn oil (% of total fatty acids)	Fish oil (% of total fatty acids)
14:0	0.23	8.43
16:0	10.40	17.30
16:1	N	10.00
18:0	1.60	3.30
18:1	26.34	9.12
18:1	N	2.77
18:2	59.7	9.00
18:3	0.98	1.33
18:3	N	3.00
20:0	0.44	0.56
20:1	0.31	0.97
20:2	N	0.88
20:4	N	1.33
20:5	N	15.50
22:5	N	2.80
22:6	N	13.70
Unsaturation Index[a]	149	234.70

[a]Unsaturation Index is the sum of the products of fatty acid concentration times the number of double bonds for each fatty acid. Values are the mean of two determinations; N, not detected.

Serum lipid peroxides and Vit E levels. Serum lipid peroxides measured as thiobarbituric acid reactive substances (TBARS) were 20% higher in n-3 PUFA-fed mice compared to n-6 PUFA-fed mice. Also, serum Vit E levels were found decreased in n-3 PUFA fed mice by 50% compared to n-6 fed mice (Table 2).

Cellular peroxides. DCFH-DA oxidation reflects a prooxidative state due to intracellular peroxide content (41). The n-3 PUFA-fed mice splenocytes contained 28.3% more peroxides than n-6 PUFA fed mice, as measured by DCFH oxidation (CO 64.24 ± 1.63 vs. FO 77.62 ± 4.05).

Apoptosis in splenocytes. Apoptosis of splenocytes was analyzed by flow cytometric detection of Annexin V and PI staining. In living cells, phosphatidylserine is exclusively located in the membrane leaflets that face the cytosol. The surface expression of phosphatidylserine is a feature of apoptosis and occurs before the loss of membrane integrity (42). Early apoptotic cells bind Annexin V, a Ca^{2+}-dependent phospholipid-binding protein with high affinity for phosphatidylserine, but exclude PI and can therefore be specifically detected and quantified by FACScan (43). Cells from n-3 PUFA-fed mice, incubated in media alone, showed a 50% increase in apoptosis compared to those of n-6 PUFA-fed mice. Furthermore, cells incubated with anti-Fas and anti-CD3 monoclonal antibodies showed an increase of 65 and 66% apoptosis and 17 and 25% necrosis, respectively, in n-3 PUFA-fed mice compared to those of n-6 PUFA-fed mice (Fig. 1).

Expression of Fas, Fas-L, and Bcl-2 in splenocytes by flow cytometry. No significant changes were observed in the levels of subsets among the dietary groups (CD4: CO 20.63 ± 0.78 vs. FO 21.87 ± 0.81; CD8: CO 7.8 ± 0.44 vs. FO 8.01 ± 0.1; CD19: CO 56.21 ± 3.83 vs. FO 59.21 ± 3.83). Flow cytometric analysis demonstrated that the Fas antigen expression in splenocytes (32.8 ± 1.11 vs. 33.1 ± 1.47 in n-6 and n-3 PUFA-fed mice, respectively) was not altered by dietary lipids. Fas-L expression was increased by 30% in splenocytes from n-3 PUFA-fed mice cultured with anti-CD3 for 24 h, compared with splenocytes from CO (n-6 PUFA)-fed mice (Fig. 2A). Interestingly, intracellular Bcl-2 levels were decreased by 21% in n-3 PUFA-fed mice compared with n-6 PUFA-fed mice (Fig. 2B).

Proliferative response of splenocytes to mitogens. The proliferative response of spleen cells to an optimal concentration

TABLE 2
Effect of Dietary Lipids on Serum Lipid Peroxides and Vitamin E Levels

	Lipid peroxides (MDA nmol/mL serum)	Vitamin E (ng/mL serum)
Corn oil	5.69 ± 0.21	190.76 ± 55.44
Fish oil	6.84 ± 0.15[a]	95.21 ± 16.29[a]

[a]Significantly different at $P < 0.05$ (corn oil vs. fish oil); values are mean ± SEM of five individual values from five mice. MDA, malondialdehyde.

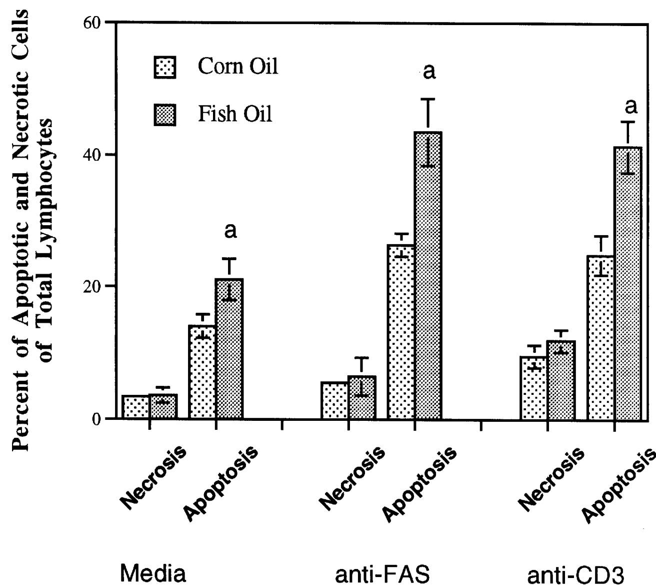

FIG. 1. Effect of dietary lipids on necrosis and apoptosis in splenocytes. Splenocytes were incubated for 8 h in medium alone or with anti-Fas or anti-CD3 and rate of apoptosis was measured as described in the Materials and Methods section. Values are mean ± SEM of five individual values of five mice. Bars marked "a" are statistically significant at $P < 0.05$.

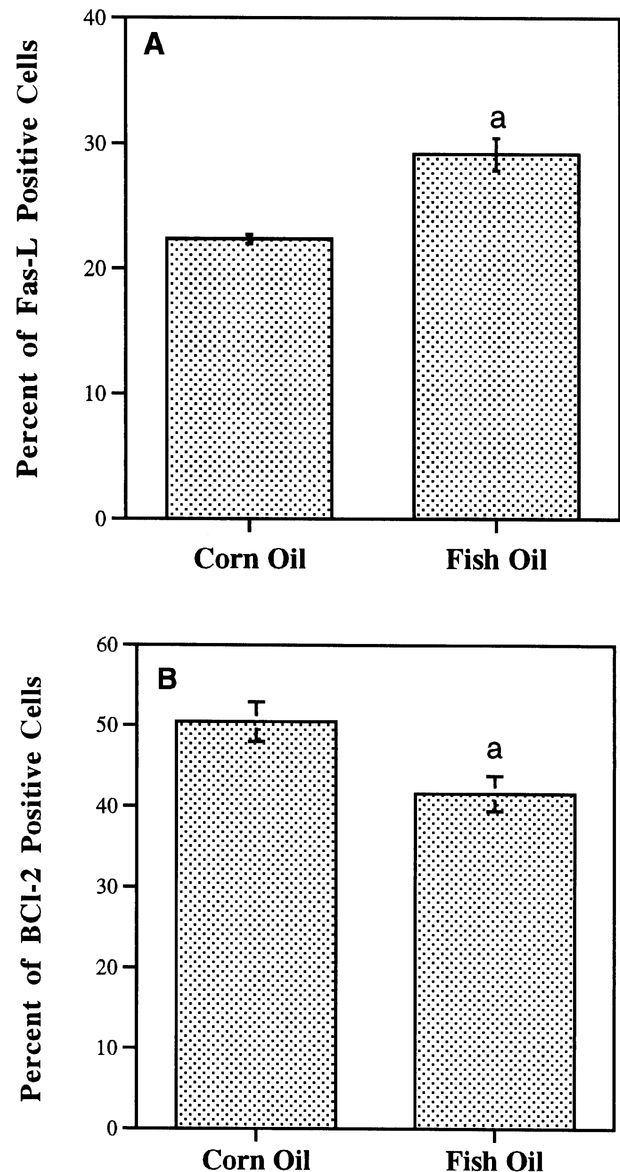

FIG. 2. Effect of dietary lipids on phenotype expression of (A) Fas-L and (B) Bcl-2 on total splenocytes. Values are mean ± SEM of five individual values of five mice; bars marked "a" are statistically significant at $P < 0.05$.

of three regularly used mitogens is summarized in Figure 3. The proliferation of unstimulated cells was decreased by 11% in n-3 PUFA-fed mice. The cell responses to Con A and anti-CD3 were significantly decreased by 16 and 23%, respectively, in n-3 PUFA-fed mice, whereas B cell response to LPS mitogen was found not altered compared to n-6 PUFA-fed mice.

DISCUSSION

Increased tissue DCFH fluorescence and plasma TBARS are the two key parameters related to the generation of reactive oxygen species. Our current findings revealed that both DCFH fluorescence in spleen cells and serum TBARS were increased in n-3 PUFA-fed mice when compared to mice fed n-6 PUFA diets. These findings are consistent with our previous results with rodents and studies in humans described by others (16,36,44,45). It is well established that the source and levels of dietary lipids influence tissue fatty acid composition and differences in immune response *in vivo* (8,36). Since the tissue fatty acid composition can be altered even by low levels of dietary lipids, the susceptibility to peroxidation and subsequent immune function appears to be closely linked to dietary lipid manipulations.

As expected, a low 5% n-3 PUFA diet significantly lowered the serum Vit E levels. In agreement with previous studies, a diet rich in n-3 PUFA increases the requirement for antioxidants such as Vit E (16,44,45). Feeding FO enriches n-3 PUFA in splenocyte membranes (8,36), lowers Vit E levels, and causes increased peroxides in both serum and spleen cells.

Our present results on apoptosis and necrosis also show that dietary FO (rich in n-3 PUFA) increases spontaneous and anti-Fas- and anti-CD3-induced cell death in long-lived nonautoimmune disease-prone mice as measured by PI and Annexin V staining. Previously, we also found that dietary n-3 PUFA increase spontaneous and dexamethazone-induced apoptosis in autoimmune-prone and -resistant strains of mice (36,46). Earlier, however, we did not measure the generation of intracellular free radicals by DCFH staining. Calviello *et al.* (47) also reported that dietary supplementation of eicosapentaenoic acid and docosahexaenoic acid increased apoptosis in Morris hepatocarcinoma 3924A tumors in rats. It has been observed that high levels of dietary FO suppress the growth of human carcinoma cell lines in athymic nude mice, presumably caused by increased accumulation of LPO prod-

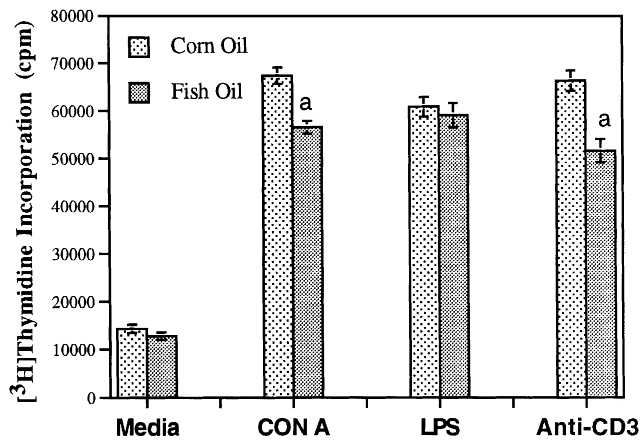

FIG. 3. Effect of dietary n-6 and n-3 polyunsaturated fatty acids on the proliferation of splenocytes. Values are mean ± SEM of five individual values of five mice; bars marked "a" are statistically significant at $P < 0.05$. ConA, concanavalin A; LPS, lipopolysaccharide.

ucts in tumor tissue (48,49), which may contribute to increased apoptosis.

Interestingly, the addition of antioxidant supplements to FO reduced the levels of tumor LPO products, which simultaneously caused an increase in tumor growth, suggesting that the tumor-suppressing activity of long-chain n-3 PUFA could be at least partly due to elevated LPO (48,50). It has been shown that with an increased number of fatty acid double bonds, increased apoptosis and necrosis, a feature of late apoptosis, were preceded by progressively increasing LPO (18). The extent of PUFA-induced LPO measured as malondialdehyde also correlated with the proportion of apoptotic cells (18). Taken together, these observations clearly indicate that highly unsaturated fatty acids are susceptible to LPO and that these lipid peroxides appear to induce apoptosis.

It is well known that the interaction of Fas-L with Fas results in intracellular signals that lead to apoptosis in lymphocytes (34,51). Activation-induced cell death is an important form of programmed cell death because it is crucial for elimination of preactivated cells and is also critical in the elimination of potentially harmful autoreactive lymphocytes (52,53). In the present study, we further showed that n-3 PUFA increase Fas-L expression in splenocytes. The Fas/Fas-L system plays a very important role in the modulation of B and T lymphocyte development and function (29). Defects in the Fas/Fas-L pathways have been shown to result in autoimmune disease (54). Further, defective antigen-mediated cell death can contribute to an increased number of activated auotoreactive cells (55). In earlier studies we showed that dietary n-3 PUFA decrease the severity of autoimmune lupus nephritis in B/W mice (8,9). Further, there is increasing evidence that alterations in cancer cell sensitivity to Fas-mediated apoptosis are a key factor in controlling tumor progression (56), and perturbations in the Fas/Fas-L cell death pathway appear to be important in determining the viability of transformed cells (57). It is well established that the functional activity of T lymphocytes is controlled by delayed sensitization of Fas/Fas-L-mediated apoptosis after activation (58). The results presented here suggest that n-3 PUFA enhance the sensitivity of splenocytes to Fas- and anti-CD3-mediated cell death leading to elimination of hyperactive lymphocytes.

Bcl-2 also plays an important role in the fate of cells committed to undergoing apoptosis (59). Bcl-2, considered a protooncogene and known to block apoptosis, is found to act as an antioxidant either by protecting cell constituents from oxidation or by decreasing the cellular generation of reactive oxygen species (39,60). Recently Hotchkiss *et al.* (61) have reported that overexpression of Bcl-2 in transgenic mice decreases apoptosis in thymic and splenic lymphocytes during sepsis. We therefore measured the basal expression of Bcl-2 in splenocytes. We observed a decreased Bcl-2 expression in n-3 PUFA-fed mice compared with n-6 PUFA-fed mice. Decreased Bcl-2 expression and higher levels of Fas-L in n-3 PUFA-fed murine splenocytes may play a role in the higher susceptibility to anti-Fas- and anti-CD3-induced apoptosis in these mice. Lymphocytes that express high levels of Fas/Fas-L and decreased levels of Bcl-2 would undergo cell death earlier (62). The anticarcinogenic and/or antitumor property of FO may be in part due to induction of elevated Fas-L, which may induce higher cell death and thereby decrease the proliferation of tumor cells.

It is well established that uncontrolled cell proliferation is closely associated with the carcinogenic process (63). Antiproliferative effects of n-3 PUFA have been shown in cancer cells *in vitro* as well as in normal cell lines (13,14). In our present study, we also found decreased proliferation of spleen cells from mice fed dietary n-3 PUFA in the presence (as well as absence) of mitogens. In agreement with our present results (18,47), it has been shown by others that n-3 (20:5 and 20:6) fatty acids are able to suppress tumor cell proliferation. In addition, the antitumor and anticancer properties of FO (n-3 PUFA), at least in part, may be due to increased apoptosis associated with increased expression of Fas-L, decreased expression of Bcl-2, and increased peroxide levels.

In conclusion, these results describe one of the possible mechanisms by which dietary n-3 PUFA enhance or increase both induced and spontaneous apoptosis compared to n-6 PUFA. n-3 PUFA increase the expression of the apoptotic inducer Fas-L and decrease the cell death inhibitor Bcl-2. The increased rate of apoptosis was also found to be closely associated with decreased *in vitro* splenocyte proliferation which is similar to previous observations in both mice and humans (64–66).

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Acyltransferase Activities in the Yolk Sac Membrane of the Chick Embryo

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ABSTRACT: The activities of some enzymes of glycerolipid synthesis and fatty acid oxidation were measured in subcellular fractions of the yolk sac membrane (YSM), an extra-embryonic tissue that mediates the transfer of lipid from the yolk to the circulation of the chick embryo. The activities of monoacylglycerol acyltransferase and carnitine palmitoyl transferase-1 in the YSM (respectively, 284.8 ± 13.2 nmol/min/mg microsomal protein and 145.6 ± 9.1 nmol/min/mg mitochondrial protein; mean ± SE; $n = 4$) at day 12 of development appear to be the highest yet reported for any animal tissue. Also, the carnitine palmitoyl transferase-1 of the YSM was very insensitive to inhibition by malonyl CoA. The maximal activities of glycerol-3-phosphate acyltransferase and diacylglycerol acyltransferase in the YSM (respectively, 26.7 ± 2.2 and 36.1 ± 2.1 nmol/min/mg microsomal protein) were also high compared with the reported values for various animal tissues. The very high enzymic capacity for glycerolipid synthesis supports the hypothesis that the yolk-derived lipids are subjected to hydrolysis followed by reesterification during transit across the YSM. The monoacylglycerol pathway appears to be the main route for glycerolipid resynthesis in the YSM. The results also suggest that the YSM has the capacity to perform simultaneously β-oxidation at a high rate in order to provide energy for the lipid transfer process.

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The transfer of lipid from the yolk to the embryo and the utilization of the various lipid moieties by the developing tissues are the predominant metabolic features of avian embryonic development. This transfer is especially intense during the second half of the developmental period when essentially all the energy needs of the embryo are provided by the β-oxidation of yolk-derived fatty acids. The uptake of lipid from the yolk is performed by the yolk sac membrane (YSM), a highly vascularized extra-embryonic structure that grows outward from the body of the embryo during the early stages of development so as to completely surround the yolk by about the midpoint of the developmental period. Within the endodermal cells of the YSM, the yolk-derived lipids are assembled into particles of the very low density lipoprotein (VLDL) type, which are then secreted into the vascular system for delivery into the embryonic circulation (1–6).

The question arises regarding the degree to which the yolk-derived lipids are modified during their transit across the endodermal cell layer of the YSM. Because the yolk essentially consists of a tightly packed mass of VLDL particles originating from synthesis in the maternal liver (7–9), the most parsimonious explanation would be the transcytosis, without alteration, of these lipoproteins across the YSM. Such a simplistic scenario is, however, very unlikely for several reasons. Most crucially, the yolk-precursor VLDL secreted by the maternal liver is a highly-specialized lipoprotein, uniquely adapted to fulfill its singular function of delivering lipid to the oocyte maturing in the ovary (1,7–11). By contrast, the primary fate of the VLDL that emerges from the YSM is to serve as a substrate for lipoprotein lipase in the capillaries which permeate the adipose tissue and muscle of the embryo, thus delivering fatty acids to the developing tissues (12). Such distinct roles must require differences in lipoprotein structure, and it is pertinent that the maternally derived VLDL particles that form the yolk are unusually small and regular (30 nm diameter) (7–9) whereas those which are exocytosed from the basal face of the YSM endodermal cells are large and polydisperse (50–150 nm diameter) (1,3–5).

In light of these considerations, the alternative situation in which the triacylglycerol (TAG), phospholipid (PL) and cholesteryl ester (CE) of the yolk are subjected to extensive hydrolysis following uptake into the YSM, thus releasing a range of products such as free fatty acids, glycerol, partial glycerides and free cholesterol into the endodermal cytoplasm, would appear to be more likely. The transfer of these hydrolytic products to the endoplasmic reticulum of the endodermal cells, followed by reesterification to re-form TAG, PL and CE in concert with the synthesis of apoproteins (13), would enable the assembly of VLDL particles with a composition suited to their functions in the embryo.

A key prediction of this "lipid remodeling" hypothesis is that the YSM should express the enzymic capacity necessary for the reesterification process. Moreover, the activities of the acyltransferases involved in glycerolipid resynthesis in the

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Abbreviations: CE, cholesteryl ester; CPT-1, carnitine palmitoyl transferase-1; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; GPAT, glycerol-3-phosphate acyltransferase; MGAT, monoacylglycerol acyltransferase; NEM, *N*-ethylmaleimide; PL, phospholipid; TAG, triacylglycerol; VLDL, very low density lipoprotein; YSM, yolk sac membrane.

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YSM would need to be commensurate with the sheer intensity of lipid transfer during the second half of the embryonic period (1). Previous work by Sansbury and coworkers (14) delineated the ontogeny of acyltransferase expression in the liver of the chick embryo. In the present study we focused on the levels of acyltransferase activity exhibited by the YSM. The activities of glycerol-3-phosphate acyltransferase (GPAT) (EC 2.3.1.15) which catalyzes the first committed step of the glycerol phosphate pathway (15,16), monoacylglycerol acyltransferase (MGAT) (EC 2.3.1.22) which acylates *sn*-2-monoacylglycerol to form *sn*-1,2-diacylglycerol and thus catalyzes the defining step of the monoacylglycerol pathway (15), and diacylglycerol acyltransferase (DGAT) (EC 2.3.1.20) which catalyzes the conversion of *sn*-1,2-diacylglycerol to TAG, a step common to both the above pathways (15), were determined in the YSM throughout the second half of development. The levels of carnitine palmitoyl transferase-1 (CPT-1) (EC 2.3.1.21) expressed in mitochondria from the YSM of the embryo were also determined as an indication of the capacity of this tissue for β-oxidation (17,18).

MATERIALS AND METHODS

Embryos. Fertile eggs of the Ross 1 broiler–breeder strain were obtained from a commercial supplier (Ross Poultry, Thornhill, Scotland). The eggs were incubated at 37.8°C and 60% relative humidity in a bench-top incubator (Brinsea Products, Banwell, United Kingdom) with automatic egg turning. At various stages throughout development, the required number of embryos were sacrificed, and the YSM and liver were collected. The YSM was washed thoroughly in 0.85% (wt/vol) NaCl at 4°C to remove any adherent yolk. Hatching occurred after 21 d of incubation, and the required number of chicks were maintained for 1 d with the provision of drinking water but with no food prior to sacrifice.

Preparation of subcellular fractions. Tissue samples were finely chopped with scissors and gently homogenized using a hand-held glass-Teflon homogenizer in 5 vol of 0.25 M sucrose containing 5 mM Tris-HCl buffer (pH 7.4) and 1 mM EGTA at 0°C. All subsequent centrifugations were performed at 4°C. The homogenate was centrifuged at 500 × *g* for 10 min, and the resultant supernatant was recentrifuged at 9,000 × *g* for 10 min to isolate mitochondria. The mitochondrial pellet was washed with homogenization medium and recentrifuged at 9,000 × *g* for 10 min. The washed mitochondria were then suspended in 5 mM Tris-HCl buffer (pH 7.4) containing 0.15 M KCl and 1 mM EGTA prior to enzyme assay. The 9,000 × *g* supernatant was then centrifuged at 100,000 × *g* for 60 min (Centrikon T-1170 Ultracentrifuge; Kontron Ltd., Watford, United Kingdom) to obtain the microsomal fraction. The microsomal pellet was resuspended in homogenization buffer and recentrifuged at 100,000 × *g* for 60 min. The washed microsomal pellet was then resuspended in the appropriate enzyme assay buffer.

The purity of the mitochondrial and microsomal fractions was assessed by marker enzyme assays. Lactate dehydrogenase, citrate synthase, and NADPH-cytochrome c reductase were determined by published methods (19–21). Both fractions were free of cytosolic contamination as indicated by the absence of lactate dehydrogenase in the final washed suspensions. The mitochondrial preparation contained no detectable NADPH-cytochrome c reductase and the microsomal preparation contained no detectable citrate synthase, indicating a lack of cross-contamination between these subcellular fractions.

Assay of MGAT. For each of the four enzymes investigated in this study, the reaction rate was linear with incubation time and with the concentration of microsomal or mitochondrial protein under the assay conditions described. Also, for each enzyme assay, the blank value obtained by stopping the reaction at time zero was subtracted from the measured incorporation. MGAT activity was determined by a previously reported method (22) with minor modification. The reaction mixture consisted of 24 mM Tris-HCl buffer (pH 7.5), 50 mM KCl, 8 mM $MgSO_4$, 0.75 mM dithiothreitol, 0.625 mg bovine serum albumin (fatty acid-free), 15 µg each of phosphatidylcholine and phosphatidylserine, 0.25 mM *sn*-2-monoolein and 25 µM palmitoyl CoA, [^{14}C] palmitoyl CoA (0.01 µCi), and 2 µg microsomal protein in a final volume of 0.5 mL. The *sn*-2-monoolein substrate, which was at least 99% pure as described by the suppliers (Sigma Chemical Co., Poole, Dorset, United Kingdom), was added as a dispersion in 0.1% (wt/vol) Tween 20; the final concentration of Tween 20 in the assay was 0.002% (wt/vol). The reaction was started by the addition of the microsomal protein, and the mixture was incubated at 37°C for 5 min. The reaction was terminated by the addition of 0.75 mL of 2-propanol/heptane/water (80:20:2, by vol). After 5 min, 0.5 mL heptane and 0.25 mL water were added, the samples were mixed and centrifuged at 600 × *g* for 5 min. The heptane layer was removed and washed twice with 1 mL of 0.5 M NaOH/ethanol/water (10:50:50, by vol). The radioactivity in a portion (0.25 mL) of the heptane layer was determined by scintillation counting. The reaction products in the heptane layer were identified as diacylglycerol (DAG) and TAG by thin-layer chromatography on silica gel G using a solvent system of heptane/isopropyl ether/acetic acid (60:40:4, by vol). The bands corresponding to DAG and TAG were scraped from the plates and the radioactivities were determined. The specific activity of MGAT was calculated from the radioactivity in DAG plus half that in TAG.

Assay of GPAT. GPAT activity was determined by a previously described method (30) with slight modification (23). The reaction mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 0.12 M KCl, 1 mg bovine serum albumin (fatty acid-free), 100 µM palmitoyl CoA, 3 mM *sn*-glycerol-3-phosphate, [^{14}C] glycerol-3-phosphate (0.2 µCi), and 50 µg of microsomal or mitochondrial protein in a final volume of 0.25 mL. The reaction was initiated by the addition of the microsomal or mitochondrial protein, and the mixture was incubated for 5 min (YSM) or 10 min (liver) at 30°C. The reaction was terminated by the addition of 2 mL water-saturated 1-butanol followed by 0.75 mL of butanol-saturated water.

After shaking, the samples were centrifuged at 600 × *g* for 10 min, and the butanol layer was removed and washed three times with butanol-saturated water. The radioactivity in 1 mL of the washed butanol layer was determined by scintillation counting. To correct for any cross-contamination between subcellular organelles, we performed the assays after pre-incubation of the subcellular fractions at 37°C for 15 min in the presence or absence of 4.5 mM *N*-ethylmaleimide (NEM). The enzyme in the mitochondrial fraction showed no detectable inhibition by NEM indicating the absence of any contamination with the microsomal form. Up to 5% of the GPAT activity present in the microsomal fraction was resistant to inhibition by NEM, suggesting some contamination of the fraction by the mitochondrial form of the enzyme; the NEM-resistant activity was subtracted from the total microsomal activity in order to provide a corrected value for the activity of microsomal GPAT.

Assay of DGAT. DGAT activity was assayed as previously described (22). The assay mixture consisted of 50 mM Tris-HCl buffer (pH 7.5), 10 mM $MgSO_4$, 0.25 mM dithiothreitol, 0.625 mg bovine serum albumin (fatty acid-free), 1.2 mM *sn*-1,2-DAG, 100 µM palmitoyl CoA, [^{14}C]palmitoyl CoA (0.025 µCi), and 10 µg microsomal protein in a final volume of 0.5 mL. The *sn*-1,2-DAG was added as a dispersion in 0.1% (wt/vol) Tween 20; the final concentration of Tween 20 in the assay was 0.004% (wt/vol). The reaction was initiated by the addition of the microsomal protein, and the mixture was incubated at 37°C for 10 min. Termination of the reaction and extraction of the products were performed as described for the MGAT assay. Radioactivity associated with the TAG fraction isolated by thin-layer chromatography was used to calculate the enzyme activity.

Assay of CPT-1. CPT-1 activity was measured in intact mitochondria as previously described (24). The assay mixture consisted of 5 mM Tris-HCl (pH 7.4), 0.15 M KCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM dithiothreitol, 5 mM ATP, 10 mg bovine serum albumin (fatty acid-free), 2 µg antimycin A, 4 µg rotenone, 250 µM palmitoyl CoA, 0.5 mM carnitine, [^{3}H]carnitine (0.18 µCi), and 0.25 mg mitochondrial protein in a volume of 1 mL. The reaction was initiated by the addition of the mitochondrial protein, with the [^{3}H]carnitine added 2 min later. The assay was for 2 min at 37°C and was terminated by the addition of 0.3 mL of 6 M HCl. The [^{3}H]palmitoylcarnitine formed was quantified as described previously (25). Maximal CPT-1 activity was determined in the presence of 250 µM palmitoyl CoA whereas the sensitivity to inhibition by malonyl CoA was determined in the presence of 35 µM palmitoyl CoA. In the latter case, the enzyme activity in the presence of a range of malonyl CoA concentrations was determined and the sensitivity to this regulatory molecule was expressed as the ID_{50}; i.e., the concentration (µM) of malonyl CoA which inhibited CPT-1 activity by 50%. In all experiments, the formation of palmitoylcarnitine was suppressed by over 90% by the highest malonyl CoA concentration (100 µM). This suggests that a high degree of membrane integrity was maintained in the mitochondrial preparations and that only CPT-1 activity was measured without any significant contribution from CPT-II.

Protein determination. Mitochondrial and microsomal protein content was determined by the method of Lowry *et al.* (26) using bovine serum albumin as standard.

Materials. L-[Methyl-^{3}H]carnitine hydrochloride and [1-^{14}C]palmitoyl CoA were obtained from Amersham International (Aylesbury, Buckinghamshire, United Kingdom). [U-^{14}C]Glycerol-3-phosphate was obtained from ICN Biomedicals Ltd. (Thame, Oxfordshire, United Kingdom). Palmitoyl CoA was purchased from Pharmacia Biotech. (St. Albans, Hertfordshire, United Kingdom), and bovine serum albumin (fatty acid-free) was supplied by Advanced Protein Products (Brierley Hill, West Midlands, United Kingdom). All other biochemicals were obtained from Sigma Chemical Co. (Poole, Dorset, United Kingdom).

Expression of results. Data are expressed as the mean ± SE of measurements on three to five replicate samples at each stage. In the case of the YSM, each replicate sample represents the subcellular fraction derived from an individual YSM. Because of the small size of the livers at days 10, 12, and 14, each replicate subcellular fraction was derived from four pooled livers. At the later developmental stages, the replicate samples were derived from individual livers. Statistical comparisons were performed using Student's *t*-test.

RESULTS

MGAT activity in the YSM. The activity of MGAT in the microsomal fraction of the YSM was determined throughout the second half of the developmental period, from day 10 of embryonic life to 1 d after hatching (i.e., day 22). Very high levels of MGAT activity were maintained in the YSM between days 10 and 19 of embryo development, with a dramatic decrease ($P < 0.001$) in this activity then occurring betweeen days 19 and 20 (Fig. 1).

GPAT activity in the YSM. The developmental changes in GPAT activity in the microsomal and mitochondrial fractions

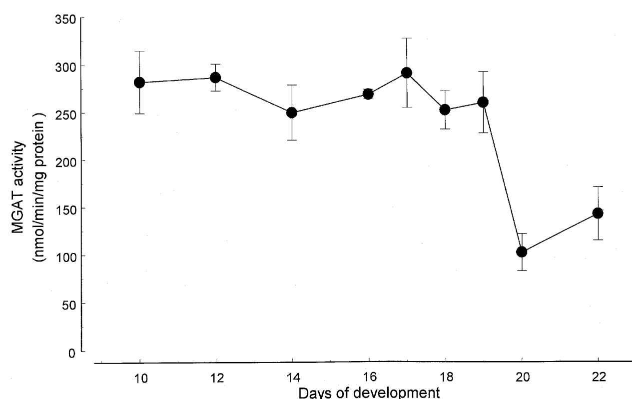

FIG. 1. Activity of monoacylglycerol acyltransferase (MGAT) in the microsomal fraction of the yolk sac membrane during development. Values are means obtained from four yolk sac membranes at each stage, and vertical bars represent SE.

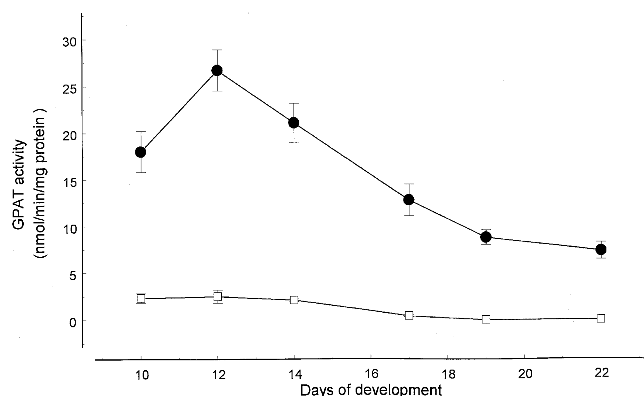

FIG. 2. Activity of glycerol-3-phosphate acyltransferase (GPAT) in the microsomal (●) and mitochondrial (□) fractions of the yolk sac membrane during development. Values are means obtained from four yolk sac membranes at each stage and vertical bars represent SE.

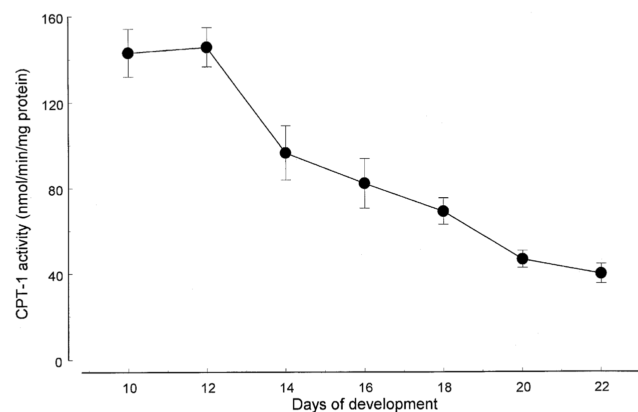

FIG. 4. Activity of carnitine palmitoyl transferase-1 (CPT-1) in the mitochondrial fraction of the yolk sac membrane during development. Values are means obtained from three to five yolk sac membranes at each stage and vertical bars represent SE.

of the YSM are shown in Figure 2. The activity of the microsomal enzyme increased between days 10 and 12 ($P < 0.05$) and decreased continuously thereafter so that the activity at day 22 was only 25% ($P < 0.001$) of the peak value at day 12. Only a small proportion of the total YSM GPAT activity was accounted for by the mitochondrial form of the enzyme at all stages. For example, at day 12 the microsomes contained 92% of the total activity with only 8% present in the mitochondria. By day 19, the mitochondrial enzyme was undetectable.

DGAT activity in the YSM. Substantial levels of DGAT activity were expressed in the microsomes of the YSM throughout the developmental period studied (Fig. 3). Although this activity appeared to reach a peak value around days 12 to 14 with a decrease thereafter, these changes were not statistically significant.

CPT-1 activity in the YSM. Very high activities of CPT-1 were expressed in the mitochondria of the YSM at days 10 and 12 (Fig. 4). After this stage the activity decreased continuously with the result that the level of this enzyme at day 22 was only 27% of the peak value at day 12 ($P < 0.001$). The sensitivity of CPT-1 from the YSM of the embryo to inhibition by malonyl CoA did not change to any major extent during the embryonic period. The ID_{50} (μM) values for this inhibitory effect were 18.5 ± 2.4, 20.0 ± 2.0, 16.0 ± 1.1, 21.0 ± 3.4, and 15.0 ± 2.2 at days 10, 12, 14, 16, and 18, respectively.

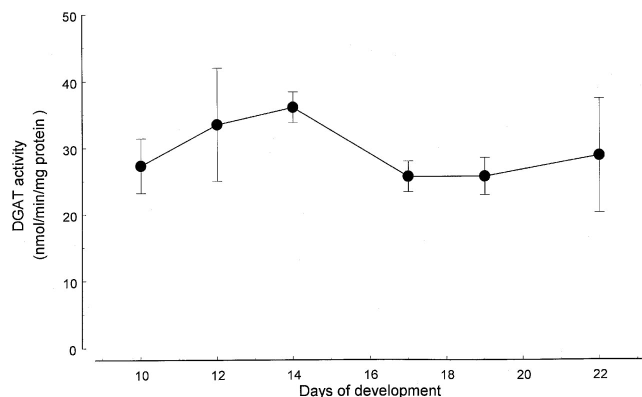

FIG. 3. Activity of diacylglycerol acyltransferase (DGAT) in the microsomal fraction of the yolk sac membrane during development. Values are means obtained from four yolk sac membranes at each stage and vertical bars represent SE.

Enzyme activities in the liver of the embryo. The activity of MGAT in the embryonic liver increased almost threefold ($P < 0.001$) between days 12 and 19 of development and then decreased by about 50% ($P < 0.001$) over the hatching period (Table 1). These hepatic activities were, however, far lower than the activities of MGAT determined in the YSM. At day 12, for example, the level of MGAT in the liver was only 9% of the activity expressed in the YSM ($P < 0.001$). The activity of GPAT in the liver microsomes showed little change from day 12 to day 22 of development, was far lower than the activity of MGAT in the liver, and was also far lower than the activity of GPAT in the YSM. About 16 to 25% of the total GPAT expressed in the liver was due to the mitochondrial form of the enzyme. The hepatic DGAT activity increased by 2.3-fold ($P < 0.05$) from day 12 to day 19, and this elevated activity was maintained over the hatching period. The activities of DGAT in the liver at days 19 and 22 were not significantly different from the concurrent activities of this enzyme in the YSM. The activity of CPT-1 in the liver mitochondria

TABLE 1
Acyltransferase Activities in the Liver of the Chick Embryo[a]

Enzyme	Day 12	Day 19	Day 22[b]
MGAT (*n* = 4)	24.9 ± 4.3	70.0 ± 2.7	38.0 ± 3.1
GPATmic (n = 6)	2.7 ± 0.1	2.5 ± 0.3	3.6 ± 0.3
GPATmit (n = 6)	0.5 ± 0.1	0.6 ± 0.1	1.2 ± 0.1
DGAT (n = 4)	9.8 ± 2.2	22.5 ± 4.1	26.4 ± 4.7
CPT-1 (n = 4)	80.3 ± 13.7	53.7 ± 6.2	52.0 ± 2.1

[a]nmol/min/mg microsomal (for MGAT, $GPAT_{mic}$, DGAT) or mitochondrial (for $GPAT_{mit}$, CPT-1) protein (mean ± SE).
[b]Represents 1 d after hatching. MGAT, monoacylglycerol acyltransferase; $GPAT_{mic}$ and $GPAT_{mit}$, glycerol-3-phosphate acyltransferase (microsomal and mitochondrial forms, respectively); DGAT, diacylglycerol acyltransferase; CPT-1, carnitine palmitoyl transferase-1.

was high at day 12, decreasing by about 33% at the later stages. The activity of CPT-1 in the liver at day 12 was, however, only about half the activity expressed in the YSM at this time ($P < 0.01$). As a measure of the sensitivity of hepatic CPT-1 to inhibition by malonyl CoA, the ID_{50} values were 11.0 ± 1.1 and 7.3 ± 0.8 μM at days 12 and 19, respectively.

DISCUSSION

A degree of caution is required in extrapolating the activities of the enzymes of glycerolipid synthesis as determined *in vitro* to the actual levels of expression of these activities *in vivo*, particularly since these assays involve membrane-bound enzymes and hydrophobic substrates/products. However, with this proviso, a major finding of the present study is that the YSM of the chick embryo displays an exceptionally high enzymic capacity for glycerolipid synthesis. This is particularly evident in the case of MGAT, which is expressed in the YSM at an unprecedented level compared with the reported activities for other avian or mammalian tissues at various developmental stages (22,27–29). Even the intestinal mucosa of adult mammals, regarded as the classic tissue for the operation of the monoacylglycerol pathway, exhibits much lower MGAT activities than those reported here for the YSM. For example, the activity (nmol/min/mg protein) of this enzyme in the intestinal mucosa of the neonatal (28) and adult (22) rat is approximately 100 compared with a peak of almost 300 in the YSM. Apart from its expression in the intestine, the tissue-specific distribution of MGAT in the adult mammal is very restricted (15). In particular, the adult liver is normally almost devoid of MGAT activity although this enzyme is transiently expressed in neonatal rat liver, attaining a level that is 30–40% of that achieved by the YSM (28). Thus the YSM of the avian embryo should be added to the select list of animal tissues possessing high MGAT activity.

The activity of GPAT in the YSM is also very high in comparison with the reported levels of this enzyme in tissues of the adult mammal. For instance, the activities of this enzyme (nmol/min/mg protein) in the liver and intestine of the adult rat are approximately 6.0 and 0.5, respectively (22), compared with 26.7 in the YSM at day 12. The activity of DGAT, the only enzyme concerned exclusively with TAG synthesis, was also relatively high in the YSM. For example, the DGAT activities (nmol/min/mg protein) in the liver and intestine of the adult rat are approximately 3.0 and 5.0, respectively (22), compared with a maximum of 36.1 in the YSM.

An additional point is that the activities of MGAT, GPAT, and DGAT are far higher in the YSM than in the developing liver, or indeed in any other tissue, of the chick embryo. These activities have previously been reported for the liver and certain other chick embryo tissues (14), and our present values obtained for the liver are consistent with this earlier study. The overall conclusion is that the YSM possesses an almost unprecedented enzymic potential for the synthesis of glycerolipids *via* the acylation of partial glycerides or glycerol-3-phosphate.

This conclusion clearly lends support to the hypothesis that, during their transit across the YSM, the lipids of the yolk are hydrolyzed to their constituent free fatty acids, glycerol, partial glycerides, free cholesterol, and the like, which are then reesterified to resynthesize TAG, PL, and CE in a process coupled to the assembly of VLDL particles. The fact that MGAT activity in the YSM was greater than that of GPAT by an order of magnitude may be taken to indicate that the monoacylglycerol pathway forms the predominant means of reesterification in this tissue, with the glycerol phosphate pathway performing a supporting role. Previous observations that the YSM also displays very high levels of acyl-CoA:cholesterol acyltransferase activity (ACAT) (30) as well as a high expression of the mRNA for apoprotein B (31) are also consistent with this view.

Complementary to these biochemical studies is the evidence from electron microscopy (1–5). In brief, this indicates that yolk droplets and granules are engulfed by the apical surface of the YSM endodermal cells and that this uptake is followed by the fusion of the phagocytotic vesicles within the cell to form large lipid-rich vacuoles. During the second half of the embryonic period, this uptake is so intense that these lipid-rich vacuoles occupy a large proportion of the cytoplasmic space and totally dominate the appearance of the cells. Meanwhile, the opposite side of the cell is observed to be extremely active in the exocytosis of lipoproteins into the vascular system of the YSM. Lipid-rich spherules corresponding to VLDL particles are packed into the cisternae of the endoplasmic reticulum and Golgi. Moreover, secretory vesicles containing VLDL particles can be observed in the process of fusion with the basal plasma menbrane, thus releasing these lipoproteins into the circulation.

The results described in the present study may help to define the biochemical processes that link the absorptive events at the apical surface with the subsequent secretory events at the basal surface of the endodermal cells. There is evidence that, as a result of fusion with lysosomes, the large lipid-rich vacuoles formed by the phagocytotic process are converted to so-called lipolysosomes in which the hydrolysis of the vacuolar contents proceeds at a high rate, catalyzed by lysosomal lipases and proteases (32). We propose that the products of this hydrolysis (free fatty acids, partial glycerides, glycerol, free cholesterol, lyso-PL, and the like) diffuse out of the large vacuoles and are transported to the endoplasmic reticulum where reesterification to synthesize TAG, PL, and CE is rapidly achieved by the very high activities of MGAT, GPAT, DGAT, acyl-CoA:acyltransferase, and presumably of the other enzymes required for complex lipid synthesis. The coordination of this reesterification process with the translation of the mRNA for apoprotein B (31) will promote the assembly of VLDL particles in the lumen of the endoplasmic reticulum (13). The exceptional activity of MGAT in the YSM implies that *sn*-2-monoacylglycerol is a major product of yolk lipid hydrolysis in the large vacuoles with the further inference that the lysosomal lipase which acts on the yolk-derived TAG preferentially hydrolyzes the ester bonds at the *sn*-1 and -3 positions.

The very high activity of CPT-1 in the mitochondria of the YSM, as reported in the present study, is indicative of an exceptionally high capacity for β-oxidation in this structure. The maximal CPT-1 activity measured in the YSM was about three times greater than the highest levels of this enzyme reported for the rat liver under any physiological state (17,18,24). Furthermore, the YSM enzyme was relatively insensitive to the physiological inhibitor, malonyl CoA. In fact, the CPT-1 activity of the adult rat liver is almost 10 times more sensitive to malonyl CoA inhibition compared with the YSM enzyme (17,18). These results suggest that a proportion of the yolk-derived lipid is oxidized in the mitochondria of the YSM in order to provide the energy for the translocation of the bulk of lipid across the endodermal layer. The present paper also reports for the first time the developmental expression of CPT-1 activity in the liver of the chick embryo; at day 12, this hepatic activity was only about half that concurrently expressed in the YSM but was nevertheless about 50% greater than the highest activities reported for the liver of the adult rat (17,18,24). CPT-1 in the mitochondria of the chick embryo liver was more sensitive than the YSM enzyme to inhibition by malonyl CoA, but was still relatively resistant to this inhibitor when compared with the enzyme from the liver of the adult rat (17,18). In the present study, we also determined the activity of CPT-1 in mitochondria isolated from the liver of the adult chicken (female, fed state). The activity of this enzyme in the adult was 20.8 ± 1.4 nmol/min/mg mitochondrial protein (n = 4 livers), far lower than the values obtained from the YSM and liver of the embryo at all stages.

The aim of the present work was to determine the activities of the acyltransferases in the YSM during the second half of the developmental period when the transfer of lipid from the yolk to the embryo is most intensive. The results indicate that the maximal or near-maximal activities of these enzymes have already been attained by day 10 of development, the earliest time-point studied. In future work, it would be of interest to measure these enzyme activities in the YSM during the first half of the developmental period in order to delineate the ontogeny of their expression and to determine the timing of their induction.

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Lipid Composition of Hepatocyte Plasma Membranes from Geese Overfed with Corn

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ABSTRACT: Twelve-week-old Landes male geese were overfed with corn for 21 d in order to induce liver steatosis (fatty liver). Lipid composition of hepatocyte plasma membranes from fatty livers was compared to that of lean livers obtained from geese fed a normal diet. The ratio cholesterol/phospholipids was higher in fatty hepatocyte plasma membranes (0.63 vs. 0.47), whereas the phospholipid/protein ratio was less than half. Overfeeding induced changes in fatty acid composition of hepatocyte plasma membranes, including a greater than twofold increase in the percentage of oleic acid (29.7 vs. 13.8%) and a somewhat lesser increase in lauric, palmitic, and palmitoleic acid contents of plasma membrane lipids of fatty livers. A concomitant reduction in the proportion of stearic acid (18.4 vs. 25.1%) was also observed. In fatty livers, the increased ratio of saturated to polyunsaturated fatty acids (PUFA) (1.5 vs. 1.0) was related to a significant decrease in PUFA content. Among all the PUFA, only the eicosatrienoic acid (20:3n-9) percentage was increased by liver steatosis. Overfeeding with corn appeared to induce competition between *de novo* synthesized and dietary fatty acids incorporated in hepatocyte plasma membranes. This resulted in an accumulation of *de novo* synthesized monounsaturated and derived fatty acids in plasma membranes from overfed birds. A defect in the incorporation of linoleic acid and linoleic- and linolenic-derived PUFA was observed despite the high proportion of these essential fatty acids in the diet. It was conclued that in overfed palmipeds, *de novo* hepatic lipogenesis prevails over dietary lipid intake to modulate lipid composition of the fatty liver plasma membrane.

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In mammals, liver steatosis generally originates from metabolic or infectious disease and is associated with an alteration of hepatic tissue structure. Fatty liver syndrome also has been described as a metabolic disease in laying chickens. When induced by overfeeding (1), this syndrome involves a twofold increase in liver weight, a liver fat content not exceeding 25%, hemorrhage, and hematoma.

In some palmiped breeds, overfeeding with a carbohydrate-rich diet also leads to the accumulation of triglycerides by hepatocytes. This susceptibility to nonhemorrhagic steatosis is used in the production of fatty liver ("foie gras"). Two or three weeks of overfeeding induces as much as a 10-fold increase in liver weight, and liver fat content can reach 60% wet weight. If overfeeding is not prolonged, tissue lesions generally do not occur (2,3), and despite overt histological fatty liver changes (2,3), metabolic activities are conserved (4). Moreover, interruption of overfeeding leads to a rapid decrease of liver weight, indicating the continuous exportation of liver fat. Histological and physiological parameters of the liver return to preoverfeeding values, showing the rapid reversibility of palmiped hepatic steatosis (5).

As in all bird species, the liver of palmipeds is the major site of *de novo* lipogenesis (6). According to Hermier *et al.*, a high lipid synthesis activity (4) and a triglyceride incorporation defect in circulating lipoproteins (7,8) could induce hepatic enlargement in overfed palmipeds. But liver enlargement also involves cellular hypertrophy and hyperplasia (9). On the other hand, geese that resist steatosis exhibit an enhanced exportation of polyunsaturated fatty acids (PUFA) in very low density lipoprotein-phospholipid (VLDL-PL) and high density lipoprotein-phospholipid (HDL-PL) which has been hypothesized to limit membrane growth and hepatocyte hypertrophy (10). The ability to use the PL for hepatocyte membrane synthesis, rather than for secretion as VLDL and HDL, could explain the differing susceptibility to steatosis among palmiped strains (11).

Thus, steatosis induced by overfeeding in palmipeds may be considered as a physiological process as opposed to hepatic steatoses usually considered as pathological in mammals or other avian species. The susceptibility of palmipeds to nonpathological liver steatosis seems to be related to a limited ability to export liver PL as lipoproteins while having continued hepatocyte membrane synthesis. This is thought to allow growth and preservation of functional and structural integrity of plasma membranes whereas triglycerides accumulate in the hepatocytes. But, to our knowledge, there are few data on the structure of hepatocyte plasma membranes of palmiped fatty liver (11), or its fatty acid composition.

The high *de novo* hepatic lipogenesis induced by carbohydrate overfeeding leads to an accumulation of the newly synthesized fatty acids in triglycerides of hepatocytes (8). Once incorporated into PL, these fatty acids can also influence the

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Abbreviations: HDL, high density lipoproteins; MUFA, monounsaturated fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PL, phospholipid; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; VLDL, very low density lipoproteins.

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plasma membrane lipid composition. On the other hand, corn is usually the only component of the diet when inducing steatosis of palmipeds. Corn lipids comprise greater than 50% linoleic acid. We can hypothesize that, as in mammals, a high intake of an essential fatty acid will change the lipid composition of hepatocyte plasma membranes.

The aim of this work was to investigate the influence of corn overfeeding on lipid and fatty acid composition of goose hepatocyte plasma membranes and to study the proportions of *de novo* synthesized and dietary lipids in altering the composition.

TABLE 1
Lipid Content (% DM) and Fatty Acid Composition of the Diets

	Control diet	Overfeeding diet
Lipids (% dry mass)	2.1	3.85
Fatty acids (wt%)		
16:0	19.9	8.8
18:0	5.1	2.0
18:1	30.5	26.2
18:2n-6	38.4	60.7
18:3n-3	4.0	1.2
18:2n-6, g/100g diet	0.81	2.31
18:3n-3, g/100g diet	0.08	0.05

MATERIALS AND METHODS

Animals and samples. Liver samples were taken from a French Landes male goose flock, bred at the Station for Goose Breeding (Asseldor, Coulaures, France) under specified conditions (12).

Eight-week-old animals were fed a commercial diet (palmiped K34, provided by COFNA, 24 Sarlat, France) (control diet). A quantitative rationing resulted in a 2.6 MJ metabolizable energy and 35 g protein intake per day. Lipid content and fatty acid composition of the diets are shown in Table 1. The intakes of 18:2n-6 and 18:3n-3 were approximately 1.70 and 0.17 g/d, respectively. After 4 wks of control diet feeding, a part of the flock was commercially processed in a slaughterhouse. (Breeding and slaughtering methods are in accordance with French regulation for welfare and humane treatments of animals). Evisceration was performed just after plucking, and liver samples were collected by taking about 50 g from the middle of lobes of five 95-g average-weight lean livers (control).

For the next 21 d, the other geese of the flock were submitted to a soaked-corn overfeeding, corresponding to an average 13 MJ metabolizable energy, 80 g protein, 18.5 g 18:2n-6, and 0.37 g 18:3n-3 intake per day. Liver samples were harvested as described above from five 950-g average-weight fatty livers. All samples were frozen in liquid nitrogen and kept at –60°C until analysis.

Preparation of plasma membranes. Plasma membranes of goose livers were prepared by an adaptation of a procedure described by Evans (13). Briefly, membranes were isolated from filtered tissue homogenate with a loose-fitting Dounce homogenizer. The use of a minimum of strokes permitted large plasma-membrane fragments to be recovered into the pellet after a low-speed centrifugation. Steatosis-affected liver tissue has a high triglyceride content. Pelleting of the plasma membrane fraction separated it from most of the triglycerides, which were recovered in the supernatant. Nevertheless, the membranes isolated from fatty livers contained 35 and 1.6% of total lipids as triglycerides and cholesteryl esters, respectively (see footnotes A, B, Table 2). Subsequent lipid class separation removed neutral lipids before PL composition determination. Plasma membranes were isolated from nuclear and mitochondrial material through a 60,000 × *g* centrifugation for 3.30 h over a discontinuous density gradient (1.076, 1.125, 1.174, 1.222 g/mL). This density gradient was prepared according to Graham *et al.* (14) by diluting Iodixanol (available commercially as OptiPrep from Nycomed Pharma, Oslo, Norway) in a solution containing 8.0% (wt/vol) sucrose, 20 mM Tris-HCl, pH 7.8, and 1 mM $MgSO_4$. Plasma membranes were collected in a strip located in a median position. This fraction was then suspended in a physiological solution (NaCl 9‰) and centrifuged to eliminate most of the related nonmembrane proteins (15). To evaluate the recovery and contamination of the plasma membrane fraction, the activities of the following enzymes were used as the markers of various subcellular organelles: 5′-nucleotidase (EC 3.1.3.5) for plasma membrane (16), succinate dehydrogenase (EC 1.3.99.1) for internal mitochondria membrane (17), NADH dehydrogenase (EC 1.6.99.3) for endoplasmic reticulum membrane (18), and acid phosphatase (EC 3.1.3.2) for lysosomal membrane (19). In the purified fraction, the recovered activity of the 5′-nucleotidase enzyme was more than 20 and 50% (in control and fatty liver, respectively) of the total homogenate activity. The contamination in control and fatty liver preparations of NADH dehydrogenase and acid phosphatase activities was lower than 10 and 5%, respectively. Succinate dehydrogenase activity was never recovered in the purified fractions.

Membrane composition. Membrane protein content was determined by the method of Lowry *et al.* (20) with bovine serum albumin as standard. Total lipids were extracted from the isolated plasma membrane preparations following the procedure of Bligh and Dyer (21). PL were estimated by an organic phosphorus assay according to the method of Vaskovsky *et al.* (22). The removal of neutral lipids and the separation of PL classes was achieved by high-performance liquid chromatography (Hewlett-Packard) according to the Hax (23) procedure. We used a Lichrosorb SI-60 chromatography column (5 µm, 25 cm × 7.5 mm) and an *n*-hexane/2-propanol/water mixture as solvent gradient system. PL absorbance was measured at 206 nm using an ultraviolet detector. Purity of each PL class was confirmed by thin-layer chromatography (24). Fatty acid composition of total PL and major PL classes was determined after transmethylation (25). Fatty acid methyl esters were analyzed using a gas chromatograph (Hewlett-Packard 5890 series II) fitted with a 0.25-µm film thickness reticulated polyethyleneglycol phase 30 m × 0.25 mm i.d. capillary column (Innowax, Hewlett-Packard),

TABLE 2
Lipid Composition of Hepatocyte Plasma Membrane of Lean (control) and Fatty Goose Liver[a] (mean ± SEM)

Criteria	Control (n = 5)	Fatty liver (n = 5)	Significance[b]
Phospholipids (µg/mg protein)	274 ± 42	127 ± 16	*
Cholesterol[c] (µg/mg protein)	59 ± 5	40 ± 4	*
Cholesterol/phospholipids (mol/mol)	0.47 ± 0.05	0.63 ± 0.04	*
Phospholipid classes (mol%)			
Phosphatidylcholine	56.9 ± 3.8	57.1 ± 4.1	NS
Phosphatidylethanolamine	38.0 ± 3.7	38.1 ± 2.5	NS
Phosphatidylserine + phosphatidylinositol	4.2 ± 1.2	3.1 ± 1.3	NS
Sphingomyelin	0.9 ± 0.18	1.7 ± 0.23	*

[a]Plasma membranes isolated from fatty liver also contained 35% of total lipids as triglycerides vs. 0.6% in membranes from control livers (determined on pooled samples).
[b]Significance of the differences: NS = nonsignificant; *$P < 0.05$.
[c]Plasma membranes isolated from fatty liver contained 1.6% of total lipids as cholesteryl esters vs. 0.1% in membranes from control livers (determined on pooled samples).

with nitrogen as carrier gas. The temperatures of the injector and the detector were 250 and 300°C, respectively, and the column temperature was set from 200 to 250°C with a 5°C/min rise. Total cholesterol in the membrane extract was quantified by a colorimetric enzymatic method using Sigma Diagnostics procedure n° 352. Removed neutral lipids were pooled and classes were separated by gas chromatography on an apolar capillary column (DB5, J&W Scientific Inc., Folsom, CA), after silylation (26). Neutral lipids, PL, and fatty acid standards were provided by Sigma Chemical (St. Louis, MO).

Statistics. Results were expressed as mean ± SEM, and their significance were analyzed using unpaired Student's *t*-test.

RESULTS

Corn overfeeding induced changes in lipid composition of goose hepatocyte plasma membranes (Table 2). Nevertheless, we considered the huge triglyceride content (35% of total lipids) of plasma membrane isolated from fatty liver as an artifact. Triglycerides accounted for 50% of fatty liver wet weight and could remain associated with plasma membrane fragments or be included into vesicles during homogenization, preventing a complete separation from plasma membranes.

Concerning the main lipids of plasma membranes, the ratio of total cholesterol to proteins was 30% lower in fatty liver than in controls whereas the PL/protein ratio exhibited a 54% decrease. This resulted in a significantly higher cholesterol/PL ratio in fatty liver than in controls. Cholesteryl esters, which were insignificant in control plasma membranes, seemed to increase in fatty liver but represented no more than 10% of total cholesterol.

As found in most animal cell membranes, phosphatidylcholine (PC) and phosphatidylethanolamine (PE) largely prevailed as they formed more than 80% of the total PL of hepatocyte plasma membrane in goose. Overfeeding did not generate any modification in the distribution of major PL classes. Only an increase in the sphingomyelin fraction content was significant.

Plasma membrane lipid fatty acid compositions of control and fatty liver are shown in Table 3. In control livers, saturated fatty acid (SFA) and PUFA contents were similar, both being around 40% of total fatty acids. The predominant SFA were stearic and palmitic acids, whereas the major PUFA were, in decreasing order, arachidonic, linoleic, and docosahexaenoic (22:6n-3) acids. Monounsaturated fatty acids were mainly represented by oleic acid (14%). The other fatty acids each represented less than 1% of total fatty acids.

Overfeeding largely modified the fatty acid profile of hepatocyte plasma membrane PL. Among SFA, the relative amounts of myristic and palmitic acids increased, whereas the

TABLE 3
Fatty Acid Composition (% by weight of total fatty acids) of Hepatocyte Plasma Membrane Phospholipids (mean ± SEM)

	Liver status		
Fatty acids	Control (n = 5)	Fatty liver (n = 5)	S[a]
14:0	0.10 ± 0.02	0.30 ± 0.05	**
16:0	16.5 ± 0.77	21.4 ± 1.21	**
16:1	0.50 ± 0.12	1.60 ± 0.29	**
18:0	25.1 ± 0.74	18.4 ± 0.90	***
18:1	13.8 ± 1.26	29.7 ± 1.81	***
18:2n-6	12.7 ± 0.24	3.10 ± 0.39	***
20:1	0.20 ± 0.01	0.30 ± 0.04	**
20:3n-9	0.30 ± 0.14	1.80 ± 0.24	***
20:4n-6	21.1 ± 1.43	17.1 ± 0.15	*
20:5n-3	0.30 ± 0.05	0.50 ± 0.12	NS
22:4n-6	0.70 ± 0.09	0.65 ± 0.12	NS
22:4n-3	0.40 ± 0.10	0.90 ± 0.52	NS
22:5n-3	0.50 ± 0.09	0.35 ± 0.11	NS
22:6n-3	5.80 ± 0.47	1.30 ± 0.32	***
MUFA	14.5 ± 1.31	31.6 ± 2.08	***
UFA/SFA[b]	1.35 ± 0.02	1.49 ± 0.06	*
UI[c]	4.00 ± 0.09	3.43 ± 0.23	*
SFA/PUFA[d]	1.00 ± 0.07	1.47 ± 0.12	**

[a]Significance of the differences: NS = nonsignificant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.
[b]Unsaturated/saturated fatty acid ratio.
[c]Unsaturation Index = Σ(% of unsaturated fatty acid × number of double bonds)/Σ(% of saturated fatty acid).
[d]Saturated/polyunsaturated fatty acid ratio.

TABLE 4
Fatty Acid Composition (% by weight of total fatty acids) of Two Major Phospholipid Classes of Hepatocyte Plasma Membranes (mean ± SEM)

	Phosphatidylethanolamine			Phosphatidylcholine		
Fatty acids	Control (n = 5)	Fatty liver (n = 5)	S[a]	Control (n = 5)	Fatty liver (n = 5)	S
16:0	11.5 ± 1.68	9.70 ± 0.98	NS	23.1 ± 1.02	28.8 ± 0.41	***
16:1	ND[b]	0.90 ± 0.65		ND	1.80 ± 1.09	
18:0	33.6 ± 1.22	29.1 ± 1.51	*	21.8 ± 0.82	13.0 ± 1.35	***
18:1	6.40 ± 1.17	26.0 ± 2.23	***	12.6 ± 2.11	33.6 ± 1.23	***
18:2n-6	7.30 ± 1.02	3.50 ± 0.61	*	11.5 ± 0.59	3.70 ± 0.75	***
20:3n-9	ND	2.20 ± 2.10		0.90 ± 0.11	2.90 ± 0.32	***
20:4n-6	29.8 ± 1.39	24.9 ± 1.10	*	25.8 ± 1.15	14.6 ± 1.41	***
22:4n-6	ND	0.90 ± 0.42		ND	ND	
22:4n-3	ND	0.30 ± 0.19	ND	ND		
22:6n-3	11.3 ± 0.59	1.20 ± 0.28	***	4.20 ± 0.52	ND	
UFA/SFA	1.20 ± 0.08	1.50 ± 0.10	*	1.20 ± 0.06	1.30 ± 0.09	NS
UI	4.60 ± 0.12	3.90 ± 0.37	NS	3.70 ± 0.12	2.60 ± 0.15	***
SFA/PUFA	0.90 ± 0.11	1.20 ± 0.23	NS	1.10 ± 0.07	2.00 ± 0.09	***

[a]Significance of the differences: NS, nonsignificant; *P < 0.05; **P < 0.01; ***P < 0.001.
[b]N.D., not detected. For other abbreviations see Table 3.

percentage of stearic acid was significantly reduced. Monounsaturated fatty acids were significantly increased, with the oleic acid content being over twofold higher in fatty than in control liver (30 vs. 14% of total fatty acids, respectively). In fatty livers, all the n-3 and n-6 PUFA proportions significantly decreased, and their total percentage was reduced from 41 to 24%. On the other hand, eicosatrienoic acid (20:3n-9) content increased about sixfold without exceeding 2%.

If the ratio of unsaturated fatty acids to SFA was slightly but significantly increased between control and fatty liver, the unsaturation index (UI), taking into consideration the number of double bonds, decreased while the SFA/PUFA ratio was markedly increased (50%).

Fatty acid compositions of both PL main species, PC and PE, were different in control and fatty liver plasma membrane (Table 4). The intensity of the modifications of fatty acid content induced by overfeeding appeared to be peculiar to each PL class. Nevertheless, fatty acid profiles for both PC and PE are modified in the same way. Thus, 18:1 and 20:3n-9 content of PC and PE was increased in fatty liver, whereas stearic acid and total n-6 and n-3 PUFA contents were greatly decreased. Only the palmitic acid proportion remained unchanged for PE and significantly increased for PC in fatty livers when compared to controls.

DISCUSSION

In palmipeds, the marked increase in liver weight after corn overfeeding corresponds to a greatly increased lipid synthesis and accumulation. In animals with fatty liver, total lipids represent over 50% of the hepatic mass. These lipids consist of more than 90% triglycerides (27). The syntheses induced by carbohydrate overfeeding also involve transport lipoproteins and structural components that are part of the cell hyperplasia and hypertrophy (9). This is highlighted by the increase in total proteins, PL and cholesterol in fatty liver (27). Nevertheless, in agreement with observations reported by Salichon *et al.* (10), our results showed that overfeeding sharply reduced the lipid content of hepatocyte plasma membranes with respect to the protein matrix. Although the proteins were not characterized, perhaps an increased level of membrane proteins is related to increases in metabolic activity of hepatocytes during overfeeding. The cholesterol/PL ratio was significantly higher in fatty liver membranes, showing that PL were relatively more reduced in plasma membranes than was total cholesterol. A decrease in liver PL retention could be also involved (28), originating in a relative deficiency in synthesis together with an enhanced secretion through VLDL and HDL (11).

By contrast, the relative proportions of the two main species of membrane PL, PC and PE, were unaffected by overfeeding. In corn-overfed ducks, Gabarrou *et al.* (29) observed a decrease in hepatic PC content, deemed to be the result of a dietary choline deficiency. Our results in geese, however, do not support a possible choline deficiency since the PC content was stable and the sphingomyelin content increased by overfeeding. Nevertheless, we had previously shown that, in the whole hepatic tissue, the content of these PL species evolved differently during the postprandial period, according to hepatic lipoprotein synthesis and flow (30). Their relative content therefore depended on the digestive status of the animals at the time of slaughter.

Palmiped overfeeding with a carbohydrate-rich diet results in high *de novo* liver lipogenesis. Palmitic and oleic acids are the main products of fatty acid synthesis, and they constituted over 80% of the hepatic triglyceride fatty acid content. Our present study showed that these two fatty acids were also the predominant species incorporated in membrane PL. Their proportion in membrane lipids increased from 30% in the control to 50% in fatty liver. Therefore, it can be concluded that increased lipogenesis induced by overfeeding in palmipeds leads to greatly increased amounts of palmitic and oleic acids and that these fatty

acids readily contribute to both PL and triglyceride synthesis. The increase in triglyceride and PL oleic acid content, together with a concomitant decrease in stearic acid content, could correspond to an increase in Δ9 desaturase enzyme activity.

In liver plasma membrane lipids of geese, the increase in palmitic and oleic acid contents is associated with a reduction in proportion of PUFA. Thus the proportion of linoleic acid was markedly reduced by steatosis. Arachidonic acid (20:4n-6), formed from linoleic acid (18:2n-6) through successive elongation and desaturation steps, also exhibited a slight but significant decrease. A similar phenomenon was observed for the main n-3 PUFA (22:6n-3) formed from elongation and desaturation of the essential fatty acid linolenic acid (18:3n-3). Only the eicosatrienoic acid (20:3n-9), produced by oleic acid elongation and desaturation, increased significantly in membrane lipids of fatty livers. These modifications occurred in both major classes of PL but to a greater extent in PC than in PE. The changes in fatty acid composition of liver plasma membrane lipids in overfed geese are comparable to those observed in hepatocyte membrane lipids of rats fed essential fatty acid-deficient diets, resulting in a decrease of n-6 and n-3 PUFA contents and increased oleic acid and n-9 PUFA (31,32).

In the present experiment, geese were overfed only with corn. The high level of carbohydrate content of corn intake was responsible for increased liver lipogenesis and probably Δ9 desaturase activity. Corn contains 4% lipids, with linoleic acid representing more than 50% of total fatty acids. Considering the very high linoleate intake during overfeeding, compared to the control period intake (18.5 vs. 1.7 g/d), the very low incorporation of linoleic acid and its elongation/desaturation products in hepatic plasma membrane lipids is even more surprising. In geese overfed with corn, Blum *et al.* (33) also reported a low linoleic acid content of hepatic lipids, but a 10-fold increased level in adipose tissue. In consequence it can be concluded that, during overfeeding, dietary and *de novo* synthesized fatty acids come into competition for their incorporation in hepatocyte membrane lipids. The high level of hepatic lipogenesis induced by overfeeding results in enrichment of hepatic membranes with oleic acid to the exclusion of dietary linoleic acid. In the same way, 22:6n-3, the main fatty acid derived from linolenic acid, was also decreased in fatty liver. However overfeeding increased the linolenate intake twofold (0.37 vs. 0.17 g/d). Hence, the same mechanism seems to limit the incorporation of both essential fatty acids. In response to overfeeding, the dramatic triglyceride accumulation in hepatocytes and the continuous secretion of lipoproteins by exocytosis could limit dietary lipid capture in hepatic pools. This limited capture could involve triglyceride-rich particles, as it was assumed in chickens with normal feeding conditions (34), as well as nonesterified fatty acids. Possibly in concert with these processes, a high *de novo* oleic acid synthesis in hepatocytes and a preferential incorporation of dietary fatty acids, especially PUFA, in VLDL and HDL, could explain the altered fatty acid profile of fatty liver plasma membranes and increased PUFA content of extrahepatic tissues (10,28).

This study shows that corn overfeeding in geese induces a modification of hepatic plasma membrane constituents, especially in cholesterol/PL, and PL/protein ratios and in fatty acid composition of PL. In general, PUFA content is significantly decreased with an increase in oleic acid content. However, changes in the fatty acid profile of hepatic plasma membranes are closely related to *de novo* lipogenesis induced by high carbohydrate intake. Consequently, the modulation of fatty liver plasma membrane composition by dietary lipid intake is masked if not strongly limited.

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Metabolism of Trideuterated *iso*-Lignoceric Acid in Rats *in Vivo* and in Human Fibroblasts in Culture[1]

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ABSTRACT: Saturated very long chain fatty acids (fatty acids with greater than 22 carbon atoms; VLCFA) accumulate in peroxisomal disorders, but there is little information on their turnover in patients. To determine the suitability of using stable isotope-labeled VLCFA in patients with these disorders, the metabolism of 22-methyl[23,23,23-$^2H_3$]tricosanoic (*iso*-lignoceric) acid was studied in rats *in vivo* and in human skin fibroblasts in culture. The deuterated *iso*-VLCFA was degraded to the corresponding 16- and 18-carbon *iso*-fatty acids by rats *in vivo* and by normal human skin fibroblasts in culture, but there was little or no degradation in peroxisome-deficient (Zellweger's syndrome) fibroblasts, indicating that its oxidation was peroxisomal. Neither the 14-, 20-, and 22-carbon *iso*-fatty acids nor the corresponding odd-chain metabolites could be detected. In the rat, the organ containing most of the *iso*-lignoceric acid, and its breakdown products, was the liver, whereas negligible amounts were detected in the brain, suggesting that little of the fatty acid crossed the blood-brain barrier. Our data indicate that VLCFA labeled with deuterium at the ω-position of the carbon chain are suitable derivatives for the *in vivo* investigation of patients with defects in peroxisomal β-oxidation because they are metabolized by the same pathways as the corresponding *n*-VLCFA. Moreover, as *iso*-VLCFA and their β-oxidation products are readily separated from the corresponding *n*-fatty acids by normal chromatographic procedures, the turnover of VLCFA can be more precisely measured.

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Saturated fatty acids with greater than 22 carbon atoms (very long chain fatty acids; VLCFA) are components of many human tissues, and, in particular, are enriched in the myelin fraction of brain (1). The VLCFA in human tissues are believed to arise from dietary sources and by *de novo* synthesis from shorter-chain fatty acids such as palmitic and stearic acids. Synthesis may take place in either microsomes or mitochondria, and there is evidence that the enzymes involved in the synthesis of VLCFA are different from those catalyzing the synthesis of fatty acids with up to 20 carbon atoms (2). Degradation of VLCFA takes place exclusively in peroxisomes in rat liver (3,4) and in human skin fibroblasts (5,6). In human skin fibroblasts, acetyl CoA released from VLCFA by peroxisomal β-oxidation is metabolized further *via* the citric acid cycle or is used for various biosynthetic reactions, including the biosynthesis of fatty acids (7). Acetyl CoA generated from peroxisomal β-oxidation in rat liver is also used for anabolic processes including incorporation into the 1-alkenyl group of ethanolamine plasmalogens (8). The role of peroxisomal β-oxidation is generally assumed to be that of chain-shortening fatty acids to products which can then be further oxidized in mitochondria (9–11).

As part of our ongoing interest in inherited disorders affecting peroxisomal β-oxidation, we synthesized a deuterated VLCFA that we hoped to use for turnover studies in patients with X-linked adrenoleukodystrophy (ALD), a rare neurodegenerative disorder caused by mutations in an ABC transporter protein (12,13), but characterized biochemically by an accumulation of saturated VLCFA (14–17), as well as in patients with peroxisomal biogenesis defects (e.g., Zellweger's syndrome; 15,16). Because ethical approval was contingent on establishing the suitability of using the deuterated VLCFA for human studies, we undertook a preliminary study of the metabolism of the fatty acid in rats *in vivo* and in human skin fibroblasts in culture. For these studies we used 22-methyl-[23,23,23-$^2H_3$]tricosanoic (*iso*-lignoceric, *iso*-24:0) acid rather than a deuterated lignoceric acid derivative as a substrate because *iso*-lignoceric acid and its metabolites are readily separated from the corresponding straight-chain fatty acids by gas chromatography and hence are more readily quantified. Our data indicate that trideuterated *iso*-lignoceric acid is oxidized in peroxisomes in human tissues and is suitable for the *in vivo* investigation of patients with defects in peroxisomal β-oxidation. There was no evidence that *iso*-lignoceric was degraded by α-oxidation in either the rat or human tissues examined.

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Abbreviations: ALD, adrenoleukodystrophy; BME, basal Eagle's medium; *iso*-fatty acids, fatty acids with a methyl branch at the ω-2 position; *iso*-lignoceric acid, *iso*-24:0, 22-methyl tricosanoic acid; lignoceric acid, 24:0, tetracosanoic acid; *n*-fatty acids, straight chain fatty acids; VLCFA, fatty acids with greater than 22 carbon atoms.

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EXPERIMENTAL PROCEDURES

Materials. Analytical grade solvents were purchased from May and Baker Pty. Ltd., Melbourne, Australia, or from Ajax Chemicals, Sydney, Australia. Basal Eagle's medium (BME) was purchased from Flow Laboratories, Irvine, Scotland, and fetal calf serum was obtained from Gibco New Zealand Ltd. [24,24,24-$^2H_3$]Tetracosanoic acid (lignoceric acid, 99% deuterium) was purchased from Larodan Fine Chemicals (Malmo, Sweden). Undecylenic acid, 11-bromoundecanoic acid, and lithium aluminum deuteride (98 atom deuterium %) were purchased from Sigma-Aldrich, Castle Hill, Australia. All other chemicals used for the synthesis of deuterated fatty acids were also obtained from Sigma-Aldrich.

Synthesis of trideuterated iso-*lignoceric acid.* 22-Methyl-[23,23,23-$^2H_3$]tricosanoic acid (trideuterated *iso*-lignoceric acid) was synthesized from 10-undecenoic acid *via* the reaction sequence outlined earlier (18). Briefly, 1-chloro-2-ketododec-10-ene was synthesized from 10-undecenoic acid and converted to 2-methyl 10-undecenoic acid *via* a base-mediated rearrangement as outlined by Johnson and Poulos (19). The acid was converted into its methyl ester, reduced to its alcohol with lithium aluminum deuteride, then converted to its mesylate. [1,1,1-$^2H_3$]-2-Methylundec-10-ene was prepared by reduction of the mesylate with lithium aluminum deuteride, hydroboration, and bromination to form [1,1,1,-$^2H_3$]-2-methyl 11-bromo-undecane. 11-Bromo-undecanoic acid methyl ester was coupled to TOSMIC (*p*-toluenesulfonylmethylisocyanide), and the product was reacted with [1,1,1,-$^2H_3$]-2-methyl 11-bromo-undecane (20). The methyl ester of 22-methyl [23,23,23-$^2H_3$]tricosanoic acid was isolated after hydrolysis of the TOSMIC ester and subsequent reduction, and converted to the free acid by alkaline hydrolysis. Combined gas chromatography–mass spectrometry of the trideuterated *iso*-lignoceric acid confirmed an abundance of >95% deuterium at the 23-position of the carbon chain, and the purity of the product was assessed as >97%.

Tissue culture. Skin fibroblast cultures were established from skin biopsies of patients with Zellweger's syndrome, and from individuals without any biochemical or clinical evidence of an abnormality in peroxisomal β-oxidation. The diagnosis of Zellweger's syndrome was based on the patient's case history, and on clinical and biochemical investigations (15,16). Human skin fibroblasts were grown in 75-cm^2 culture flasks in 10 mL BME containing 10% fetal calf serum until confluent. The medium was then removed and replaced with 10 mL BME containing 0.5% fetal calf serum. After incubation for 24 h the medium was replaced with BME containing 0.5% fetal calf serum and 136 nmol trideuterated *iso*-lignoceric acid, or 68 nmol trideuterated lignoceric acid plus 68 nmol trideuterated *iso*-lignoceric acid, dissolved in ethanol, and the cells were maintained in culture for a further 3 d. At the end of this period, the medium was removed and 5 mL of water was added; and the cells were scraped from the flask, sonicated, and stored at –20°C until ready for lipid and protein analysis. Protein assays were carried out as described by Bradford (21).

Animal feeding experiments. Four young Sprague-Dawley rats (purchased from the Institute of Medical and Veterinary Science Field Station, Adelaide, Australia) (weighing 133–151 g) were gavaged with an aqueous suspension of trideuterated *iso*-24:0 (20 µmol) and egg yolk lecithin (10 µmol) per 100 g body weight (Experiment A). Single animals were sacrificed by exposure to carbon dioxide gas after 1, 4, 7, and 10 d. Brain, liver, kidney, and heart were removed, weighed, and stored at –20°C until ready for analysis. Two adult animals weighing 465 and 495 g were fed a similar suspension of trideuterated *iso*-24:0 and egg yolk phosphatidylcholine (Experiment B) and were sacrificed using carbon dioxide gas after 1 and 2 d, respectively. Organs were removed, weighed, and stored at –20°C until ready for analysis.

To test for the degree of gut absorption of the trideuterated *iso*-24:0, four young rats (average weight 150 g) were placed in metabolism cages (Tecniplast, Sieper and Company, Strathfield, Australia) for 2 d. The feces which were produced over two successive 24-h periods were collected and stored until ready for analysis.

Lipid extraction and analysis. Cell suspensions were extracted according to the Bligh and Dyer method (22) after the addition of nonadecanoic acid (19:0, 80 nmol) as internal standard. Lipids were extracted from 0.5 g of tissue or pulverized feces (23) after the addition of appropriate amounts (150–750 nmol) of octacosanoic acid (28:0) as internal standard. The lipid extracts were transesterified with 1.5% (vol/vol) sulfuric acid in methanol for 4 h at 100°C, and the fatty acid methyl esters were purified by thin-layer chromatography (7). The saturated fatty acid methyl esters were isolated from the total fatty acid methyl esters by argentation chromatography (7) and dissolved in a small volume of hexane. Aliquots were injected into a Hewlett-Packard 5890 gas chromatograph equipped with a 25 m × 0.22 mm (internal diameter) (0.25 µm phase thickness) BP-1 capillary column, and the temperature was programmed from 160 to 320°C at 4°C/min. Quantitation of the *iso*-fatty acid methyl esters (16:0, 18:0, 24:0, 26:0) was performed by reference to the internal standard. The fatty acid methyl esters were then subjected to gas chromatography–mass spectrometry analysis using a JEOL DX-303 mass spectrometer and a Hewlett-Packard 5890 gas chromatograph equipped with an identical BP-1 capillary column. The ratio of the trideuterated fatty acid to total trideuterated and unlabeled fatty acid for each *iso*-fatty acid was obtained from the peak height ratio of the molecular ions of the trideuterated and unlabeled methyl esters. Quantitation of the deuterated fatty acids was then determined by multiplying the mass spectrometric ratio for each *iso*-fatty acid by its total weight obtained by gas chromatography.

RESULTS

The exact uptake of trideuterated *iso*-lignoceric acid by skin fibroblasts in culture could not be established because of the difficulties in assessing the proportion of the fatty acid that was completely oxidized to CO_2. However, the data obtained

using the peroxisome-deficient (Zellweger's syndrome) cell lines, where there was negligible oxidation of the fatty acid, indicated that, at least in these lines, <5% of the total trideuterated *iso*-lignoceric acid recovered from the cells and medium was taken up into the cells during the incubation period. It is likely that similar amounts were taken up by control cells in this period because the combined amounts of the substrate and its various products in control cells were not greatly different from the values obtained in the mutant cell lines (Table 1).

Trideuterated *iso*-24:0 was relatively slowly metabolized by normal cells, and there was considerable variability in the turnover of the fatty acid. This variability may reflect the combined effects of the poor uptake of *iso*-24:0 as well as cell line differences. Similar variability was reported earlier when using lignoceric acid as a substrate (24). The substrate and the main products, i.e., the corresponding 16- and 18-carbon *iso*-fatty acids, eluted faster from the gas chromatographic column than the corresponding straight-chain fatty acids and were well separated from them (Fig. 1, Table 1). Despite very careful analysis of the extracts, 22- and 20-carbon homologs were not detected. Moreover, α-oxidation products, for example, the corresponding *iso*-23:0, -21:0, -19:0, and -17:0 fatty acids, could also not be detected. Analysis of the extracts of peroxisome-deficient cells incubated with trideuterated *iso*-24:0 showed the expected marked reduction in the levels of the *iso*-16:0 and *iso*-18:0 products, indicating a block in peroxisomal β-oxidation (6), which is consistent with the nearly complete or total absence of peroxisomes in this syndrome (25). Instead, peroxisome-deficient cells contained significant amounts of trideuterated *iso*-26:0, the elongation product of *iso*-24:0 (Table 1 and Fig. 1). The difference between Zellweger's cells and control cells in the metabolism of *iso*-lignoceric acid is highlighted when the amount of elongation product (*iso*-26:0) is expressed as a ratio of the amount of oxidized products (*iso*-18:0 and *iso*-16:0), with the Zellweger's cells having a ratio >6.5 and control cells a ratio <0.14. It is likely that the variability in the levels of trideuterated *iso*-24:0 in peroxisome-deficient cell lines is associated with differences in uptake of the fatty acid, which may reflect differences in the genotype and/or growth characteristics of the individual cell lines.

To study the influence of the n-2 methyl group on the metabolism of 24:0, normal fibroblasts were incubated with a mixture of equal amounts of trideuterated *n*-24:0 and *iso*-24:0. Trideuterated *n*- and *iso*-derivatives of 16:0 and 18:0, products of the β-oxidation of *n*-24:0 and *iso*-24:0, respectively, were detected in the cell extracts. While the trideuterated *n*-24:0/*iso*-24:0 fatty acid ratios for two normal cell lines were around 1 (0.96 and 0.91), the corresponding trideuterated *n*-16:0/*iso*-16:0 (3.37 and 3.0) and *n*-18:0/*iso*-18:0 (3.37 and 2.65) fatty acid ratios, formed by β-oxidation of the deuterated VLCFA, were much greater, indicating that the *n*-24:0 was preferentially metabolized. Although we were unable to estimate the *n*-16:0/*iso*-16:0 and *n*-18:0/*iso*-18:0 fatty acid ratios in a Zellweger cell line because of the very low conversions, the ratio of trideuterated *n*-26:0/*iso*-26:0, the major product in these cells, was close to 1.

On average, 58% (range, 45 to 75%) of the trideuterated *iso*-24:0 administered to rats was recovered in the feces in a 2-d period, and greater than 90% of the recovered fatty acid was detected within the first 24 h. Analysis of fecal lipids confirmed that the fatty acid had not been metabolized during passage through the gut. Analysis of liver, brain, kidney, and heart taken from rats fed trideuterated *iso*-lignoceric acid confirmed the presence of small amounts of endogenous (i.e., nondeuterated) *iso*-lignoceric acid, with liver containing the most and brain the least. Other *iso*-fatty acids (also nondeuterated), notably *iso*-16:0 and -18:0, were also detected in all tissues examined. Once again, the brain contained the lowest amounts of these fatty acids and liver the most.

Trideuterated *iso*-lignoceric acid was detected in liver, kidney, and heart up to 10 d after a single dose of the fatty acid was fed (by gavage) to young rats (Table 2, Experiment A). However, the fatty acid was detected in the brain only in the early time period and only in trace amounts (<1% of the labeled fatty acid recovered from the tissues). The data indicate a drop in the levels of trideuterated *iso*-lignoceric acid, at least in the liver, over the 10-d period. One day after the gavage, and assuming an even distribution of the fatty acid, around 8% of trideuterated *iso*-24:0 was recovered in liver, kidney, and heart, with about a further 4% of the deuterium label recovered in *iso*-16:0 and *iso*-18:0. A similar result was obtained by feeding the fatty acid to adult animals (Table 2, Experiment B), although the amount of *iso*-24:0 recovered in the younger animals was greater. It is possible that this difference relates to the age of the animals. As around 58% of the labeled fatty acid passed through the gut without being absorbed, approximately one-third was not accounted for, but

TABLE 1
Oxidation of Trideuterated *Iso*-Lignoceric Acid by Human Skin Fibroblasts[a]

	(nmoles trideuterated fatty acid/mg protein)			
Cell line	*Iso*-16:0	*Iso*-18:0	*Iso*-24:0	*Iso*-26:0
Controls				
1	2.18	2.43	17.66	0.66
2	2.42	3.13	12.24	0
3	1.91	4.39	24.66	0.18
4	1.52	3.27	26.94	0
Mean ± SD	2.01 ± 0.39	3.31 ± 0.81	20.39 ± 6.72	0.21 ± 0.31
Zellweger's				
1	0.04	0.53	34.81	3.71
2	0.43	0.32	39.42	4.94
3	0.20	0.25	26.13	3.63
4	0	0	14.73	1.33
Mean ± SD	0.17 ± 0.20	0.28 ± 0.22	28.78 ± 10.86	3.40 ± 1.51

[a]Human skin fibroblasts were incubated with trideuterated *iso*-24:0, and the fatty acid products were determined by gas chromatography–mass spectrometry as described in the text. The data shown here represent the mean of duplicate assays carried out on four separate control and Zellweger syndrome cell lines. The mean ± the standard deviation are also shown.

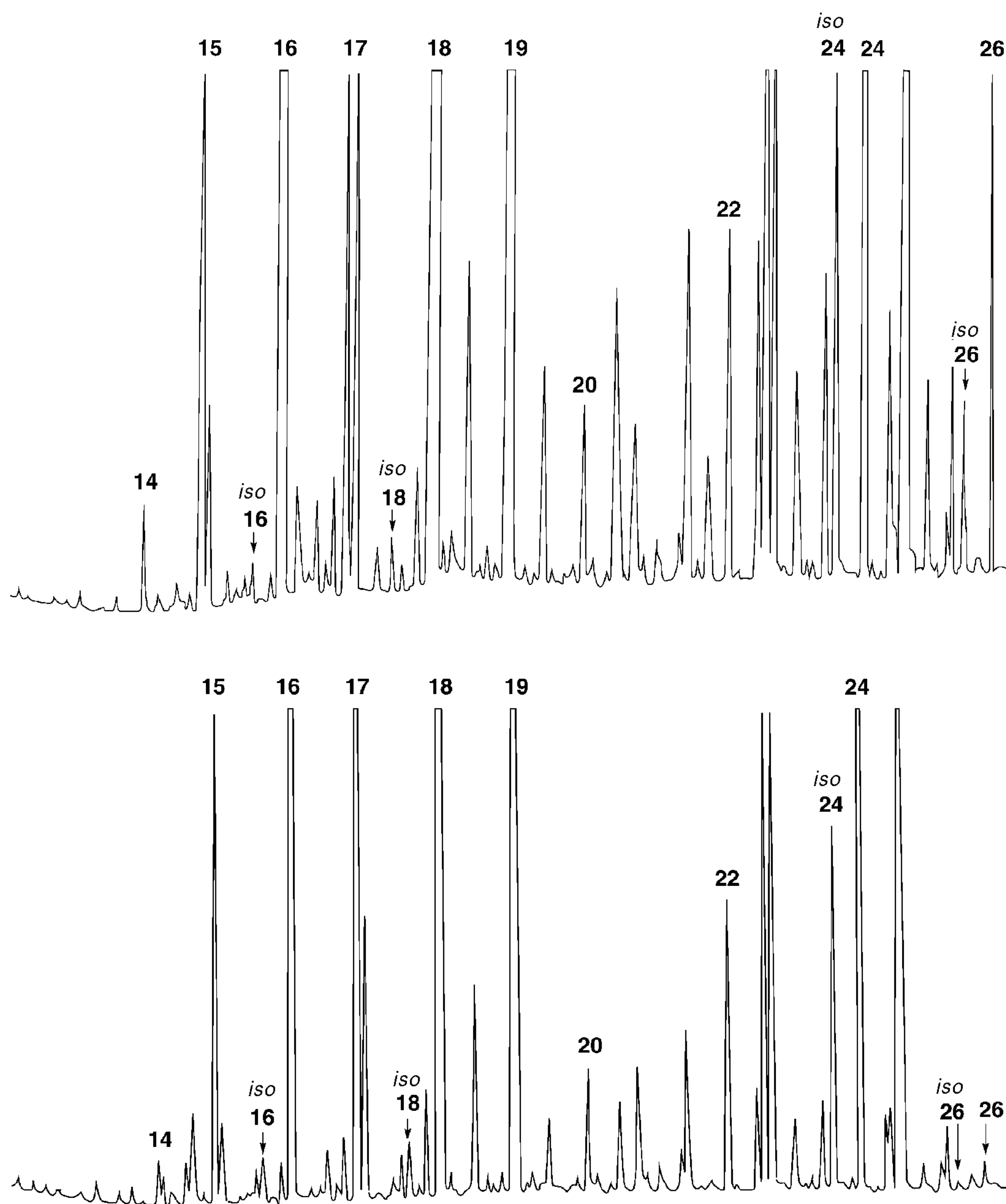

FIG. 1. Gas chromatography of *iso*-fatty acids in human skin fibroblasts was carried out as described in the text. The carbon chain length of the various fatty acids is shown. Top panel, Zellweger syndrome; bottom panel, control fibroblasts.

much of this may represent fatty acid taken up into other tissues and blood.

Metabolites of deuterated *iso*-24:0 were detected in all six animals investigated, indicating that the substrate was oxidized *in vivo*. In particular, oxidation products were detected in the liver and heart up to 10 d after feeding the deuterated fatty acid to the animals. These included 16- and 18-carbon fatty acids, presumably formed by β-oxidation of *iso*-24:0. As observed with skin fibroblasts, *iso*-22:0 and *iso*-20:0, presumed intermediates in *iso*-16:0 and *iso*-18:0 formation, were either not detected or observed only in small amounts (*iso*-22:0 was detected only at day 1 in Experiment A in much smaller amounts than the other metabolites, Table 2).

DISCUSSION

The oxidation of *iso*-lignoceric acid was greatly diminished in peroxisome-deficient human cells, indicating that, as with lignoceric acid, this *iso*-fatty acid is oxidized exclusively in peroxisomes. The metabolic products formed from *iso*-24:0 in the rat were similar to those produced in control human fibroblasts, implying that its oxidation was also peroxisomal in rat tissues. Our studies indicate that the fatty acid is readily

TABLE 2
Oxidation of Trideuterated *Iso*-Lignoceric Acid by Rats *in Vivo*[a]

	(μmoles trideuterated fatty acid/g tissue wet weight)		
	Iso-16:0	*Iso*-18:0	*Iso*-24:0
Experiment A			
Liver			
Day 1[b]	0.038	0.098	0.316
Day 4	0.006	0.034	0.092
Day 7	0.007	0.008	0.019
Day 10	ND	0.008	0.007
Heart			
Day 1	0.007	0.023	0.026
Day 4	0.005	0.018	0.016
Day 7	0.004	0.037	0.012
Day 10	ND	0.025	0.012
Kidney			
Day 1	0.003	0.007	0.008
Day 4	ND	0.010	0.008
Day 7	ND	ND	0.004
Day 10	ND	ND	0.004
Brain			
Day 1	ND	ND	0.003
Day 4	ND	ND	ND
Day 7	ND	ND	ND
Day 10	ND	ND	ND
Experiment B			
Liver			
Day 1	0.018	0.058	0.121
Day 2	0.018	0.071	0.160
Heart			
Day 1	0.002	0.018	0.010
Day 2	0.007	0.030	0.020
Kidney			
Day 1	0.004	0.006	0.004
Day 2	0.003	0.009	0.005
Brain			
Day 1	ND	ND	ND
Day 2	ND	ND	ND

[a]Animals were fed trideuterated *iso*-lignoceric acid and sacrificed at the times shown. Analysis of *iso*-fatty acids was performed as outlined in the Experimental Procedures section. Animals used for Experiment A were younger than those used for Experiment B (see Experimental Procedures section). ND, <0.001 μmol/g.
[b]Traces of *iso*-22:0 (0.021 μmol/g) were also detected in liver on day 1.

taken up from the gut in rats with, of the tissues examined, most ending up in the liver. Whereas liver, kidney, and heart had easily demonstrable quantities of *iso*-24:0 (and its metabolites), the fatty acid was not detected in the brains of the animals, except in trace amounts, indicating that little crossed the blood-brain barrier over the experimental period. Although the effect of repeated doses over a longer period is not known, under our conditions, there is little evidence that exogenous VLCFA contribute to the pool of endogenous fatty acids in the brain. Earlier studies indicated that exogenous saturated VLCFA could cross into the brain, but these were based on experiments carried out on a single terminally ill patient with X-linked ALD (26). It is entirely possible therefore that this does not occur in normal individuals or in all patients. Support for this conclusion is provided by more recent studies showing that erucic acid (22:1), which has a carbon chain length approaching that of *iso*-lignoceric acid, does not enter the brain of ALD or adrenomyeloneuropathy patients even after long-term dietary supplementation (27,28).

A number of other tentative conclusions can be drawn from our studies. First, the almost complete absence of trideuterated *iso*-22 and *iso*-20 carbon intermediates in normal human cells, and in rat tissues, indicates that 18- and 16-carbon fatty acids are major products of peroxisomal β-oxidation of saturated VLCFA. If production of 20- and 22-carbon fatty acids *via* the peroxisomal β-oxidation pathway is limited, and given that VLCFA oxidation does not take place in mitochondria, synthesis of these fatty acids must occur *via* the elongation of shorter-chain precursors. Since the acetate released from the peroxisomal β-oxidation of VLCFA is used for chain elongation of fatty acids (7), the puzzling reduction in 22:0 in patients with abnormalities in peroxisomal biogenesis (15) could be explained by a reduced elongation of shorter-chain fatty acids to form 22:0. Second, as we were unable to detect odd-chain breakdown products of lignoceric acid, our data demonstrate that VLCFA are poor substrates for the α-oxidation system in cultured skin fibroblasts. Earlier studies by Tsuji *et al.* (29) had indicated that odd-chain fatty acids (17-, 19-, 21-, 23-, and 25-carbon saturated fatty acids) were formed from [^{3}H]24:0 and -26:0, and it was suggested that they were formed by α-oxidation. These earlier studies by Tsuji *et al.* (29) utilized 24:0 and 26:0 fatty acids, labeled with tritium in positions 15,16 and 17,18 of the carbon chain, respectively. One possible explanation for the differences between our data and theirs is that the tritium attached to the 15,16- and 17,18-carbons is more readily exchangeable than the deuterium bound to the ω-carbon atom. Another possibility is that tritium released by β-oxidation of the fatty acid was reutilized for the synthesis of odd carbon-number chain fatty acids. There is also the possibility that at least some of the odd carbon-number chain metabolites reported by Tsuji *et al.* (29) had been derived from the appreciable amounts of odd carbon-number chain VLCFA and other radiolabeled contaminants present in the substrates used (up to 7.5% of the total label). Although confirmation of the structure of the various metabolites formed in our investigations was based on rigorous gas chromatography–mass spectrometry procedures, in the earlier studies of Tsuji *et al.* (29) identification of odd carbon-number chain metabolites was based largely on gas chromatographic retention times, and there may have also been greater errors in structural assignment. Our observed lack of α-oxidation of *iso*-24:0 by human skin fibroblasts in culture is supported by other studies showing that formic acid, a major product of α-oxidation of fatty acids, is not formed from lignoceric acid (30; Poulos, A., and Sharp, P., unpublished data). Moreover, there is no indication that odd carbon-number chain fatty acids accumulate in patients with defects in peroxisomal β-oxidation (24). Finally, our data obtained using equimolar mixtures of lignoceric and *iso*-lignoceric acids indicate that the insertion of a methyl group, at a position in the carbon chain far removed from the carboxyl group, can have a marked effect on the oxidation of a fatty acid. It is not surprising that the β-oxidation activity

toward *iso*-24:0 is less than the activity toward lignoceric acid as a number of peroxisomal enzyme proteins are involved in this process, and the affinity of many of these enzymes for different substrates may vary. Similarly, fatty acid elongation is also a complex process requiring a number of enzyme proteins. Our earlier studies demonstrated elongation of a straight-chain fatty acid, but not a branched-chain fatty acid with a γ-methyl group (31), suggesting that there is some substrate specificity for the elongation system. However, our present data indicate that the elongation system does not distinguish between straight-chain VLCFA and VLCFA with a methyl branch at the ω-end of the molecule.

Our *in vivo* studies with rats, and the earlier studies of Kishimoto *et al.* (26) carried out on a single ALD patient, demonstrate that unesterified deuterated straight-chain VLCFA, and deuterated *iso*-VLCFA, are absorbed from the gut. However, for *in vivo* investigations, either with patients or animals, deuterated *iso*-VLCFA have an advantage as they separate from the corresponding straight-chain VLCFA on gas chromatography (Fig. 1). As straight-chain VLCFA are significant components of plasma and patient tissues, precise determination of the level of deuterium is difficult, particularly if the levels of the deuterated VLCFA are small in comparison with the corresponding endogenous VLCFA. In addition, the shorter-chain deuterated *iso*-fatty acids which are formed from deuterated *iso*-VLCFA by peroxisomal β-oxidation, such as for example the *iso*-18:0 and *iso*-16:0 formed from *iso*-24:0, are completely separated from the corresponding *n*-fatty acids (Fig. 1). As the latter are major fatty acid components of normal tissues and body fluids (see Fig. 1), the measurement of small levels of deuterium enrichment in these fatty acids is technically more difficult than the corresponding measurements in shorter chain *iso*-fatty acids derived from *iso*-VLCFA. We conclude therefore that *iso*-24:0 is a potentially useful substrate for studying turnover of VLCFA in human subjects whose peroxisomal β-oxidation pathway is compromised. In particular, following the turnover of deuterated *iso*-lignoceric in plasma would be a useful tool for monitoring the immediate effectiveness of various new drug therapies (e.g., phenylbutyrate (32) and lovastatin (33) treatments for ALD patients) in stimulating VLCFA oxidation *in vivo.*

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Eicosapentaenoic and Docosahexaenoic Acid Affect Mitochondrial and Peroxisomal Fatty Acid Oxidation in Relation to Substrate Preference

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ABSTRACT: Decreased triacylglycerol synthesis within hepatocytes due to decreased diacylglycerol acyltransferase (DGAT) activity has been suggested to be an important mechanism by which diets rich in fish oil lower plasma triacylglycerol levels. New findings suggest that eicosapentaenoic acid (EPA), and not docosahexaenoic acid (DHA), lowers plasma triacylglycerol by increased mitochondrial fatty acid oxidation and decreased availability of fatty acids for triacylglycerol synthesis. To contribute to the understanding of the triacylglycerol-lowering mechanism of fish oil, the different metabolic properties of EPA and DHA were studied in rat liver parenchymal cells and isolated rat liver organelles. EPA-CoA was a poorer substrate than DHA-CoA for DGAT in isolated rat liver microsomes, and in the presence of EPA, a markedly lower value for the triacyl[^{3}H]glycerol/diacyl[^{3}H]glycerol ratio was observed. The distribution of [1-^{14}C]palmitic acid was shifted from incorporation into secreted glycerolipids toward oxidation in the presence of EPA (but not DHA) in rat liver parenchymal cells. [1-^{14}C]EPA was oxidized to a much greater extent than [1-^{14}C]DHA in rat liver parenchymal cells, isolated peroxisomes, and especially in purified mitochondria. As the oxidation of EPA was more effective and sensitive to the CPT-I inhibitor, etomoxir, when measured in a combination of both mitochondria and peroxisomes, we hypothesized that both are involved in EPA oxidation, whereas DHA mainly is oxidized in peroxisomes. In rats, EPA treatment lowered plasma triacylglycerol and increased hepatic mitochondrial fatty acid oxidation and carnitine palmitoyltransferase (CPT)-I activity in both the presence and absence of malonyl-CoA. Whereas only EPA treatment increased the mRNA levels of CPT-I, DHA treatment increased the mRNA levels of peroxisomal fatty acyl-CoA oxidase and fatty acid binding protein more effectively than EPA treatment. In conclusion, EPA and DHA affect cellular organelles in relation to their substrate preference. The present study strongly supports the hypothesis that EPA, and not DHA, lowers plasma triacylglycerol by increased mitochondrial fatty acid oxidation.

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Abbreviations: BSA, bovine serum albumin; CMC, carboxymethyl cellulose; CPT, carnitine palmitoyltransferase; DGAT, diacylglycerol acyltransferase; DHA, docosahexaenoic acid; DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FABP, fatty acid binding protein; FAO, fatty acyl-CoA oxidase; PPAR, peroxisome proliferator activated receptor.

The hypolipidemic effect of fish oils enriched in eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has been extensively studied in both humans and different animal models (1,2). Reduced triacylglycerol synthesis due to reduced diacylglycerol acyltransferase (DGAT) activity, diminished production, and/or increased removal of lipoprotein particles has been proposed as contributing to the hypolipidemic effect (3–9). Moreover, results from both *in vivo* and *in vitro* experiments indicate that the instant hypotriacylglycerolidemic effect observed after fish oil or EPA administration could, at least in part, be attributed to sudden increased mitochondrial fatty acid oxidation (10–15). The availability of triacylglycerol is a major driving force in the secretion of very low density lipoproteins by the liver (16,17). Factors influencing the balance between triacylglycerol biosynthesis and/or fatty acid oxidation may therefore ultimately influence plasma lipoprotein levels and metabolism (18). The finding that triacylglycerol synthesis, and not cholesterol ester synthesis, correlated with apolipoprotein B-100 secretion in HepG2 cells further supports this hypothesis (19).

The two components of fish oil attracting most attention, namely EPA and DHA, are still often referred to as n-3 fatty acids without any further distinction. Nearly all studies that have been conducted have used a mixture of EPA and DHA. However, several studies have reported that EPA and DHA possess different metabolic properties in rats and humans (20–26).

We earlier demonstrated that EPA treatment, and not DHA, lowers plasma triacylglycerol levels in rats (14,15,24). This has also been demonstrated in humans (23,27). In contrast, Hirai *et al.* (28) showed a significant reduction of postprandial serum triacylglycerol in hyperlipidemic patients and Grimsgaard *et al.* (29) showed a significant drop in plasma triacylglycerol after both EPA and DHA treatment in healthy Norwegians. The cause of these differences is not clear. However, retroconversion of DHA to EPA has been reported to occur in cultured rat hepatocytes (30), rats (25), and man (31). This implies that it will be difficult to study the unique effects of DHA, as some of it will always be converted to EPA. Moreover, EPA is more rapidly released from different tissues than DHA (32). The mol% of DHA is more than 10-fold

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higher than EPA in rat liver (25). Standard chow for rats does not contain EPA or DHA (25), and the daily intake of these fatty acids is therefore easier to control in rats than humans. Also, in countries with low intake levels of n-3 fatty acids, the level of EPA in plasma phospholipids is often one-fifth of the normal concentration of EPA (22). Subsequently, basal levels of EPA and DHA and the dose and length of treatment may influence the results.

Short-time regulation of fatty acid oxidation after EPA and 3-thia fatty acid treatment is well documented (14,33,34). Therefore, to contribute to the understanding of the triacylglycerol-lowering mechanism of fish oil, the different metabolic and hypolipidemic properties of EPA and DHA were studied in rat liver parenchymal cells. By using [1-^{14}C]-labeled fatty acids dissolved in an accurately defined culture medium, the flux of fatty acids and more precise metabolic properties of the fatty acids can be observed. Moreover, as it has previously been demonstrated that EPA and its CoA ester are poor substrates for triacylglycerol synthesis and DGAT compared to oleic acid (4,5), decreased triacylglycerol synthesis within the hepatocytes has been suggested to be an important mechanism by which diets rich in fish oil lower plasma triacylglycerol. Therefore, the effect of both EPA-CoA and DHA-CoA on DGAT activity was studied.

MATERIALS AND METHODS

Chemicals and drugs. [α-^{32}P]dCTP, L-[methyl-^{14}C]carnitine hydrochloride, [1-^{14}C]palmitoyl-CoA, and L-3-phosphatidylcholine,1,2-di[1-^{14}C]oleoylglycerol (114 mCi/mmol) were purchased from the Radiochemical Centre (Amersham, England). [1-^{14}C]Palmitic acid (50 mCi/mmol), [1-^{14}C]oleic acid (50 mCi/mmol), [1-^{14}C]EPA (50 mCi/mmol), [1-^{14}C]DHA (50 mCi/mmol), [1-^{14}C]oleoyl-CoA (59 mCi/mmol), and [4,5-^{3}H]DHA were obtained from New England Nuclear (Boston, MA). [1.2.3-^{3}H]glycerol (5.0 mCi/mmol) was purchased from New England Nuclear (Buckinghamshire, United Kingdom). [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA was from American Radiolabeled Chemicals Inc. (St. Louis, MO). Nylon membranes (NY ^{13}N) were obtained from Schleicher & Schuell (Dassel, Germany). The sulfur-substituted fatty acids were prepared at the Department of Chemistry, University of Bergen, as previously described (35). EPA and DHA as ethyl esters (97 and 91.2% pure, respectively) were obtained from Norsk Hydro AS Research Centre (Porsgrunn, Norway). EPA acyl-CoA and DHA acyl-CoA were synthesized as described by Kawaguchi *et al*. (36). Etomoxir was from Research Biochemicals International (Natick, MA) and dissolved in water before used. All other chemicals and solvents were of reagent grade from common commercial sources.

In vivo *experiments. (i) Treatment of animals.* Male Wistar rats from Möllegaard Breeding Laboratory (Ejby, Denmark), weighing about 250 g, were housed in pairs in metal-wire cages and maintained on a 12-h cycle of light and dark at 20 ± 3°C. The animals were acclimatized under these conditions for at least 1 wk before the experiments. The fatty acids were suspended in 0.5% sodium carboxymethyl cellulose (CMC) and administered by orogastric intubation once a day for 3–7 d, at a dose of 1500 mg/d/kg body weight. The control animals received CMC only. Control and fatty acid-treated animals had free access to water and standard rat pellet food during the experiment. Each test and control group consisted of four animals. After 12 h of fasting, the rats were anesthetized with 0.2 mL of Hypnorm-Dormicum (fluanisone-fentylmidazolam)/100 g body weight. Cardiac puncture was performed and blood was collected in vacutainers. The livers were removed, samples immediately freeze-clamped, weighed, and stored at −80°C. Other pieces of the liver were chilled on ice and weighed. The Norwegian State Board of Biological Experiments with Living Animals approved the use of the animals.

(ii) Preparation of subcellular fractions and protein measurements. The livers from individual rats were homogenized in ice-cold sucrose medium [0.25 M sucrose, 10 mM HEPES (pH 7.4), and 2 mM EDTA]. Subcellular fractions were prepared according to DeDuve *et al*. (37) using preparative differential centrifugation. Modifications, purity, and yield were as previously described (38). The mitochondrial preparations were further purified by Percoll-gradient centrifugation to minimize contamination (39). A Bio-Rad protein kit (Bio-Rad, Richmond, CA) was used for protein measurement. Bovine serum albumin (BSA) dissolved in distilled water was used as a standard.

(iii) Preparation of plasma and measurement of plasma triacylglycerol. Plasma was prepared from whole blood by centrifugation at 10 × *g* for 10 min. Analysis of triacylglycerol was carried out with a Biopak triacylglycerol enzymatic kit (Bristol, France).

(iv) Measurement of enzyme activities. Acid-soluble products were measured in the mitochondrial-enriched fractions using palmitoyl-CoA as substrate (24). Briefly, the assay mix (0.3 mL) contained 12 mM HEPES buffer (pH 7.3), 11 mM $MgCl_2$, 12 mM dithiothreitol, 5.6 mM ADP, 0.2 mM NAD^+, 0.6 mM EDTA, 125 mM KCl, and 1.0 mg mitochondrial protein. Palmitoyl-L-carnitine oxidation was measured with 80 μM [1-^{14}C]palmitoyl-L-carnitine, and the palmitoyl-CoA oxidation was measured with 80 μM [1-^{14}C]palmitoyl-CoA supplemented with 1 mM L-carnitine. When included, malonyl-CoA was added prior to the start of the reaction. After a 2-min incubation at 30°C, the reaction was terminated with 150 μL 1.5 M KOH. Then, 2.5 mg BSA and 500 μL 4 M $HClO_4$ were added. The test tubes were centrifuged at 1880 × *g* for 10 min and 500 μL of the supernatant was assayed for radioactivity. Acid-soluble products also were measured using fatty acids as substrates (26). The assay medium (0.25 mL) contained 13.3 mM HEPES, pH 7.3, 83.3 mM KCl, 16.7 mM $MgCl_2$, 13.3 mM dithiothreitol, 0.7 mM EDTA, 1.25 mM L-carnitine, 0.2 mM NAD^+, 2.0 mM ATP, 0.4 mM CoASH, 150 μM [1-^{14}C]fatty acid, and 0.25–0.5 mg protein. All samples were preincubated for 3 min at 30°C. After incubation for 4 min (mitochondrial fraction) or 10 min (peroxisomal frac-

tion), the reaction was terminated by addition of 150 µL 1.5 M KOH. Fatty acid-free BSA (25 µL; 100 mg/mL) was added to the suspension in order to bind nonoxidized substrates. $HClO_4$ (4M; 500 µL) was added to precipitate protein and nonoxidized substrates bound to BSA. The total solution was centrifuged at 2010 × *g* for 10 min. Aliquots of 500 µL were assayed for radioactivity.

Carnitine palmitoyltransferase (CPT)-I and -II activities were measured as described by Bremer with minor modification (40). The assay for CPT-I contained 20 mM HEPES, pH, 7.5, 70 mM KCl, 5 mM KCN, 100 µM palmitoyl-CoA, 10 mg BSA/mL, and 0.6 mg mitochondrial protein/mL. The reaction was started with 200 µM [methyl-^{14}C]-L-carnitine (200 cpm/nmol). When included, malonyl-CoA was added prior to the start of the reaction. Assay conditions for CPT-II were identical except that BSA was omitted and 0.01% Triton X-100 was included. Mitochondrial protein concentration was 0.1 mg/mL.

Acyl-CoA synthase activity was measured according to Bar-Tana *et al.* (41) with some modifications. The reaction mixture at a total volume of 250 µL contained 120 mM Tris-HCl buffer, pH 7.4, 0.05% Triton X-100, 2 mM EDTA, 5 mM $MgCl_2$, 2 mM ATP, 0.5 mM CoA, 8 mM dithiothreitol, and 10 to 20 µg protein. The reaction was started with 25 µL, 1.5 mM [1-^{14}C]fatty acid. Incubations were carried out for 3 min at 37°C and terminated with 3.25 mL methanol/chloroform/heptane (141:125:100, by vol). The contents were mixed for 5 min and centrifuged, and 0.5 mL of the water phase was assayed for radioactivity.

Fatty acyl-CoA oxidase activity was measured in the peroxisomal fraction by the coupled assay described by Small *et al.* (42). The production of H_2O_2 was measured by monitoring the increase in dichlorofluorescein absorbance in the presence of palmitoyl-CoA.

DGAT was performed as described by Coleman and Bell (43) and by Rustan *et al.* (5). The assay was performed at 23°C in a shaking water bath in a total volume of 0.5 mL of Tris-buffer (175 mM, pH 7.8) containing BSA (15 µM, fatty acid-free), $MgCl_2$ (8 mM), and oleoyl-CoA in the presence or absence of different acyl-CoA derivatives. After a 10-min preincubation, the reaction was started by adding rat liver microsomes (10–20 µg protein/mL) followed immediately by 1,2-di[1-^{14}C]oleoylglycerol (125 µM, dissolved in absolute ethanol, 10% vol/vol final concentration), and blended in a vortex mixer. The reaction was terminated after 10 min by the addition of 10 mL chloroform/methanol (2:1, vol/vol) and the lipids were extracted and separated by thin-layer chromatography as explained above. The triacylglycerol band was cut out, and the amount of ^{14}C was determined by scintillation counting. 1,2-di[1-^{14}C]oleoylglycerol was prepared from L-3-phosphatidylcholine,1,2-Di[1-^{14}C]oleoyl by treatment with purified phospholipase C as described elsewhere (44). More than 98% of the labeled phosphatidylcholine,1,2-dioleoyl, was converted to 1,2-dioleoylglycerol (0.92–1.1 µCi/mL) by this method. The dioleoylglycerol was extracted twice with diethyl ether, redissolved in absolute ethanol, and stored at ~20°C.

(vi) Purification of RNA and hybridization analysis. Total RNA was isolated using the guanidinium thiocyanate–phenol method (45) and the RNA concentrations were determined by measuring absorbance at 260 nm. Northern- and slot-blotting were performed as previously described (46). Three different RNA concentrations were applied. Hybridization reactions were performed as described by Sambrook *et al.* (47), and the membranes were stringently washed three times. Kodak XAR-5 X-ray films (Rochester, NY) were exposed to the membranes and autoradiograms were obtained as described elsewhere (46). The relative levels of mRNA expression were estimated as the amount of radioactive probe hybridized to each sample of RNA relative to the levels of 28S rRNA in each sample.

(vii) Preparation of hybridization probes. The appropriate DNA fragments were cut from plasmids by restriction enzymes. Purified fragments were then [^{32}P]-labeled using the oligolabeling technique (48), resulting in specific activities ranging from 0.8–5 × 10^9 cpm/mg. The probes were purified fragments of CPT-I, 2600 bp *Eco*RI fragment of pBK2-CPT-I (49); partial CPT-II, 1600 bp *Xho* I/*Xba*I fragment of pBKS-CPT-II.4 (50); fatty acid-binding protein (FABP), 334 bp *Pvu*II-*Eco*RI fragment in pJG418 (51); and fatty acyl-CoA oxidase (FAO), 1400 bp Pst fragment of pMJ125 (52). As the control, we used 28S rRNA; 1.4 kb *Bam* HI insert in pA (53).

In vitro *experiments. (i) Preparation of cultured hepatocytes.* Rat liver parenchymal cells were isolated as described by Berry and Friend (54) with modifications according to Seglen (55). The hepatocytes were plated at a density of 2.0 × 10^6/dish (1–1.5 mg cell protein) in 2 mL of Dulbecco's modified Eagle's medium (DMEM) containing 20 mM HEPES and 2% Ultroser G. When indicated, 500 µM L-carnitine or 50 µM L-aminocarnitine was added. Cultures were maintained in a humidified incubator at 37°C in an atmosphere containing 5% CO_2. After overnight incubation, the medium was replaced with DMEM (2 mL/dish) containing [1-^{14}C]-labeled fatty acids (0.25 µCi/mL) bound to BSA. Additions of other fatty acids or [1.2.3-^{3}H]glycerol (5 µCi/mL) to cell cultures are stated in the legends to figures and tables. The molar ratio between fatty acids and BSA was 2.5:1. The cells were further incubated for 4 h.

(ii) Termination of incubation and harvesting of cells. Incubations were terminated by cooling the culture dishes on ice. Incubation medium was collected, centrifuged at 600 × *g* for 5 min, and the cell-free supernatant was treated as described below. The cells were scraped off the dish by a rubber policeman into 2 mL of phosphate-buffered saline (NaCl/Pi), pH 7.4, containing 137 mM NaCl, 2.7 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4. The culture dishes were washed once with 2 mL of NaCl/Pi. The cell suspensions were centrifuged and the cells were resuspended in 0.5 or 1.0 mL distilled water and frozen. A Bio-Rad protein kit (Bio-Rad, Richmond, CA) was used for protein measurement in the cell suspensions. BSA dissolved in distilled water was used as a standard.

(iii) Determination of acid-soluble products and CO_2. The cell-free supernatant (250 µM) was precipitated with 1.0 mL

of ice-cold 0.75 M $HClO_4$. To secure complete precipitation of free fatty acids and lipids, 0.45% BSA was added. The extract was centrifuged at 1800 × *g* for 10 min, and 0.5 mL of the supernatant was assayed for radioactivity by liquid scintillation counting. $^{14}CO_2$ was trapped from sealed culture flasks essentially as described by Christiansen and Davies (56).

(iv) Lipid extraction and quantitation. Cellular lipids were extracted from the cell suspensions and according to Folch *et al.* (57). The cell suspension was mixed with 20 vol of chloroform/methanol (2:1, vol/vol), 4 vol of 0.9% NaCl (pH 2) were added, and the mixture was allowed to separate into two phases. The organic phase was evaporated under N_2 and the extracted lipids were dissolved in *n*-hexane and separated by thin-layer chromatography on silica gel plates developed in hexane/diethyl ether/acetic acid (80:20:1, by vol). The bands were visualized by iodine vapor, cut into pieces, and assayed for radioactivity by scintillation counting. Four volumes of chloroform/methanol (2:1) and 2% serum as lipid carrier were added to 1 mL of the cell-free medium. The water phase of the medium was reextracted once with 4 vol of chloroform/methanol (2:1, vol/vol), and the combined organic phases were further treated as the cells.

Statistical analysis and presentation of data. The data are presented as mean ± SD from four animals or at least three independent cell experiments. The results were evaluated by a two-sample variance Student's *t*-test (two-tailed distribution) where relevant. The level of statistical significance was set at $P < 0.05$.

RESULTS

Metabolism of [1-^{14}C]EPA and [1-^{14}C]DHA in cultured hepatocytes. To investigate the mechanism by which fish oils exert their hypolipidemic effect, the metabolism and flux of [1-^{14}C]-labeled EPA, DHA, oleic acid, and palmitic acid were studied in cultured hepatocytes. [1-^{14}C]-Labeled EPA, DHA, oleic acid, and palmitic acid were taken up by the hepatocytes to the same extent. The total metabolism (sum of incorporation into glycerolipids, cellular free fatty acids, and oxidation) of these fatty acids was not significantly different at equal concentrations (data not shown). Figure 1 shows that EPA and DHA were incorporated into cell-associated and secreted glycerolipids at a rate similar to that of palmitic and oleic acids up to 100 μM. At higher concentrations, i.e., 500 μM, DHA was incorporated more efficiently than EPA ($P < 0.005$) into cell-associated glycerolipids (Fig. 1A). On the other hand, at this concentration, EPA was more enriched in secreted glycerolipids than DHA ($P < 0.005$) (Fig. 1B).

Figure 2 summarizes the incorporation of different fatty acids (100 μM) into triacylglycerol and phospholipids (both secreted and cell-associated). Palmitic and oleic acids were equally distributed between triacylglycerol and phospholipids (Fig. 2). EPA, however, was incorporated more efficiently than DHA into triacylglycerol ($P < 0.05$). In contrast, DHA was preferentially incorporated into cell-associated phospholipids (Fig. 2). However, the most striking difference between the metabolism of EPA and DHA was that less than 1% of DHA was oxidized, independent of the concentration used, while EPA was easily oxidized (Fig. 1C and Fig. 2).

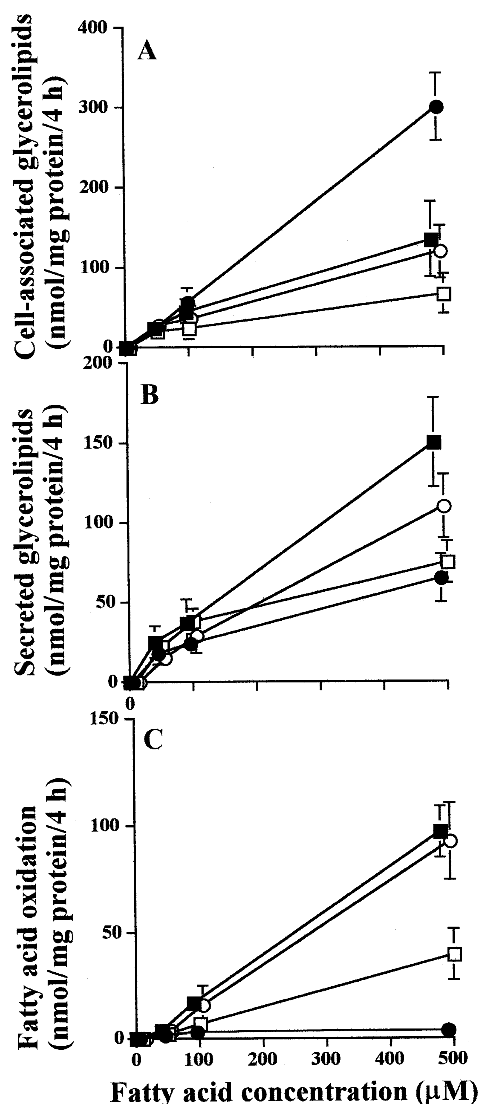

FIG. 1. Metabolism of 50, 100, and 500 μM [1-^{14}C]eicosapentaenoic acid (EPA) (■), [1-^{14}C]docosahexaenoic acid (DHA) (●), [1-^{14}C]oleic (□) and [1-^{14}C]palmitic acids (○) in hepatocytes plated overnight in Dulbecco's modified Eagle's medium (DMEM) and incubated for 4 h as described in the Materials and Methods section. The results were calculated as (A) the sum of [1-^{14}C]glycerolipids within the cells (synthesized), (B) the sum of [1-^{14}C]glycerolipids in the medium (secreted), and (C) the sum of acid-soluble ^{14}C in the medium. Experiments were run in triplicate, and the results are given as mean ± SD from four different rats.

EPA and DHA as substrates for mitochondrial and peroxisomal fatty acid oxidation. To further investigate the different oxidation of EPA and DHA, mitochondria and peroxisomes were isolated from rat liver. [1-^{14}C]EPA produced 10 times more acid-soluble products than [1-^{14}C]DHA in purified mitochondria (Table 1). The difference was, however, less pronounced when [^{3}H]-labeled fatty acids were used as substrates. As the labeled carbon atom is placed in the carboxyl

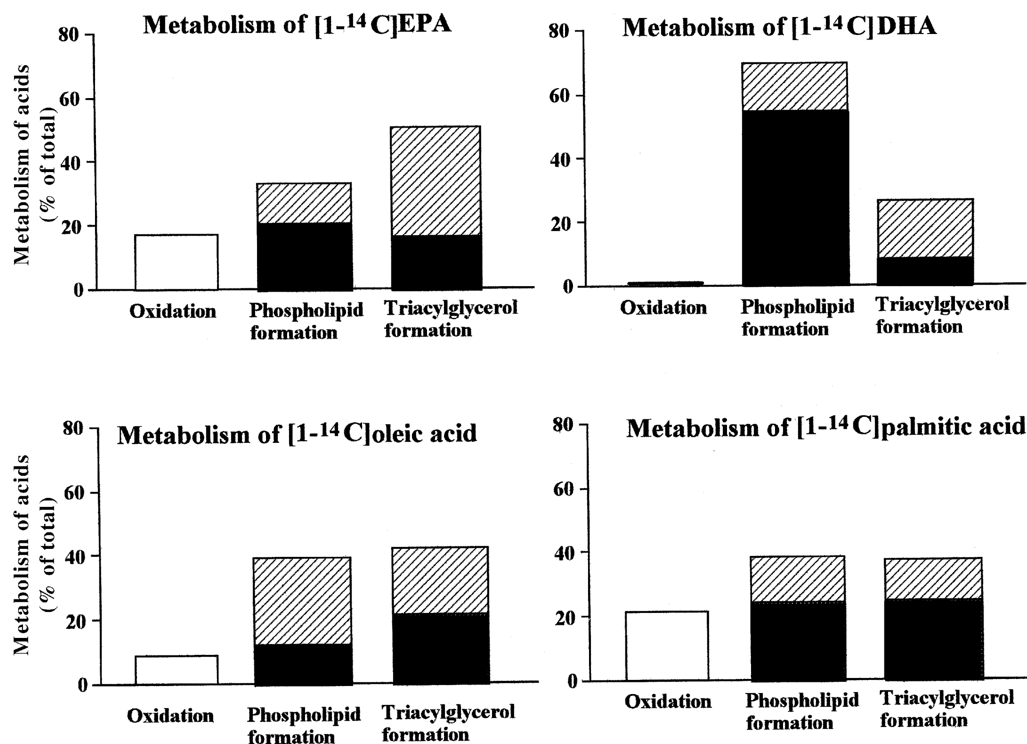

FIG. 2. Metabolism of 100 μM [1-^{14}C]EPA, [1-^{14}C]DHA, [1-^{14}C]oleic acid, and [1-^{14}C]palmitic acid in hepatocytes plated overnight in DMEM and incubated for 4 h as described in the Materials and Methods section. The results were calculated as acid-soluble ^{14}C in the medium (open bar) and [1-^{14}C]fatty acid-labeled cell-associated (closed bar) and secreted (ruled bar) glycerolipids (triacylglycerols and phospholipids). Experiments were run in triplicate, and the results are given as percentage of total metabolized fatty acids as mean from four different rats. The total metabolism (sum of incorporation into glycerolipids, cellular free fatty acids, and oxidation) of [1-^{14}C]EPA, [1-^{14}C]DHA, [1-^{14}C]oleic acid, and [1-^{14}C]palmitic acid was 99 ± 20, 82 ± 10, 72 ± 14, and 76 ± 18 nmol/mg protein/4 h, respectively. The levels of cellular [1-^{14}C]-labeled fatty acids and incorporation into mono- and diacylglycerol were less than 5%. See Figure 1 for abbreviations.

unit, oxidation of [1-^{14}C]fatty acids only takes the first round of oxidation into account. On the other hand, the [^{3}H]fatty acids are labeled beyond the third position. Thus, a second round of the fatty acid oxidation cycle, dependent on 2.4-dienoyl-CoA reductase and the enoyl-CoA isomerase, is necessary to detect activity.

TABLE 1
Oxidation of [1-^{14}C]EPA, [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA, [1-^{14}C]DHA, and [4,5-^{3}H]DHA in Purified Mitochondria and Peroxisomes[a]

	[^{14}C]EPA	[^{3}H]EPA	[^{14}C]DHA	[^{3}H]DHA[b]
		(nmol/min/mg protein)		
Mitochondria	2.92	0.46	0.28	0.24
Peroxisomes	0.39	0.05	0.12	0.06
		Net dpm		
Net dpm summarized[c]	1388	261	176	137
Mitochondria and peroxisomes	2460	1134	223	209
+ 50 mM etomoxir	1585	857	202	248

[a]Mitochondria and peroxisomes were purified from rat liver; formation of acid-soluble products was measured in 0.24 mg pure mitochondria, 0.5 mg peroxisomes, and in a combination of both mitochondria and peroxisomes, as described in the Materials and Methods section.
[b]Values represent labeled acid-soluble products in 0.5 mL solution; the specific activity of [1-^{14}C]eicosapentaenoic acid (EPA), [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA, [1-^{14}C]docosahexaenoic acid (DHA), and [4,5-^{3}H]DHA were 1648, 2083, 934, and 1987 disintegrations per minute (dpm)/nmol, respectively.
[c]Disintegrations per minutre summarized from two separate reactions using peroxisomes and mitochondria separately.

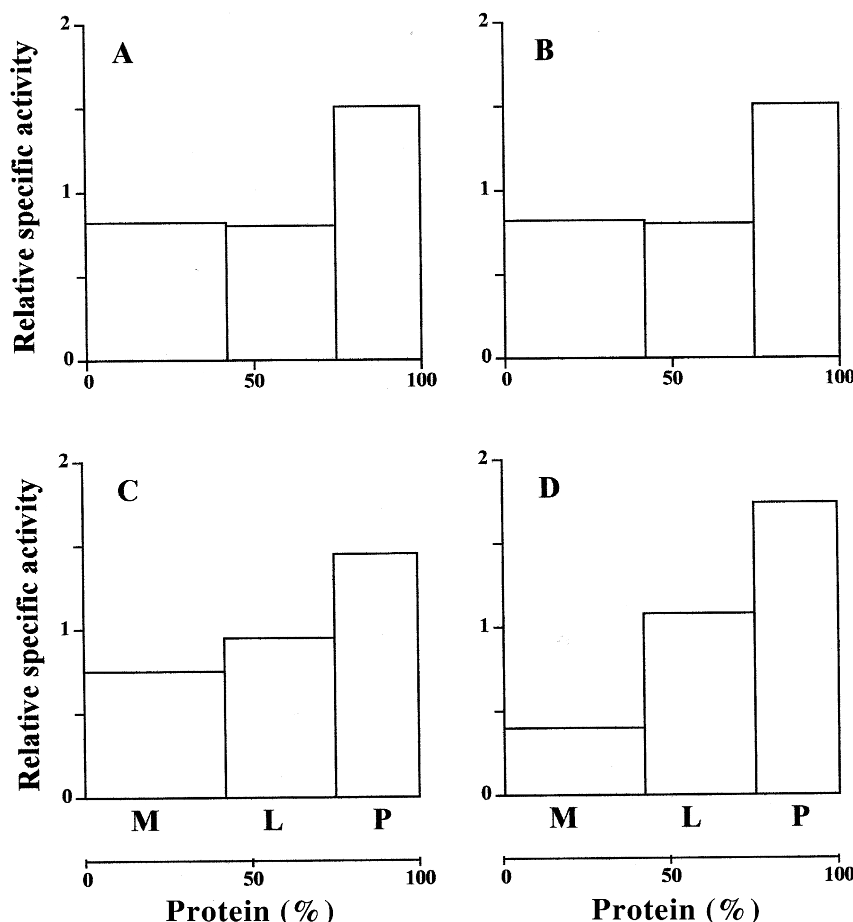

FIG. 3. Rats were fed palmitic acid at a dose of 1500 mg/d/kg body weight suspended in 0.5% carboxymethyl cellulose (CMC) for 7 d. The postnuclear homogenates from the livers of four individual rats were fractionated into mitochondrial (M), peroxisomal (L), and microsomal (P) fractions, and the acyl-CoA synthase activities were measured using (A) [1-^{14}C]palmitic acid, (B) [1-^{14}C]oleic acid, (C) [1-^{14}C]EPA, and (D) [1-^{14}C]DHA as substrates, as described in the Materials and Methods section. The abscissa represents the cumulative protein content for each fraction as a percentage of the total protein (M + L + P). The ordinate represents relative specific activity, i.e., the percentage of the total enzyme activity in the fraction over the percentage of total protein in the fraction. The respective specific activities, using [1-^{14}C]palmitic acid, [1-^{14}C]oleic acid, [1-^{14}C]EPA and [1-^{14}C]DHA as substrates, were 79 ± 9, 69 ± 6, 41 ± 5, and 9 ± 2 nmol/mg protein/min in the M fraction, 102 ± 20, 90 ± 15, 56 ± 24, and 19 ± 4 nmol/mg protein/min in the L fraction, and 153 ± 7, 142 ± 11, 101 ± 12, and 28 ± 2 nmol/mg protein/min in the P fraction. See Figure 1 for other abbreviations.

The formation of acid-soluble products from [1-^{14}C]EPA was also higher than from [1-^{14}C]DHA in peroxisomes, but the difference was much less pronounced. Oxidation of both [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA and [4,5-^{3}H]DHA was almost negligible in the peroxisomes, suggesting that only one cycle of oxidation takes place in this organelle (Table 1). In a combination of mitochondria and peroxisomes, the formation of acid-soluble products from both [^{14}C]DHA and [^{3}H]DHA was comparable to the sum of acid-soluble products formed in the two organelles separately, and the reaction was not inhibited by the mitochondrial CPT-I inhibitor, etomoxir (Table 1). On the other hand, the formation of acid-soluble products from both [1-^{14}C]EPA and especially [5,6,8,9,11,12,14,15,17,18-^{3}H]EPA was much higher in a combination of mitochondria and peroxisomes, suggesting synergetic oxidation (Table 1).

Fatty acid activation. Activation to CoA esters is necessary prior to fatty acid oxidation. We earlier demonstrated that DHA is a much poorer substrate than EPA for the acyl-CoA synthetases (26). The present study shows the subcellular distribution of acyl-CoA synthetase using EPA, DHA, palmitic and oleic acids as substrates (Fig. 3). The activity was equally distributed between the mitochondrial and peroxisomal fractions when palmitic or oleic acid was used as substrate, but shifted toward the peroxisomes when EPA and especially DHA were used (Fig. 3). Neither EPA nor DHA treatment significantly changed the subcellular distribution of the acyl-CoA synthetases (not shown).

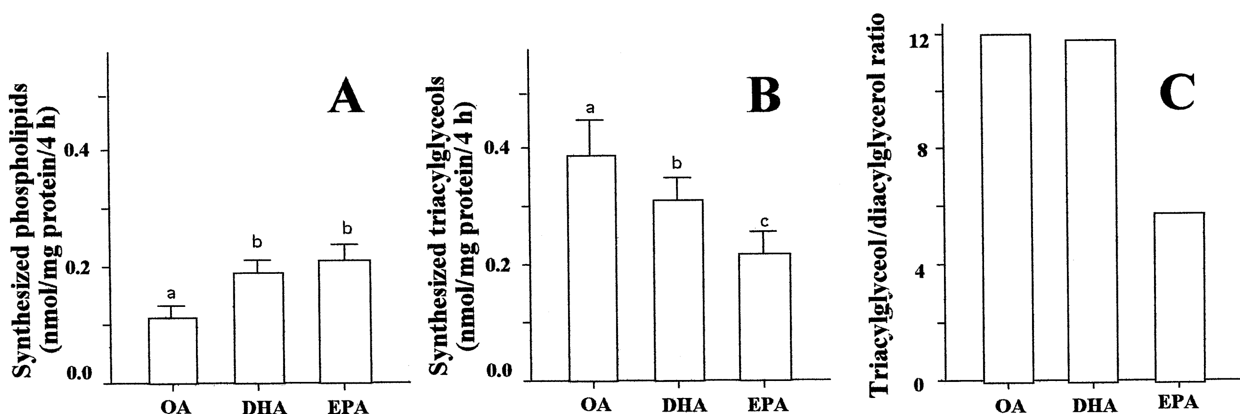

FIG. 4. Cells were plated overnight in DMEM supplemented with 0.5 mM L-carnitine and incubated for 4 h with 200 μM palmitic acid and 25 μM [^{3}H]glycerol in the presence of 200 μM EPA, DHA, or oleic acid (OA) as described in the Materials and Methods section. The results are presented as (A) formation of [^{3}H]phospholipids, (B) formation of triacyl[^{3}H]glycerol, and (C) as ratio of triacyl[^{3}H]glycerol/diacyl[^{3}H]glycerol. The experiments were run in triplicate, and the results are given as mean from three to five different rats. Columns with different superscript letters are significantly different at the $P = 0.05$ level. See Figure 1 for abbreviations.

Effects of oleic acid, EPA, and DHA on [^{3}H]glycerol incorporation into glycerolipids. The poor oxidation of labeled DHA confirmed that DHA was preferentially incorporated into glycerolipids as indicated in Figures 1 and 2. To further investigate the incorporation of EPA and DHA into glycerolipids, the metabolism of [^{3}H]glycerol was studied in rat hepatocytes. The formation of [^{3}H]phospholipids was not significantly different in the presence of EPA and DHA (Fig. 4A). However, compared to oleic acid, both EPA and DHA ($P < 0.05$) stimulated phospholipid formation (Fig. 4A). In contrast, the formation of triacyl[^{3}H]glycerols was reduced in the presence of both EPA and DHA, compared to oleic acid ($P < 0.05$) (Fig. 4B). The formation of triacyl[^{3}H]glycerols was, however, significantly lower in the presence of EPA compared to DHA ($P < 0.05$) (Fig. 4B). The finding that EPA reduced formation of triacyl[^{3}H]glycerols and stimulated formation of [^{3}H]phospholipids suggested specific inhibition of triacylglycerol synthesis. Moreover, compared to DHA and oleic acid addition, a markedly lower value for the triacyl[^{3}H]glycerol/diacyl[^{3}H]glycerol ratio was observed in the presence of EPA ($P < 0.05$), indicating that the last step in triacylglycerol synthesis was inhibited (Fig. 4C).

EPA-CoA and DHA-CoA as substrates for DGAT. The final step of the triacylglycerol synthetic pathway involves the conversion of diacylglycerol and acyl-CoA into triacylglycerol catalyzed by DGAT. In isolated rat liver microsomes, incorporation of oleoyl-CoA, palmitoyl-CoA, and DHA-CoA increased in a dose-dependent manner with 1,2-di[1-^{14}C]oleoylglycerol conversion into triacylglycerol, whereas EPA-CoA slightly increased triacylglycerol formation up to 10 μM and no further increase was seen (Fig. 5A). Triacylglycerol formation in the presence of EPA-CoA at 20 μM was less than 20%

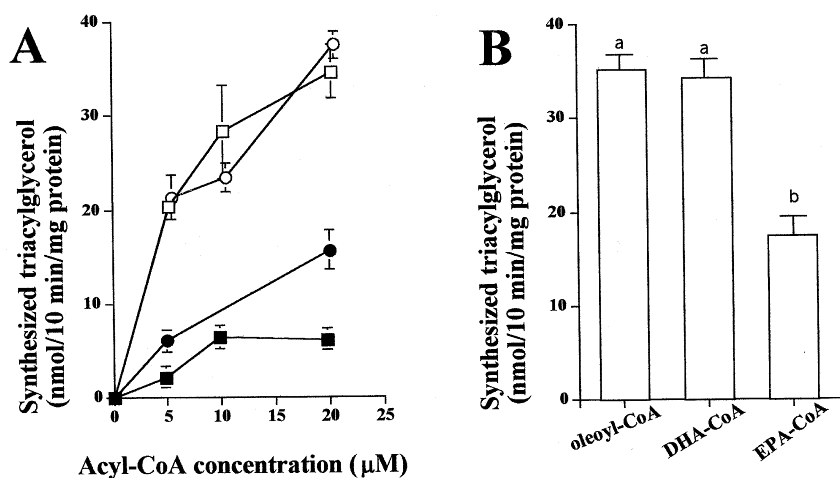

FIG. 5. Microsomes were isolated from four individual rat livers as described in the Materials and Methods section. The results are presented as formation of nmol [1-^{14}C]-labeled triacylglycerol formated per mg microsomal protein in 10 min from (A) 1,2-di[1-^{14}C]oleoylglycerol and different concentrations of EPA-CoA (■), DHA-CoA (●), oleoyl-CoA (□), and palmitoyl-CoA (○); and (B) 1,2-dioleoylglycerol and [1-^{14}C]oleoyl-CoA, in the presence of EPA-CoA, DHA-CoA or oleoyl-CoA. The values represent mean ± SD. Columns with different superscript letters are significantly different at the $P = 0.05$ level. See Figure 1 for abbreviations.

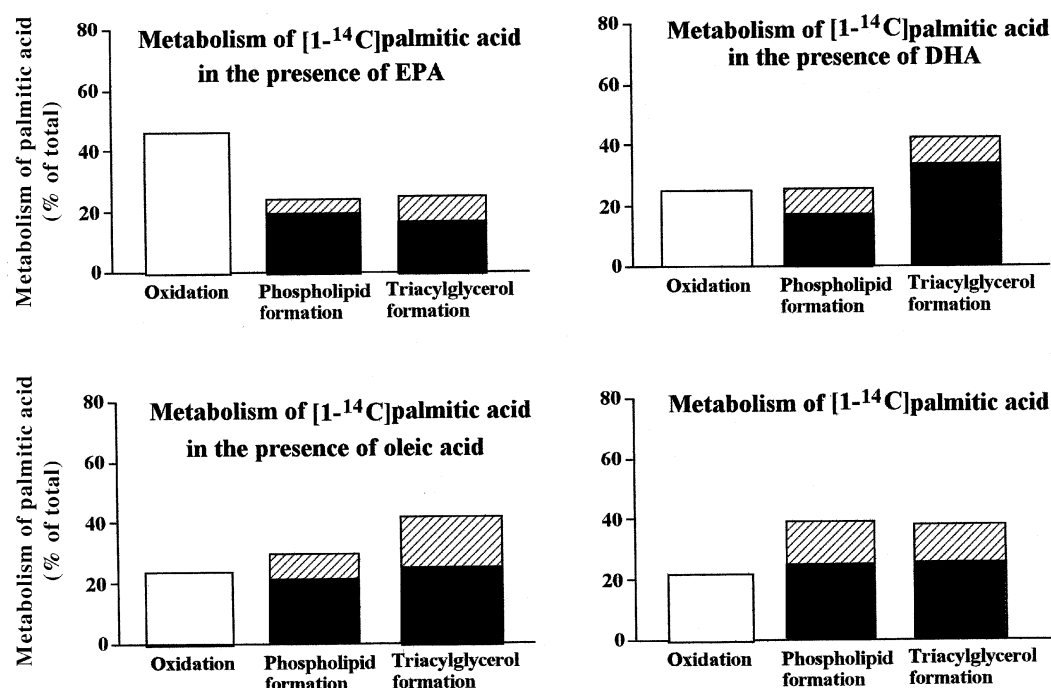

FIG. 6. Metabolism of 200 µM [1-^{14}C]palmitic acid in hepatocytes plated overnight in DMEM and incubated for 4 h in the absence and presence of 200 µM EPA, DHA, and oleic acid as described in the Materials and Methods section. The results were calculated as acid-soluble ^{14}C in the medium (open bar), [1-^{14}C]palmitic acid-labeled cell-associated (closed bar), and secreted (ruled bar) glycerolipids (triacylglycerols and phospholipids). Experiments were run in triplicate, and the results are given as the percentage of total metabolized fatty acids as mean from four different rats. The total metabolism (sum of incorporation into glycerolipids, cellular free fatty acids, and oxidation) of [1-^{14}C]palmitic acid in the absence of other fatty acids was 227 ± 25 nmol/mg protein/4 h. In the presence of EPA, DHA, and oleic acid, the total metabolism of [1-^{14}C]palmitic acid was 223 ± 27, 211 ± 26, and 172 ± 31, nmol/mg protein/4 h, respectively. The levels of cellular [1-^{14}C]palmitic acid and incorporation into mono- and diacylglycerol were less than 5%.

when compared to oleoyl-CoA ($P < 0.005$) and palmitoyl-CoA ($P < 0.005$), and 40% in comparison to DHA-CoA ($P < 0.05$) (Fig. 5A). Furthermore, [1-^{14}C]oleoyl-CoA was incorporated into triacylglycerol to a lesser extent when EPA-CoA, but not DHA-CoA, was added (Fig. 5B), suggesting that EPA, but not DHA, influenced triacylglycerol metabolism.

Effects of oleic acid, EPA, and DHA on [1-^{14}C]palmitic acid oxidation, glycerolipid synthesis, and secretion. To further investigate the different effects of EPA and DHA on triacylglycerol metabolism, the flux of [1-^{14}C]palmitic acid was studied in rat hepatocytes incubated 4 h in the presence of oleic acid, EPA, and DHA at 200 µM. Figure 6 shows that the proportion of [1-^{14}C]palmitic acid incorporated into triacylglycerols was reduced in the presence of EPA ($P < 0.05$, compared to DHA, oleic acid, and nonaddition). Moreover, in the presence of EPA, palmitic acid was oxidized to a greater extent than in presence of DHA, oleic acid, and nonaddition (Fig. 6). Table 2 illustrates the oxidation of [1-^{14}C]palmitic acid into acid-soluble products. CO_2 is lower in the presence of DHA than in the presence of EPA ($P < 0.005$), indicating that EPA, and not DHA, influences mitochondrial β-oxidation.

TABLE 2
Oxidation of [1-^{14}C]Palmitic Acid in the Presence of EPA and DHA[a]

	4-h incubation		9-h incubation	
Fatty acid	Acid-soluble products	CO_2	Acid-soluble products	CO_2
	(nmol/mg protein)			
EPA	62 ± 9	18 ± 3	79 ± 13	27 ± 5
DHA	38 ± 7	5± 2	51 ± 15	6 ± 1

[a]Cells were plated overnight in medium supplemented with L-carnitine (0.5 mM) and incubated for 4 or 9 h with labeled palmitic acid and 200 µM EPA or DHA at different concentrations, as described in the Materials and Methods section; values are presented as means ± SD from at least four independent experiments. See Table 1 for abbreviations.

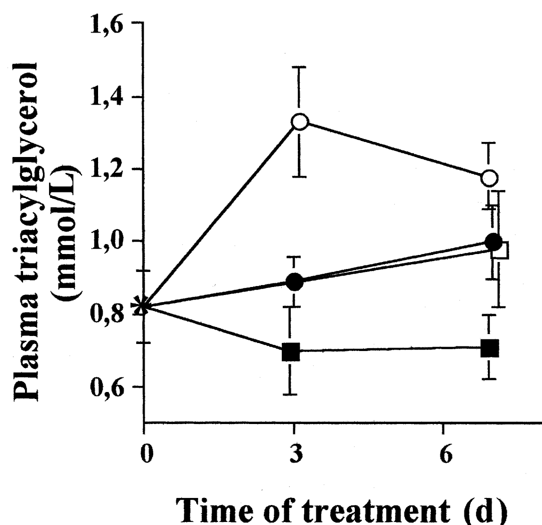

FIG. 7. Rats were fed EPA (■), DHA (●), oleic (□) and palmitic (○) acids at a dose of 1500 mg/d/kg body weight suspended in 0.5% CMC for 3 or 7 d. Control animals received CMC only (✱). The triacylglycerol was measured in plasma prepared from each rat. The values are presented as mean ± SD. See Figures 1 and 3 for other abbreviations.

Effect of EPA and DHA treatment on plasma triacylglycerol and fatty acid oxidation in rats. Confirming earlier studies, EPA treatment reduced plasma triacylglycerol levels in rats (Fig. 7). Moreover, compared to untreated rats, EPA treatment, in contrast to DHA treatment, significantly ($P < 0.05$) increased the oxidation of palmitoyl-CoA and the CPT-I activity in the presence and absence of malonyl-CoA in isolated mitochondria (Fig. 8). Also the CPT-I mRNA levels were significantly increased after EPA treatment (Table 3). It was interesting, however, that both EPA and DHA treatment increased the activities and mRNA levels of peroxisomal FAO and mitochondrial CPT-II, as well as FABP mRNA (Table 3).

DISCUSSION

The mechanism by which diets enriched in EPA and DHA reduce plasma triacylglycerol is still a matter of dispute, and the available literature is not consistent concerning the different effects. We earlier demonstrated that EPA reduces plasma triacylglycerol in rats (12,24). However, in most studies performed, a combination or a mixture containing both EPA and DHA is used. Different basal levels, retroconversion of DHA to EPA (25,30,31), and divergent release of EPA and DHA from different tissues (32) complicate the interpretation of animal and especially human studies. Even though retroconversion of DHA to EPA also has been demonstrated in cultured rat hepatocytes (30), most of these complicating factors are excluded when cultured hepatocytes are used.

In our system, the levels of total metabolism and flux of [1-^{14}C]-labeled EPA and DHA were similar at equal concentrations, but different metabolic pathways were preferred (Figs. 1 and 2). Reduced triacylglycerol synthesis has been suggested as a mechanism by which diets enriched in fish oil reduce plasma triacylglycerol, as EPA and EPA-CoA are poor substrates for DGAT (5,6). In the present study, we demonstrated, using labeled 1,2-di[1-^{14}C]oleoylglycerol or [1-^{14}C]-oleoyl-CoA as a precursor, that EPA-CoA reduced DGAT activity (Fig. 5). Moreover, as the formation of triacyl[^{3}H]glycerol (Fig. 4B) and the triacyl[^{3}H]glycerol/diacyl[^{3}H]glycerol ratio (Fig. 4C) from [^{3}H]glycerol were reduced in the presence of EPA compared to DHA, it is likely that EPA, and not DHA, influenced DGAT.

It has previously been shown that secretion of very low density lipoprotein particles is dependent on triacylglycerol synthesis and the availability of triacylglycerol within the hepatocytes (58,59). However, studies by Al-Shurbaji *et al.* (10) and Ikeda *et al.* (60) found that dietary treatment of rats with a mixture of n-3 fatty acids or EPA alone did not change hepatic DGAT activity. Willumsen *et al.* (12) have indicated that

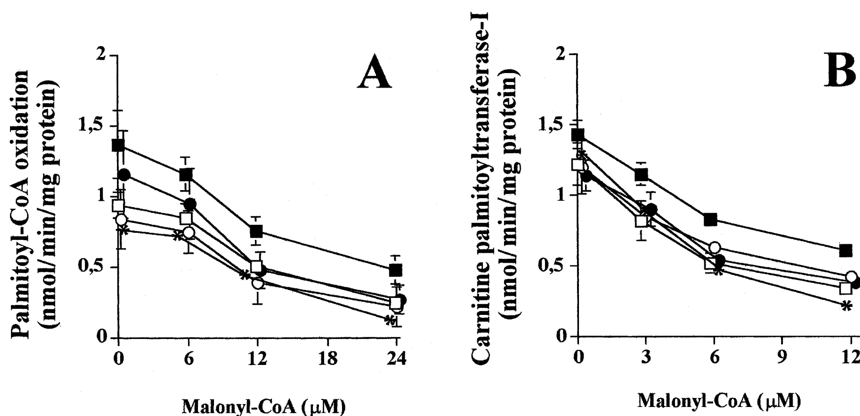

FIG. 8. Rats were fed EPA (■), DHA (●), oleic (□) and palmitic (○) acids, at a dose of 1500 mg/d/kg body weight, suspended in 0.5% CMC for 7 d. Control animals received CMC only (✱). (A) Palmitoyl-CoA oxidation and (B) carnitine palmitoyltransferase-I activity were measured in isolated mitochondria as described in the Materials and Methods section in four individual rat livers in the absence and presence of malonyl-CoA. The values are presented as mean ± SD. See Figures 1 and 3 for abbreviations.

TABLE 3
Effects of EPA, DHA, Oleic Acid, and Palmitic Acid on Hepatic Carnitine Palmitoyltransferase (CPT)-I and -II, Fatty Acid Binding Protein (FABP), and Fatty Acyl-CoA (FAO) Oxidase[a]

Treatment[b]	Control	Palmitic acid	Oleic acid	EPA	DHA
CPT-I mRNA (fold increase)	1.0 ± 0.1	1.1 ± 0.1	1.0 ± 0.1	1.5 ± 0.2[c]	1.2 ± 0.1
CPT-II mRNA (fold increase)	1.0 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.3 ± 0.1[c]	1.7 ± 0.1[c]
CPT-II activity (nmol/mg protein)	26 ± 3	32 ± 3	33 ± 4	37 ± 4[c]	42 ± 5[c]
FABP mRNA (fold increase)	1.0 ± 0.1	0.7 ± 0.3	0.8 ± 0.1	1.3 ± 0.1[c]	1.6 ± 0.1[c]
FAO mRNA (fold increase)	1.0 ± 0.1	0.7 ± 0.2	1.0 ± 0.1	1.6 ± 0.3[c]	2.1 ± 0.5[c]
FAO activity (nmol/mg protein)	16 ± 5	20 ± 3	18 ± 3	29 ± 5[c]	33 ± 6[c]

[a]Animals were treated with different fatty acids at a dose of 1500 mg/d/kg body weight for 7 d; CPT-II and FAO activities were measured in mitochondrial and peroxisomal fractions prepared from individual rat livers as described in the Materials and Methods section.
[b]The mRNA were extracted from the livers of four different rats and hybridized with different cDNA as described in the Materials and Methods section; each value (mean ± SD) was normalized to the corresponding 28S rRNA levels, and the mean of the controls was set to 1.0. See Table 2 for abbreviations.
[c]Significantly different from controls ($P < 0.05$).

dietary EPA treatment stimulates DGAT activity, whereas Geelen *et al.* (61) demonstrated that hepatic DGAT activity in rats was significantly reduced when compared to rats fed corn oil. It is also noteworthy that treatment of rats with hypotriacylglycerolemic 3-thia fatty acids, which also leads to a reduced hepatic content of triacylglycerol (62), increased DGAT activity in the liver (63). On the other hand, 4-thia fatty acid treatment, which induced fatty liver and increased plasma triacylglycerol (62), strongly inhibited DGAT activity (63). Thus, changed DGAT activity does not appear to be coordinated with the triacylglycerol-lowering effect.

The oxidation of [1-^{14}C]palmitic acid into both CO_2 and acid-soluble products was significantly higher in the presence of EPA than DHA (Table 2). Moreover, the distribution of [1-^{14}C]palmitic acid shifted from incorporation into triacylglycerol to oxidation when EPA was added to cells (Fig. 6). Acute stimulation of fatty acid oxidation by EPA might thereby reduce fatty acid availability for synthesis and secretion of triacylglycerol.

The logical consequence of our *in vitro* findings would be that EPA, and not DHA, is the hypolipidemic component in fish oil. Indeed, in confirming earlier studies (12,24), there was a significant reduction in the plasma triacylglycerol levels after 7 d of treatment with pure EPA when compared to DHA in these rats (Fig. 7). Moreover, in agreement with a recent study by Ikeda *et al*. (60), EPA treatment increased the mitochondrial palmitoyl-CoA oxidation and CPT-I activity in the absence and presence of malonyl-CoA (Fig. 8).

The molecular mechanism involved in gene regulation by fatty acids remains unknown. It is, however, suggested that they act through the peroxisome proliferating activated receptor (PPAR). This is supported by the recent finding that PPARα is required for induction of FAO and cytochrome P450 4A2 but not for suppression of fatty acid synthase by polyunsaturated fatty acids (64). These findings suggest that PPARα activation upregulates both mitochondrial and peroxisomal fatty acid oxidation and provide evidence for two distinct pathways for polyunsaturated fatty acid control of hepatic lipid metabolism. A peroxisome proliferator responding element is located upstream the FABP gene (65), and it is likely that EPA and DHA treatment induces the FABP and FAO mRNA levels (Table 3) *via* PPARα activation. The increased mRNA levels of CPT-II (Table 3) after EPA, and especially DHA treatment, which is a stronger peroxisome proliferator than EPA (15), might occur by a similar mechanism. The recent finding that treatment with the classic peroxisome proliferator, Wy-14.643, increases CPT-II mRNA as well as FAO mRNA in wild type, but not PPARα-null mice (66), supports this hypothesis.

In contrast, EPA increased the mRNA levels of CPT-I (Table 3). Hepatic CPT-I, which is considered to be rate limiting for mitochondrial fatty acid oxidation, is to our knowledge not under the regulation of PPARα. In case of the CPT-I gene, the work by Chatelain *et al*. (67) suggested that peroxisome proliferators and long-chain fatty acids mediate their action on CPT-I through different mechanisms.

EPA was a far better substrate for fatty acid oxidation than DHA in rat parenchymal cells (Figs. 1C and 2), isolated mitochondria, and peroxisomes (Table 2). We have demonstrated that oxidation of EPA, and not DHA, is sensitive to CPT-II inhibition in rat parenchymal cells (46), suggesting peroxisomal, not mitochondrial, oxidation of DHA. The finding that oxidation of DHA in a combination of mitochondria and peroxisomes is not inhibited by the CPT-I inhibitor, etomoxir, further supports this hypothesis (Table 1), as the entry and oxidation of fatty acids in the peroxisomes are independent of carnitine (68). In addition, compared to EPA-CoA, DHA-CoA was more effectively synthesized in the microso-

mal and peroxisomal fractions than the mitochondrial fraction (Fig. 3). It is likely that peroxisomal oxidation of DHA, which is quantitatively less effective than mitochondrial oxidation, might divert intracellular DHA into the glycerolipids. Indeed, [1-^{14}C]DHA is preferentially incorporated into cell-associated phospholipids in rat parenchymal cells (Fig. 2).

In contrast, the formation of acid-soluble products from EPA was sensitive to CPT-I inhibition, suggesting mitochondrial oxidation (Table 1). However, the formation of acid-soluble products, especially from carbon atoms beyond the third position of EPA, was far more effective when peroxisomes were added (Table 1). Whereas the formation of acid-soluble products from DHA was comparable to the sum of acid-soluble products formed in the two organelles separately, EPA oxidation was much higher in a combination of mitochondria and peroxisomes, suggesting synergetic oxidation (Table 1). A possible explanation for those results would be that the peroxisomal chain-shortened EPA is delivered to mitochondria for further oxidation. The first round of β-oxidation might occur in both mitochondria and peroxisomes, in addition to plain mitochondrial oxidation.

Polyunsaturated fatty acids are more difficult to oxidize than their saturated counterparts due to their double bonds. Accumulation of DHA-CoA and EPA-CoA esters in the peroxisomes and mitochondria, respectively, might give a "fatty acid overload" signal, leading to increased fatty acid oxidation of palmitic acid. Thus, different metabolic properties of these polyunsaturated n-3 fatty acids also imply different effects, i.e., they affect organelle oxidation in relation to the substrate preference. This is consistent with the finding that EPA, but not DHA, treatment increased the mRNA levels of CPT-I (Table 3) and caused proliferation of mitochondria (15). On the other hand, DHA is a stronger peroxisome proliferator than EPA (15) and more effectively increases the mRNA levels of FAO (Table 3). However, stimulation of this quantitatively less effective peroxisomal fatty acid oxidation system might not be sufficient to influence the balance between fatty acid oxidation and triacylglycerol biosynthesis.

In conclusion, the different metabolic properties of EPA and DHA as shown in this study might be related to their different hypolipidemic properties. Our results suggest that EPA is the more metabolically active fatty acid, whereas DHA might have structural functions. The present study supports the hypothesis that EPA, and not DHA, lowers plasma triacylglycerol by increased mitochondrial β-oxidation. Evidently, EPA is the primary hypolipidemic component of fish oil.

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Effects of Conjugated Linoleic Acid Isomers on the Hepatic Microsomal Desaturation Activities *in Vitro*

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ABSTRACT: The influence of individual conjugated linoleic acid (CLA) isomers on the Δ6 desaturation of linoleic and α-linolenic acids and on the Δ9 desaturation of stearic acid was investigated *in vitro*, using rat liver microsomes. The Δ6 desaturation of 18:2n-6 was decreased from 23 to 38% when the ratio of 9*cis*,11*trans*-18:2 to 18:2n-6 increased from 0.5 to 2. The compound 10*trans*,12*cis*-18:2 exhibited a similar effect only at the highest concentration. The Δ6 desaturation of α-linolenic acid was slightly affected by the presence of CLA isomers. The sole isomer to induce an inhibitory effect on the Δ9 desaturation of stearic acid was 10*trans*,12*cis*-18:2.

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Conjugated linoleic acid (CLA) is a generic term that encompasses several isomers of linoleic acid with conjugated double bonds. CLA has been reported to have beneficial effects on different aspects of health, including cancer (1–3), body mass composition (4,5), and cardiovascular risk factors (6,7). However, most of the studies reported to date used mixtures of different CLA isomers, including 7,9-, 8,10-, 9,11-, 10,12-, and 11,13-18:2, with different combinations of *cis* and *trans* double bonds (8,9). On the other hand, the main dietary source of CLA is ruminant fat and dairy products (10), in which the main CLA isomer is 9*cis*,11*trans*-18:2. CLA has been found in different human tissues, including milk (11,12), bile (13,14), adipose tissue (15,16), and serum (17,18). It recently has been demonstrated in rats that only the 9*cis*,11*trans* and the 10*trans*,12*cis* isomers are desaturated and elongated into 18:3 and 20:3 conjugated fatty acids (19). The biosynthesis of conjugated 20:4 was low except when rats were fed a linoleic acid-poor diet (19).

Different mechanisms of action have been hypothesized, including alteration of eicosanoid synthesis even if CLA isomers and metabolites are poorly incorporated in tissue phospholipids (20). However, CLA intake modifies the fatty acid profile of tissues, particularly the long-chain polyunsaturated content. CLA decrease the expression of stearoyl-CoA desaturase mRNA in the liver of mice (21), and the authors of this research suggested the active form may be an isomer other than 9*cis*,11*trans*. However, the effects of CLA on the different desaturation steps involved in the biosynthesis of unsaturated fatty acids have not yet been investigated. In the present work, we studied the effects of different CLA isomers on the Δ6 desaturation of linoleic and α-linolenic acids, as well as on the Δ9 desaturation of stearic acid.

MATERIALS AND METHODS

Animals and diet. Male Wistar rats (IFFA CREDO, L'Arbresle, France) were fed a fat-free semisynthetic diet for 1 wk, as previously described (22).

Chemicals. [1-^{14}C]-18:0, [1-^{14}C]-18:2n-6, and [1-^{14}C]-18:3n-3 were purchased from NEN Life Science Products (Paris, France) and diluted to a specific activity of 370 M·mmol^{-1} using the corresponding unlabeled fatty acids from Sigma (L'Isle D'Abeau, France). The 9*cis*,11*trans*- and the 10*trans*,12*cis*-18:2 were obtained by total synthesis (23,24). All the fatty acids were dissolved in ethanol. Coenzymes were purchased from Sigma.

Preparation of microsomes. The animals were anesthetized with diethyl ether at 8:30 A.M. in order to avoid variation in enzyme activities due to circadian rhythm (25), exsanguinated, and the livers were quickly excised. Microsomes were prepared using a slight modification of the previously described method (26). Briefly, each liver (12.5 ± 0.33 g, mean ± SEM) was homogenized in sucrose and phosphate buffer (pH 7.4). The homogenate was first centrifuged at 400 × *g* for 5 min to pellet cellular debris. The supernatant was then centrifuged at 15,000 × *g* for 15 min to eliminate nuclei, mitochondria, lysosomes, and peroxysomes. Finally, the supernanant was ultracentrifuged at 105,000 × *g* for 60 min. The pellet was then resuspended into saccharose-phosphate buffer and cytosol. Microsomal proteins were quantified according to Lowry *et al.* (27).

Microsomal suspensions containing 5 mg of protein were immediately incubated with [1-^{14}C]-18:0 (40 nmoles) or [1-^{14}C]-18:2 or -18:3 (60 nmoles) in the presence of increasing concentrations of 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2, in order to obtain inhibitor/substrate (I/S) ratios from 0 to 2. The incubations were carried out at 37°C for 15 min, as previously described (26), with the following modifications. Each incu-

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Abbreviations: CLA, conjugated linoleic acid; I/S, inhibitor/substrate.

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bation medium contained 72 mM phosphate buffer, 4.8 mM $MgCl_2$, 0.5 mM CoA, 3.8 mM ATP, 1.2 mM NADPH, and 1.2 mM reduced glutathione, in a final volume of 2 mL.

Analysis of the conversion products. The reactions were stopped by addition of KOH in ethanol. The lipids were saponified by heating at 100°C for 1 h under nitrogen. Fatty acid methyl esters (FAME) were prepared according to Morrison and Smith (28). The FAME were then extracted with hexane and fractionated using reversed-phase high-performance liquid chromatography. The FAME were dissolved in 100 µL of acetone and analyzed using a Nucleosil C18 column packing (5 µm particle size, 250 × 4.6 mm i.d., Interchim, Montluçon, France) and a high-performance liquid chromatographic system (model 600, Waters, Saint Quentin en Yvelines, France). The mobile phase was acetonitrile at a flow rate of 0.5 mL·min^{-1}. The radioactivity was measured in the counting cell of a radiochromatographic Flo-One β detector (Series A-100, Radiomatic Instruments, Tampa, FL) after mixing the column effluent with a Uniscint BD scintillation cocktail (National Diagnostic, Atlanta, GA) in a ratio of 1:1.2 (effluent/scintillation cocktail).

Some samples were also analyzed by gas chromatography coupled with radioactive detection, as previously described (26). Briefly, microsomal methyl esters were analyzed on a Hewlett-Packard 5890 series II gas chromatograph (Palo Alto, CA), equipped with a splitless injector and a fused Stabilwax wide-bore silica column (60 m × 0.53 mm i.d.; film thickness; 0.50 µm; Restek, Evry, France). The outflow from the column was split between a flame-ionization detector (10%) and a copper oxide oven heated at 700°C in order to transform the labeled fatty acids into $^{14}CO_2$ (90%). The radioactivity was determined with a radiodetector (GC-RAM; Lablogic, Sheffield, United Kingdom) by counting $^{14}CO_2$ after mixing it with a 9:1 ratio of argon/methane. The data were computed using Laura software (Lablogic).

Statistical analysis. Results are expressed as means ± SD (n = 3). The comparisons between values were done by an analysis of variance and the Newman-Keuls' test using the NCSS software (Cork, Ireland).

RESULTS

To examine the effects of individual CLA isomers on the desaturation of C_{18} fatty acids, we incubated stearic, linoleic, and α-linolenic acids in the presence of increasing concentrations of CLA isomers and rat liver microsomes.

Analysis of the microsomal methyl esters by radio-gas chromatography was performed in order to ascertain the conversion of stearic acid into oleic acid and of linoleic and α-linolenic acids into their corresponding Δ6 desaturated metabolites. Indeed, stearidonic, γ-linolenic, and oleic acids were detected (Fig. 1).

The control microsomes exhibited a Δ9 desaturation activity toward stearic acid of 385 pmol·min^{-1}·mg protein^{-1} and a Δ6 desaturation activity toward linoleic and α-linolenic acids of 115 and 400 pmol·min^{-1}·mg^{-1} protein, respectively. These

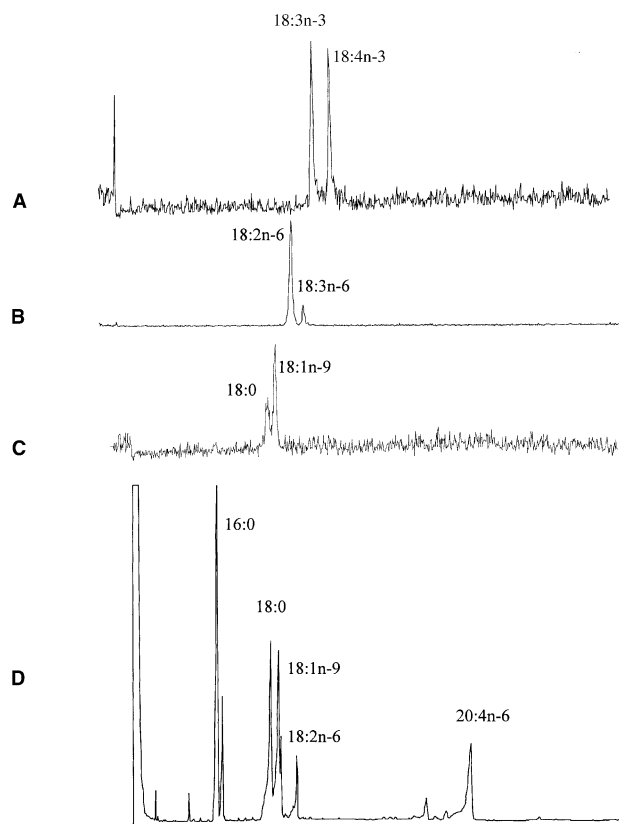

FIG. 1. Radiochromatograms and flame-ionization detector (FID) signal of methyl esters obtained from microsomes (5 mg of protein) incubated during 15 min at 37°C under control conditions [without conjugated linoleic acid (CLA) isomers] with 60 nmol of α-linolenic acid [1-^{14}C] (trace A), 60 nmol of linoleic acid [1-^{14}C] (trace B) or 40 nmol of stearic acid [1-^{14}C] (trace C). The D-trace corresponds to the FID signal (for radiochromatographic conditions, see the Material and Methods section).

values were comparable to those reported by several authors (29–32). Figure 2 presents the Δ9 desaturation of stearic acid in the absence (control) or presence of increasing concentrations of 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2. Coincubation of 9*cis*,11*trans*-18:2 did not significantly modify the desaturation of stearic acid, whereas 10*trans*,12*cis*-18:2 significantly inhibited the conversion of stearic acid. This inhibition of conversion was 39% when the I/S ratio (CLA/radiolabeled substrate ratio) was 1 and reached 50% when the I/S ratio was 2; microsome concentration was constant.

The Δ6 desaturation of linoleic and α-linolenic acids is presented in Figures 3 and 4, respectively. The desaturation of linoleic acid was significantly decreased when 9*cis*,11*trans*-18:2 was present in the incubation medium. This inhibition ranges between 23 and 38% when the I/S ratio varies from 0.5 to 2. On the other hand, 10*trans*,12*cis*-18:2 inhibits the Δ6 desaturation of linoleic acid only at the highest I/S ratio. The desaturation of α-linolenic acid was only slightly affected by the presence of CLA isomers in the incubation medium.

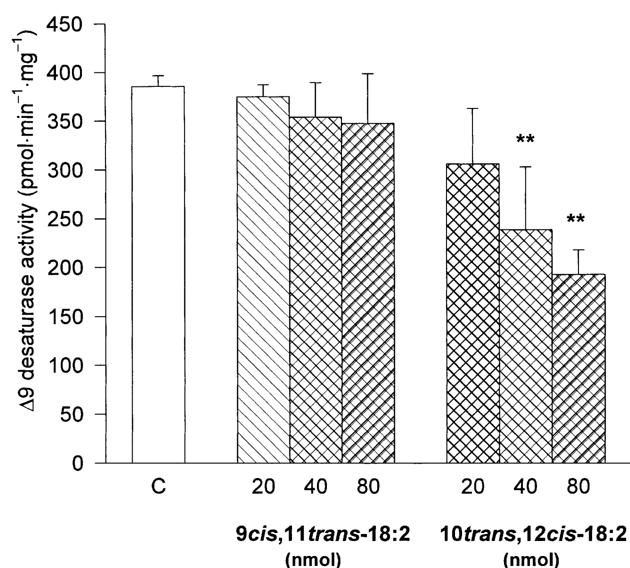

FIG. 2. Δ9 Desaturase activity of stearic acid (18:0, 40 nmol) by rat liver microsomes (5 mg protein) incubated under control conditions (without CLA) or in the presence of increasing concentrations of 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2. For abbreviations see Figure 1. Vertical bars represent standard deviation ($n = 3$). **, significantly different from control (C) at $P < 0.01$.

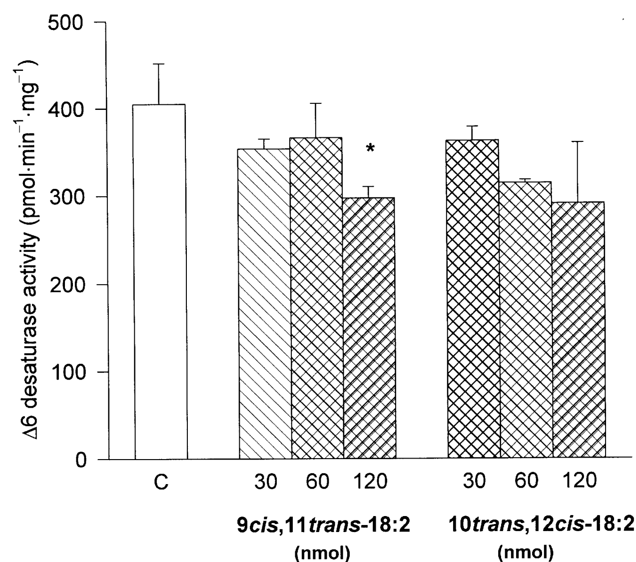

FIG. 4. Δ6 Desaturase activity of α-linolenic acid acid (18:3n-3, 60 nmol) by rat liver microsomes (5 mg protein) incubated under control conditions (without CLA) or in the presence of increasing concentrations of 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2. For abbreviations see Figure 1. Vertical bars represent SD ($n = 3$). *, significantly different from control (C) at $P < 0.05$.

DISCUSSION

The interconversion of nonconjugated fatty acids may be implicated in different pathologies in which CLA has been reported to have beneficial effects. The present study reports selective effects of individual isomers of CLA on desaturation steps involved in the biosynthesis of unsaturated fatty acids. It has previously been suggested (33) that CLA isomers may alter the desaturation of saturated fatty acids, but no clear evidence for the role of each isomer has been reported. A recent study (21) indicated that CLA alter the expression of stearoyl-CoA desaturase mRNA in the liver from mice. The CLA isomer responsible for this effects has not been clearly identified.

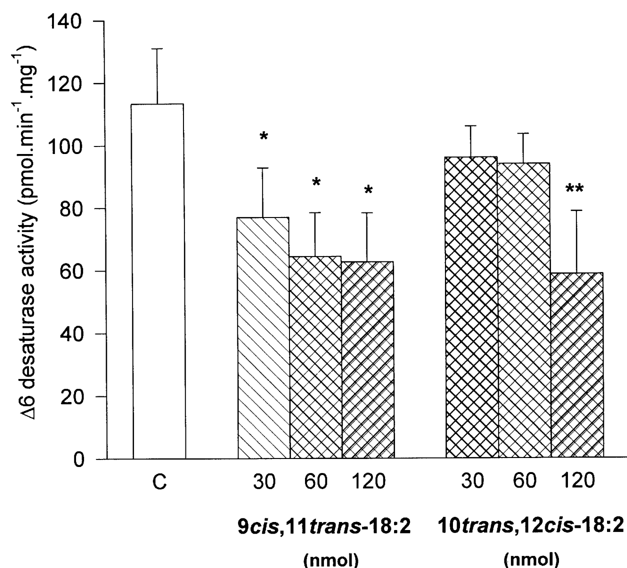

FIG. 3. Δ6 Desaturase activity of linoleic acid (18:2n-6, 60 nmol) by rat liver microsomes (5 mg protein) incubated under control conditions (without CLA) or in the presence of increasing concentrations of 9*cis*,11*trans*- or 10*trans*,12*cis*-18:2. For abbreviation see Figure 1. Vertical bar represents SD ($n = 3$). * and **, significantly different from control (C) at $P < 0.05$ and 0.01, respectively.

Data from Belury and Kempa-Stedzko (34) suggested that liver microsomes may desaturate linoleate as well as CLA. Our present work suggests that the 9*cis*,11*trans* isomer alters the Δ6 desaturation of linoleic acid, whereas the 10*trans*,12*cis* isomer is effective only when present at a high I/S ratio. Dietary CLA has been reported to modulate the fatty acid profile of different tissues (33,34). This might be explained by the alteration of desaturation activities by dietary CLA intake.

The Δ6 desaturation of linoleic acid is the well-known regulatory step in the biosynthesis of arachidonic acid, which is further released from phospholipids to be converted into bioactive lipids, i.e., eicosanoids. Eicosanoids are involved in cancer, immune function, and cardiovascular diseases, conditions in which CLA reportedly have beneficial effects. The present data show that the 9*cis*,11*trans* isomer is more active in this desaturation step than the 10*trans*,12*cis*-18:2. On the other hand, both CLA isomers studied here modify the Δ6 desaturation of α-linoleic acid only slightly, suggesting that they are not active on the n-3 polyunsaturated fatty acid (PUFA) biosynthesis cascade. The enrichment in long-chain n-3 PUFA by using pure dietary isomers (Sébédio, J.L., Juaneda, P., and Chardigny, J.M., unpublished data) and by using CLA

mixtures (34) might be due to an alteration of other enzymatic activities. Alternatively, such an inhibition of the n-6 PUFA biosynthesis, in conjunction with a preferential acylation of n-3 fatty acids over n-6, as previously shown (35), may be a valuable explanation of this result.

Previous reports indicated that dietary CLA may alter the Δ9 desaturase activity (21,33). However, no information on the specific effects of individual isomers was then available. Our present data clearly show that only the 10*trans*,12*cis* isomer is able to antagonize the desaturation of stearic acid into oleic acid, suggesting that it is the isomer responsible for the modification of saturated and monounsaturated levels in tissues from animals fed a CLA mixture. These data substantiate what was observed by Lee *et al.* (21), who suggested that the effect on the expression of stearoyl-CoA desaturase was not due to the 9*cis*,11*trans* isomer. It is suggested that the 9*cis*,11*trans*-18:2 mainly alters the Δ6 desaturation of linoleic acid whereas the 10*trans*,12*cis*-18:2 decreased the Δ9 desaturation of stearic acid, both of which are regulatory steps in the conversion of 18-carbon fatty acids.

The data reported here indicate that, *in vitro*, different CLA isomers alter the desaturation steps involved in the biosynthesis of unsaturated fatty acids differently. Further studies are needed to clarify the *in vivo* situation. In particular, it should be interesting to elucidate the influence of CLA on lipoprotein metabolism in light of the clear relationship, reported by Legrand *et al.* (36), between the inhibition of Δ9 desaturase activity and the decrease in the secretion of very low density lipoprotein-triacylglycerols in cultured chicken hepatocytes.

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Δ^9 Desaturase Activity in Bovine Subcutaneous Adipose Tissue of Different Fatty Acid Composition

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ABSTRACT: Two experiments were conducted to investigate the relationship between Δ^9 desaturase (stearoyl-coenzyme A desaturase) activity and fatty acid composition in subcutaneous adipose tissue from cattle of different backgrounds. In Experiment 1, subcutaneous adipose tissue samples were taken from carcasses of pasture-fed cattle and feedlot cattle fed for 100, 200, or 300 d. Adipose tissue from pasture-fed cattle had significantly lower total saturated fatty acids and higher total unsaturated fatty acids than feedlot cattle. Desaturase activity correspondingly was 60–85% higher in pasture-fed cattle than in feedlot cattle. There was no difference in the fatty acid composition or desaturase activity among samples from the 100-, 200-, and 300-d feedlot cattle. In Experiment 2, adipose tissue samples were collected from carcasses of feedlot cattle fed for 180 d with either a standard feedlot ration (control group), or a ration containing rumen-protected cottonseed oil (CSO) for the last 70–80 d. Adipose tissue from the CSO-fed cattle was more saturated than that from the control group, having significantly more 18:0 and less 16:1 and 18:1. Correspondingly, adipose tissue from the CSO group had significantly lower desaturase activity. The elevated 18:2 in adipose tissue from the CSO group confirmed that unsaturated fatty acids (including cyclopropenoid fatty acids) were protected from biohydrogenation. Further studies are needed to determine whether the repression of desaturase activity results from direct inhibition by cyclopropenoic acids or by higher dietary contents of 18:2.

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Grain-feeding of beef cattle markedly improves meat marbling, fat color, and product uniformity. This is particularly important for the marketing of beef to certain countries such as Japan and Korea, where significant levels of marbling and white fat color are demanded. However, with certain dietary regimens, it has been observed that there is an increase in the degree of saturation of fatty acids in adipose tissues, resulting in hard fat (1). Often, this has been observed when whole cottonseed or cottonseed products are fed as a component of the feed. Feedlot cattle, particularly cattle on long-term grain feeding, generally produce large carcasses with excessive fat thickness and fat that often is very hard when chilled. This hard fat is a problem confronting the meat industry for a number of reasons. Japan is a valuable export market for beef, and this market highly regards fat that is soft, finely textured, and satin-like in appearance. Furthermore, in some countries, hard fat results in processing difficulties, safety issues, and inefficiencies, and attempts to overcome this hardness often result in a reduction in ultimate meat quality attributes. Finally, health authorities recommend reduced consumption of saturated fat, particularly that of animal origin.

The hardness of carcass fat at a given temperature is determined mainly by its overall fatty acid composition as the overall melting point of fat depends largely on the proportion of these individual fatty acids. There have been many studies of fatty acid composition of bovine adipose tissue (2–4), and generally, for a particular body location, composition has been shown to be fairly intransigent to change, as a result of rumen hydrogenation of dietary fatty acids. However, composition is affected to some degree by factors such as breed, sex, diet, weight, age, and environmental effects, such as climate and season (3–9). The observed difference in fat hardness between pasture-fed and feedlot beef also suggests a difference in their ability to synthesize monounsaturated fatty acids. Fatty acyl coenzyme A (CoA) desaturase (stearoyl-CoA desaturase) (EC 1.14.99.5) catalyzes the conversion of saturated fatty acids to Δ^9 monounsaturated fatty acids. Our previous results suggested that grain-feeding cattle leads to inhibition of desaturase activity (9). We therefore conducted two experiments to document the relationship between the fatty acyl CoA desaturase activity and fatty acid composition in adipose tissue from cattle of different backgrounds.

MATERIALS AND METHODS

Sample collection. Two experiments were conducted in this study. In Experiment 1, subcutaneous adipose tissue samples were taken from the loin region of carcasses from nine pasture-fed cattle and 30 feedlot cattle fed for either 100, 200, or 300 d at one feedlot (I). Feed consisted of mainly sorghum with 5% whole cottonseed together with roughage. Adipose tissue samples also were collected from the same anatomical location of carcasses from 10 feedlot cattle fed for 300 d at a

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Abbreviations: CoA, coenzyme A; CSO, cottonseed oil.

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different feedlot (II). These cattle were fed a similar grain-based diet, but the ration contained a higher percentage of silage. All cattle were Angus-cross. In Experiment 2, subcutaneous fat samples were collected at two abattoirs from carcasses of feedlot cattle fed for 180 d. The animals were fed either a standard feedlot ration (control group), or a similar ration containing rumen-protected cottonseed oil (CSO) for the last 70 (Abattoir I) or 80 (Abattoir II) d of the feedlotting period before slaughter. The protected cottonseed lipid was included at 8.7% of dry matter. The grain used was dry-rolled sorghum with 6% whole cottonseed.

For both experiments, subcutaneous adipose tissue samples from the mid-loin region were collected on the slaughter floor, placed on dry ice within 30 min of stunning, and kept at −70°C until used for analyses.

Fatty acid composition. Adipose tissue (50–100 mg) was placed in 3 mL of 0.25 M sodium methoxide/diethyl ether (1:1, vol/vol) at 60°C for 2 min. After allowing to cool at room temperature, 5 mL 5% NaCl was added, and tubes were sealed and vortexed. Fatty acid methyl esters were extracted with 5 mL petroleum ether (b.p. 60–80°C). After the phases separated and the top phase became clear, a 1.5-mL gas chromatography sample bottle was filled with the top phase. The separation and distribution of fatty acid methyl esters were obtained using gas–liquid chromatography with a capillary column (CP-Sil-88, ChromPak, Middelburg, The Netherlands) as described previously (9). As *trans* 18:1 acids behave in a manner physiologically similar to saturated fatty acids and have a higher melting point than their *cis* equivalents, we included them in the calculation of total saturated fatty acids.

Desaturase activity. The method of Cameron *et al.* (10) was used with modifications. Frozen subcutaneous adipose tissue samples (~50 g) were diced and homogenized for 1 min in 2 vol of 0.02 M phosphate buffer (pH 7.4) containing 0.15 M KCl, 1 mM EDTA, and 1 mM dithiothreitol and filtered through cheesecloth into a flask. The homogenate was centrifuged at 1,500 × *g* for 10 min and filtered through glass wool. The fat-free supernate was centrifuged at 17,300 × *g* for 30 min, and the supernate was filtered through glass wool. The microsomal fraction was isolated by centrifugation of the resultant supernate at 104,000 × *g* for 60 min, and the microsomal pellet was resuspended in 1 mL cold buffer. Protein concentration was determined using the method of Bradford (11).

The reaction mixture for the measurement of desaturase assay (final volume 1.0 mL) contained 5 mM $MgCl_2$, 50 mM potassium phosphate, 5 mM ATP, 0.5 mM NADH, and 0.05 mM [1-^{14}C]palmitoyl-CoA (specific activity = 57.1 μCi/mmol) and was performed in duplicate. The reaction was initiated by the addition of 1 mg of the microsomal protein and was incubated at 37°C for 5 min before being stopped with 2 mL 10% KOH in methanol. Blanks were obtained by adding 2 mL 10% KOH in methanol to the reaction mixture prior to the addition of the microsomal protein. The tubes were then heated at 70°C for 30 min, and the solution was acidified with 6 mL 3 N HCl. Fatty acids were extracted three times with 6 mL petroleum ether, and the combined extract was washed with 6 mL acidic water (pH 3.0). The pooled upper layer was evaporated to dryness, and the fatty acids were methylated in 1 mL 5% H_2SO_4 in methanol at 60°C overnight. On the following day, 1 mL distilled water was added, and the methyl esters were extracted three times with 2 mL petroleum ether. The combined upper phase was then evaporated under nitrogen and redissolved in 100 μL petroleum ether. Aliquots of the methyl esters were separated on freshly activated thin-layer plates (silica G) impregnated with 3% $AgNO_3$, and the plates were developed in petroleum ether/diethyl ether (97:3, vol/vol) for about 30 min and sprayed with 0.02% dichlorofluorescein. Saturated and unsaturated species were identified under ultraviolet light, then cut and counted in 4 mL Opti-Fluor O (Packard, Downers Grove, IL) using a Packard Tri-Carb liquid scintillation counter (model 4530). The desaturase activity was calculated as the percentage of the counts for the unsaturated species over that for the unsaturated and saturated species and expressed as nmol palmitoleic acid formed per mg protein per min.

Source of chemicals and radioisotopes. All biochemicals were purchased from Sigma Chemical Co. (Sigma-Aldrich, Sydney, Australia). The radioisotope, [1-^{14}C]palmitoyl-CoA, was purchased from Dupont NEN (Boston, MA).

Statistical analysis. The results are expressed as mean ± SE and all data were analyzed as one-way analysis of variance (12).

RESULTS

Experiment 1—Pasture vs. grain feeding. Subcutaneous adipose tissue of pasture-fed cattle had significantly lower total saturated fatty acids and higher total unsaturated fatty acids compared with that from the 100-, 200-, or 300-d feedlot cattle, resulting in a higher unsaturated/saturated fatty acid ratio (Table 1). The adipose tissue from a 300-d fed group from another feedlot also contained significantly more saturated fatty acids than the pasture-fed group. Generally, the adipose tissue from pasture-fed cattle had lower percentages of 16:0 and 18:0 and higher percentages of 16:1, 18:1*c*9, and 18:3. These significant differences in fatty acid composition between pasture- and grain-fed cattle were supported by desaturase activities, which were 60–85% higher in pasture-fed cattle. There was no difference in either the fatty acid composition or desaturase activities among the 100-, 200-, and 300-d fed cattle from Feedlot I. Overall, adipose tissue from cattle of Feedlot II was similar to that from the groups fed in Feedlot I, except that it had a lower percentage of 18:0.

The fatty acid composition of the microsomal fraction of the adipose tissues is given in Table 2. For pasture-fed cattle, the total saturated fatty acids and unsaturation ratios for the membrane fraction were similar to those observed for whole adipose tissue (Table 1). However, the content of total monounsaturated fatty acids was lower, and there was a higher concentration of polyunsaturated fatty acids, especially 20:4. For the 300-d feedlot cattle, the total saturated fatty acids and monounsaturated fatty acids of the microsomal fraction were both significantly lower than those observed in whole subcu-

TABLE 1
Fatty Acid Composition (wt%) and Desaturase Activity of Subcutaneous Adipose Tissue from Pasture-Fed and Feedlot Cattle

	Pasture-fed[a]	Grain-fed			
		I			II
		100 d	200 d	300 d	300 d
Fatty acids	n^b = 9	n^b = 10	n^b = 10	n^b = 10	n^b = 10
14:0	3.3 ± 0.22	3.4 ± 0.21	3.3 ± 0.16	3.4 ± 0.16	2.8 ± 0.12
14:1	1.5 ± 0.20[a]	0.8 ± 0.10[b]	1.2 ± 0.10[a]	1.2 ± 0.10[a]	1.1 ± 0.12[a,b]
15:0	0.5 ± 0.03	0.7 ± 0.07	0.4 ± 0.02	0.4 ± 0.02	0.6 ± 0.02
16:0	23.4 ± 0.93[b]	26.2 ± 0.72[a]	26.3 ± 0.54[a]	26.7 ± 0.67[a]	25.2 ± 0.38[a,b]
16:1	5.5 ± 0.34[a]	3.2 ± 0.17[c]	4.1 ± 0.30[b]	3.8 ± 0.27[b,c]	4.2 ± 0.39[b]
17:0	0.9 ± 0.04	1.9 ± 0.14	1.0 ± 0.06	1.0 ± 0.04	1.4 ± 0.10
17:1	1.1 ± 0.06	1.3 ± 0.10	0.8 ± 0.03	0.8 ± 0.03	1.3 ± 0.04
18:0	11.1 ± 0.97[b,c]	13.7 ± 0.43[a]	12.5 ± 0.61[a,b]	13.3 ± 0.50[a]	10.1 ± 0.61[c]
18:1*t*11	3.1 ± 0.15[b]	2.9 ± 0.30[b]	3.7 ± 0.18[b]	3.6 ± 0.35[b]	5.3 ± 0.51[a]
18:1*c*9	43.6 ± 1.27	41.7 ± 0.86	41.7 ± 0.90	41.2 ± 0.93	42.5 ± 0.63
18:1*c*11	1.5 ± 0.15	1.3 ± 0.03	1.5 ± 0.10	1.4 ± 0.05	1.9 ± 0.06
19:0	0.6 ± 0.09	0.5 ± 0.04	0.8 ± 0.05	0.7 ± 0.02	0.8 ± 0.06
18:2	0.7 ± 0.05	1.0 ± 0.08	1.1 ± 0.08	1.2 ± 0.06	1.1 ± 0.06
18:3	0.42 ± 0.02	0.13 ± 0.01	0.13 ± 0.02	0.08 ± 0.01	0.14 ± 0.01
Saturated (S)	42.8 ± 1.72[b]	49.2 ± 0.72[a]	48.0 ± 1.24[a]	49.1 ± 1.18[a]	46.2 ± 0.89[a]
Monounsaturated (M)	53.2 ± 1.60[a]	48.2 ± 0.70[b]	49.3 ± 1.20[b]	48.3 ± 1.21[b]	51.0 ± 0.87[a,b]
Polyunsaturated (P)	1.13 ± 0.06	1.15 ± 0.08	1.21 ± 0.10	1.27 ± 0.06	1.24 ± 0.06
(M + P)/S ratio	1.30 ± 0.09[a]	1.01 ± 0.03[b]	1.06 ± 0.05[b]	1.02 ± 0.05[b]	1.14 ± 0.04[b]
M/S ratio	1.27 ± 0.09[a]	0.98 ± 0.03[b]	1.04 ± 0.05[b]	0.99 ± 0.05[b]	1.11 ± 0.04[b]
Desaturase activity[c]	1.48 ± 0.17[a]	0.82 ± 0.05[b]	0.82 ± 0.08[b]	0.83 ± 0.09[b]	0.92 ± 0.04[b]

[a]Means within the same row with the same roman superscript are not statistically different ($P > 0.05$).
[b]Number of cattle; fat tissue from each animal was analyzed in duplicate.
[c]Palmitoleic acid formed (nmol/mg protein/min).

taneous adipose tissue. The polyunsaturated fatty acids constituted up to 16.5% of the membrane fatty acids. There was no difference in the saturated/unsaturated fatty acid ratios between adipose tissues from pasture-fed and 300-d feedlot cattle, although samples from the pasture-fed cattle had significantly higher percentages of monounsaturated fatty acids and lower polyunsaturated fatty acids.

Experiment 2—Effect of protected CSO. The adipose tissue from cattle fed the protected CSO formulation contained significantly more saturated fatty acids than adipose tissue from control cattle (Table 3). Correspondingly, the unsaturated/saturated fatty acid ratios also were lower for both groups fed the protected CSO (0.92 vs. 1.18 for Abattoir I and 0.81 vs. 1.10 for Abattoir II). Feeding the protected CSO supplement caused a large increase in 18:0 and 18:2, a decrease in 18:1*c*9, and small but significant decreases in most of the other monounsaturated fatty acids. Desaturase activity was reduced by over 50% in adipose tissue from cattle fed protected CSO (Table 3).

Feeding the protected CSO significantly reduced the microsomal concentrations of 16:1, 17:1, 18:1*c*9, and 18:1*c*11, and increased concentrations of 18:0 and 18:2 in adipose tissue from cattle slaughtered at Abattoir II (Table 4). However, only 18:2 was affected significantly in microsomes from adipose tissue of cattle slaughtered in Abattoir I.

The 18:2 content of the microsomes from Experiment 2 control cattle (17.3 and 18.7%; Table 4) was approximately twice as high as that from the pasture-fed cattle in Experiment 1 (9.5%; Table 2). The 18:2 content of the microsomes from Experiment 2 control cattle also was as high as or higher than that of the 300-d feedlot cattle of Experiment 1 (14.6 and 16.5%; Table 2).

Relationship between fatty acid composition and desaturase activity. Highly significant correlations (r^2) were observed between the desaturase activity and percentage contents of a number of fatty acids in adipose tissue (Fig. 1). Desaturase activity correlated positively with the major monounsaturated fatty acids (0.35 with 14:1, 0.55 with 16:1, 0.31 with 18:1*c*9, and 0.48 with total monounsaturated fatty acids), and negatively with the major saturated fatty acids (−0.49 with 16:0, −0.34 with 18:0, and −0.49 with the total saturated fatty acids). This resulted in a significantly positive correlation (0.49) of desaturase activity with the monounsaturated/saturated fatty acid ratio (Fig. 1G).

The relationship between desaturase activity and percentage of 18:2 was not significant in adipose tissue from cattle from Experiment 1. In contrast, there was a strong negative correlation ($r^2 = -0.51$) between desaturase activity and adipose tissue 18:2 in Experiment 2 (Fig. 1E).

DISCUSSION

We recently documented that subcutaneous adipose tissue from cattle fed whole cottonseed or protected CSO contained unusually high levels of saturated fatty acids (as much as 26% 18:0) and low concentrations of monounsaturated fatty acids

TABLE 2
Fatty Acid Composition (wt%) of the Microsomal Fraction of Subcutaneous Adipose Tissue from Pasture-Fed and 300-d Feedlot Cattle (n = 8)[a]

Fatty acids	Pasture-fed	300-d Grain-fed	
		Feedlot I	Feedlot II
14:0	3.5 ± 0.39	2.8 ± 0.15	3.2 ± 0.18
14:1	0.8 ± 0.11	0.6 ± 0.07	0.7 ± 0.72
15:0	0.4 ± 0.02[a]	0.4 ± 0.02[a]	0.3 ± 0.02[b]
16:0	20.2 ± 0.46	20.4 ± 0.57	21.2 ± 0.73
16:1	4.5 ± 0.29[a]	3.7 ± 0.20[b]	3.3 ± 0.23[b]
17:0	0.7 ± 0.03[b]	1.1 ± 0.06[a]	0.7 ± 0.03[b]
17:1	0.9 ± 0.05[b]	1.1 ± 0.06[a]	0.7 ± 0.02[c]
18:0	13.6 ± 0.65	13.1 ± 0.33	14.8 ± 0.42
18:1*t*11	2.4 ± 0.19[b]	4.0 ± 0.27[a]	2.6 ± 0.23[b]
18:1*c*9	35.2 ± 0.84[a]	32.6 ± 0.57[b]	31.4 ± 0.64[b]
18:1*c*11	1.7 ± 0.13[a]	1.8 ± 0.10[a]	1.4 ± 0.07[b]
19:0	0.4 ± 0.06	0.5 ± 0.05	0.4 ± 0.04
18:2	1.5 ± 0.08[b]	2.6 ± 0.20[a]	2.9 ± 0.30[a]
20:0	0.23 ± 0.02[a]	0.13 ± 0.01[c]	0.17 ± 0.01[b]
20:1	0.4 ± 0.03[a]	0.3 ± 0.02[b]	0.2 ± 0.03[b]
18:3	0.6 ± 0.02[a]	0.2 ± 0.01[b]	0.1 ± 0.00[c]
20:3	1.6 ± 0.09[b]	3.5 ± 0.20[a]	3.6 ± 0.19[a]
20:4	5.5 ± 0.23[c]	7.5 ± 0.29[b]	9.1 ± 0.64[a]
24:0	1.1 ± 0.07[a]	0.3 ± 0.02[b]	0.2 ± 0.02[b]
24:1	0.8 ± 0.15	0.7 ± 0.07	0.7 ± 0.06
20:5	0.3 ± 0.02[b]	0.8 ± 0.04[a]	0.8 ± 0.06[a]
Saturated (S)	42.6 ± 1.02	42.6 ± 0.52	43.4 ± 0.56
Monounsaturated (M)	44.2 ± 0.97[a]	40.8 ± 0.46[b]	38.4 ± 0.77[c]
Polyunsaturated (P)	9.5 ± 0.36[b]	14.6 ± 0.49[a]	16.5 ± 1.04[a]
(M + P)/S ratio	1.27 ± 0.06	1.30 ± 0.03	1.27 ± 0.03
M/S ratio	1.05 ± 0.03[a]	0.96 ± 0.02[a,b]	0.89 ± 0.03[b]

[a]Means within the same row with the same roman superscript are not statistically different ($P > 0.05$).

(9). This caused a migration of 18:0 from the *sn*-2 triacylglycerol position to the outer *sn*-1,3 triacylglycerol positions. More importantly, the reduction in monounsaturated fatty acids suggested that dietary whole cottonseed or protected CSO increased the saturated fatty acid concentration of adipose tissue by reducing Δ^9 desaturase activity. The effect of this was a significant increase in fat hardness, judged subjectively at 10°C, and an increase in the slip point of the isolated fat (9).

Whole cottonseed and some meals contain significant quantities of cyclopropenoid fatty acids, ranging from 0.6 to 2.1% of total fatty acids in oil (13). Although cyclopropenoid fatty acids are themselves unsaturated, they have been shown to increase the saturation of adipose tissue lipids 2.5-fold when fed to rats (14). This increase in saturation is caused by the inhibition of stearoyl-CoA desaturase activity in adipose tissue (15). The effect of cyclopropenoid fatty acids on desaturase activity in cattle apparently is not as great as in nonruminants, as these acids can be hydrogenated in the rumen and lose their inhibitory effect (16). Some cycloprenoid fatty acids escape hydrogenation, and this may be a contributing factor to the higher saturation of carcass fat when feeding whole cottonseed in commercial feedlots. Similarly, feeding rumen-protected cyclopropenoid acids to ruminants led to higher ratios of stearic/oleic acids in milk, suggesting that this resulted from an inhibition of mammary Δ^9 desaturase activity (17).

In the present study we were unable to detect the presence of sterculic or malvalic acids in adipose tissues, but that may be because of relatively low contents in the feeds. We have analyzed many samples of CSO and have found the contents of total cyclopropenoic acids to be about 0.6% of the total

TABLE 3
Effect of Protected Cottonseed Oil (CSO) on Fatty Acid Composition (wt%) and Desaturase Activity of Bovine Adipose Tissue

Fatty acids	Abattoir I			Abattoir II		
	Control n^b = 10	CSO n^b = 10	Statistics[a]	Control n^b = 13	CSO n^b = 10	Statistics
14:0	3.2 ± 0.18	3.1 ± 0.16		3.1 ± 0.16	4.1 ± 0.18	***
14:1	1.4 ± 0.18	0.8 ± 0.09	***	1.0 ± 0.11	0.6 ± 0.06	*
16:0	25.4 ± 0.45	25.9 ± 0.56		26.3 ± 0.58	27.9 ± 0.67	
16:1	4.9 ± 0.61	2.8 ± 0.24	***	3.6 ± 0.32	2.1 ± 0.14	***
17:0	1.0 ± 0.07	1.1 ± 0.07		0.8 ± 0.09	0.9 ± 0.04	
17:1	1.0 ± 0.06	0.7 ± 0.05	***	0.7 ± 0.03	0.4 ± 0.02	***
18:0	11.7 ± 1.31	15.1 ± 0.91	***	12.8 ± 0.67	18.0 ± 0.67	***
18:1t11	2.4 ± 0.24	3.3 ± 0.31	*	3.2 ± 0.19	3.1 ± 0.19	
18:1c9	42.2 ± 1.29	39.5 ± 0.67	***	42.5 ± 0.99	33.0 ± 0.65	***
18:1c11	1.8 ± 0.20	1.3 ± 0.06	***	1.7 ± 0.11	1.1 ± 0.04	***
19:0	0.9 ± 0.08	0.7 ± 0.06	***	0.8 ± 0.03	0.5 ± 0.02	***
18:2	1.5 ± 0.14	4.1 ± 0.34	***	1.6 ± 0.11	7.1 ± 0.39	***
18:3	0.2 ± 0.01	0.1 ± 0.01	***	0.1 ± 0.01	0.1 ± 0.01	
Saturated (S)	45.2 ± 1.95	49.5 ± 0.75	***	47.4 ± 1.23	54.7 ± 0.51	***
Monounsaturated (M)	51.3 ± 2.05	45.0 ± 0.92	***	49.5 ± 1.25	37.2 ± 0.69	***
Polyunsaturated (P)	1.6 ± 0.15	4.2 ± 0.33	***	1.7 ± 0.12	7.2 ± 0.39	***
(M + P)/S ratio	1.18 ± 0.10	0.92 ± 0.03	***	1.10 ± 0.05	0.81 ± 0.02	***
M/S ratio	1.17 ± 0.10	0.91 ± 0.03	***	1.06 ± 0.05	0.68 ± 0.02	***
Desaturase activity[c]	1.44 ± 0.12	0.62 ± 0.55	***	0.83 ± 0.07	0.40 ± 0.04	***

[a] *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
[b]Number of cattle; fat tissue from each animal was analyzed in duplicate.
[c]Palmitoleic acid formed (nmol/mg protein/min).

TABLE 4
Fatty Acid Composition (wt%) of the Microsomal Preparation from Bovine Subcutaneous Adipose Tissue from Cattle Fed Diets with or without Protected Cottonseed Oil (CSO)

	Abattoir I			Abattoir II		
Fatty acids	Control $n^b = 9$	CSO $n^b = 8$	Statistics[a]	Control $n^b = 6$	CSO $n^b = 6$	Statistics
14:0	5.4 ± 1.21	5.1 ± 1.44		1.4 ± 0.26	1.7 ± 0.38	
14:1	0.4 ± 0.09	0.3 ± 0.09		0.2 ± 0.08	0.1 ± 0.05	
16:0	15.6 ± 0.55	17.3 ± 0.73		15.9 ± 0.77	17.9 ± 1.05	
16:1	3.2 ± 0.32	2.4 ± 0.26		2.2 ± 0.27	1.2 ± 0.24	*
17:0	0.5 ± 0.11	0.6 ± 0.09		0.6 ± 0.08	0.5 ± 0.11	
17:1	0.7 ± 0.11	0.6 ± 0.05		0.6 ± 0.04	0.2 ± 0.07	***
18:0	16.4 ± 0.78	15.9 ± 0.73		15.9 ± 0.51	22.3 ± 0.94	***
18:1*t*11	1.8 ± 0.18	1.7 ± 0.11		2.2 ± 0.22	1.9 ± 0.21	
18:1*c*9	33.3 ± 2.08	32.3 ± 1.30		36.8 ± 1.14	24.4 ± 0.95	***
18:1*c*11	1.4 ± 0.13	1.2 ± 0.09		1.1 ± 0.05	0.5 ± 0.13	**
18:2	4.2 ± 0.37	8.0 ± 0.72	***	4.3 ± 0.31	12.1 ± 0.71	***
18:3	0.7 ± 0.10	0.5 ± 0.09		0.4 ± 0.17	0.2 ± 0.07	
20:3	4.6 ± 0.55	4.1 ± 0.24		5.0 ± 0.28	4.5 ± 0.38	
20:4	7.5 ± 0.76	7.5 ± 0.92		8.9 ± 0.63	9.3 ± 0.85	
20:5	0.4 ± 0.13	0.1 ± 0.05		0.1 ± 0.05	0	
Saturated (S)	39.7 ± 1.14	40.5 ± 1.55		35.9 ± 0.81	44.3 ± 0.92	***
Monounsaturated (M)	39.1 ± 2.15	36.7 ± 1.62		40.9 ± 1.24	26.4 ± 1.30	***
Polyunsaturated (P)	17.3 ± 1.48	20.2 ± 1.82		18.7 ± 0.54	26.0 ± 1.70	**
(M + P)/S ratio	1.44 ± 0.08	1.43 ± 0.08		1.66 ± 0.07	1.19 ± 0.04	***
M/S ratio	1.00 ± 0.07	0.92 ± 0.07		0.68 ± 0.05	1.06 ± 0.04	***

[a] $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.
[b] Number of cattle; fat tissue from each animal was analyzed in duplicate.

fatty acids present. In view of this, it may not be expected that we could detect these acids in adipose tissue. Where cyclopropenoic acids were detected in adipose tissues of rats, it was fed at subacute levels (14).

Feeding formaldehyde-protected fatty acid supplements such as CSO has been reported to improve growth performance and to alter the fat composition of cattle quite dramatically (18). CSO is highly unsaturated, having more than 50% 18:2 (19), and high percentages of 18:2 (up to 10%) were found in adipose tissue of cattle fed protected CSO (18). As significant amounts of dietary 18:2 were deposited in fat tissues, clearly some portion of these polyunsaturated fatty acids were protected from hydrogenation in the rumen. This was particularly obvious in samples from Abattoir II, where the cattle were on protected CSO ration for 10 d longer, resulting in more 18:2 being deposited in adipose tissue than in samples from Abattoir I. There was a highly significant negative correlation between the stearoyl-CoA desaturase activity and 18:2 in this experiment ($r^2 = -0.51$), which was not observed in Experiment 1.

There are a number of reports demonstrating that certain polyunsaturated fatty acids, including 18:2, repress stearoyl-CoA desaturase activity in adipocytes by depressing expression of stearoyl-CoA desaturase gene 1 (20,21). Waters and Ntambi (22) demonstrated that 18:2 inhibits stearoyl-CoA desaturase gene 1 transcription in hepatic tissue, possibly by elevating the prostaglandin $F_{2\alpha}$ concentration as reported in adipose tissue (23). Alternatively, enriching the microsomal membranes with 18:2 may cause a conformational change in stearoyl-CoA desaturase and a consequent loss in activity.

Gulati *et al.* (18) and Ashes *et al.* (24) recently have drawn attention to the observation that differences in fatty acid composition result from feeding protected canola and protected cottonseeds. When oilseeds such as sunflower seed meal were fed in protected form (18), the content of 18:2 in subcutaneous adipose tissue increased from 1.5% to 5.3%, but there was no significant effect on 18:0, which remained about 14%. When protected CSO was fed to cattle (18), there was a significant increase in the percentage of 18:0; the percentage of 18:2 increased much more than when unprotected plant oils were fed. Results of an early study in cattle (25), which also employed the feeding of protected 18:2 (in the absence of cyclopropenoic acids) demonstrated that, whereas feeding protected 18:2 substantially increased the concentration of 18:2 in adipose tissue, it did not alter the monounsaturated/saturated fatty acid ratio.

On the basis of the present work, we were unable to conclude that the reduction in desaturase activity was due entirely to cyclopropenoid fatty acids contained in whole cottonseed and in protected CSO. In fact, comparison of Experiments 1 and 2 suggests that 18:2 content, especially in the microsomal fraction, contributed to the reduction in Δ^9 desaturase activity. The highest desaturase activity and monounsaturated/saturated fatty acid ratio and lowest microsomal 18:2 concentration were observed in adipose tissue from the pasture-fed cattle of Experiment 1. Adipose tissue microsomes from the control cattle of Experiment 2 contained substantially more 18:2 than microsomes from either the pasture-fed or the feedlot cattle of Experiment 1, and desaturase activities

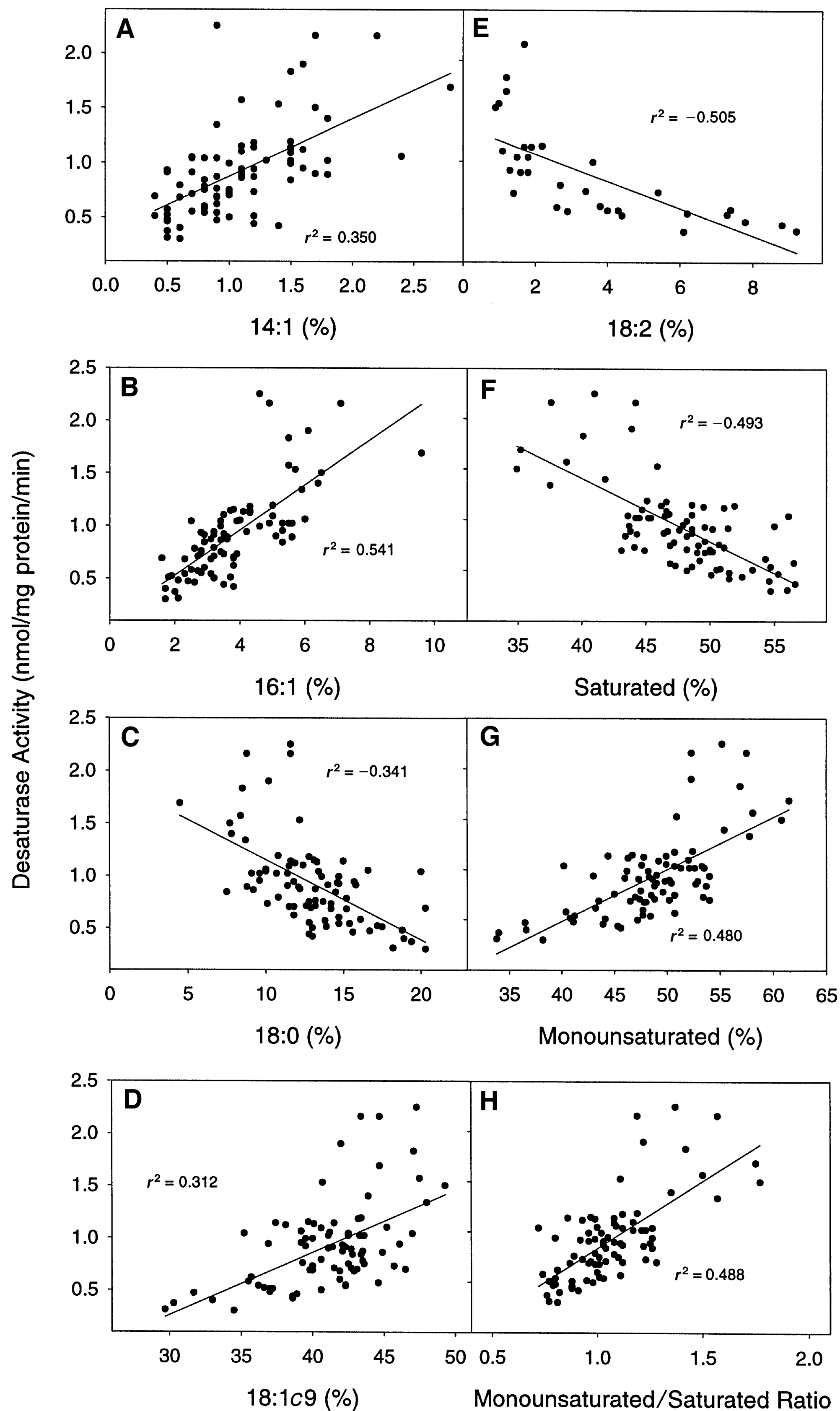

FIG. 1. Relationship between the desaturase activity and (A) 14:1, (B) 16:1, (C) 18:0, (D) 18:1*c*9, (E) 18:2, (F) total saturated fatty acids, (G) total monounsaturated fatty acids, and (H) the ratio between the monounsaturated and saturated fatty acids [n = 82 for all except (E) where n = 32].

and monounsaturated/saturated fatty acid ratios typically were lower in adipose tissue samples from Experiment 2.

If cyclopropenoids in whole cottonseed are reducing desaturase activity, then this phenomenon may be dependent upon the variety of whole cottonseed used for feeding. In experiments in the United States, feeding cattle up to 30% whole cottonseed for 54 d did not increase the concentration of 18:0, nor did it alter the monounsaturated/saturated fatty acid ratio (26). Furthermore, in other experiments in the United States, feeding 30% whole cottonseed for 180 d had no effect on stearoyl-CoA desaturase activity in subcutaneous adipose tissue, although it reduced the rate of lipogenesis from acetate by 30% compared to rates measured in adipose tissue from control cattle (27). The results of the present investigation indicate that feeding cattle for as little as 100 d was sufficient to elicit maximal inhibition of desaturase activity. Clearly there is a need to determine the content of cyclopropenoic fatty acids in the commercial cultivars of whole cottonseed used for feed. Also to be considered is that, in the United States, most of the metabolizable energy in finishing diets for cattle is derived either from corn or sorghum. In Australia, wheat, barley, and sorghum are fed in place of corn. Therefore, future studies will be designed to document the interaction between whole cottonseed and grain type on desaturase activity and fatty acid composition of bovine adipose tissue.

In summary, in this work we have demonstrated that feeding whole cottonseed or protected CSO to cattle increases the saturated fatty acid concentration of adipose tissue by reducing the activity of stearoyl-CoA desaturase. Whether this repression of activity resulted from direct inhibition by cyclopropenoic acids or by higher contents of 18:2 needs to be determined unequivocally. In addition, practices of feeding whole cottonseed or protected CSO need to be evaluated, and the consequent biochemical outcomes determined so as to avoid the problem of hard fat in beef carcasses.

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METHOD

Lipase-Catalyzed Fractionation of Conjugated Linoleic Acid Isomers

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ABSTRACT: The abilities of lipases produced by the fungus *Geotrichum candidum* to selectively fractionate mixtures of conjugated linoleic acid (CLA) isomers during esterification of mixed CLA free fatty acids and during hydrolysis of mixed CLA methyl esters were examined. The enzymes were highly selective for *cis*-9,*trans*-11-18:2. A commercial CLA methyl ester preparation, containing at least 12 species representing four positional CLA isomers, was incubated in aqueous solution with either a commercial *G. candidum* lipase preparation (Amano GC-4) or lipase produced from a cloned high-selectivity *G. candidum* lipase B gene. In both instances selective hydrolysis of the *cis-9,trans*-11-18:2 methyl ester occurred, with negligible hydrolysis of other CLA isomers. The content of *cis*-9,*trans*-11-18:2 in the resulting free fatty acid fraction was between 94 (lipase B reaction) and 77% (GC-4 reaction). The commercial CLA mixture contained only trace amounts of *trans*-9,*cis*-11-18:2, and there was no evidence that this isomer was hydrolyzed by the enzyme. Analogous results were obtained with these enzymes in the esterification in organic solvent of a commercial preparation of CLA free fatty acids containing at least 12 CLA isomers. In this case, *G. candidum* lipase B generated a methyl ester fraction that contained >98% *cis*-9,*trans*-11-18:2. *Geotrichum candidum* lipases B and GC-4 also demonstrated high selectivity in the esterification of CLA with ethanol, generating ethyl ester fractions containing 96 and 80%, respectively, of the *cis*-9,*trans*-11 isomer. In a second set of experiments, CLA synthesized from pure linoleic acid, composed essentially of two isomers, *cis*-9,*trans*-11 and *trans*-10,*cis*-12, was utilized. This was subjected to esterification with octanol in an aqueous reaction system using Amano GC-4 lipase as catalyst. The resulting ester fraction contained up to 97% of the *cis*-9,*trans*-11 isomer. After adjustment of the reaction conditions, a concentration of 85% *trans*-10,*cis*-12-18:2 could be obtained in the unreacted free fatty acid fraction. These lipase-catalyzed reactions provide a means for the preparative-scale production of high-purity *cis*-9,*trans*-11-18:2, and a corresponding CLA fraction depleted of this isomer.

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The identification of dietary components that have the potential to reduce the incidence of cancer is a topic of considerable interest to the research and medical communities and the general population. Pariza and colleagues identified a lipid fraction from beef fat that possessed anticarcinogenic properties and demonstrated that it was composed of conjugated double-bond isomers (designated CLA) of *cis*-9,*cis*-12-octadecadienoic (18:2) acid (linoleic acid) (1). It was shown that this fraction was similar to the CLA isomer mixture produced under some conditions by alkali isomerization of linoleic acid (2). Subsequent studies with animal models showed that consumption of CLA reduced body fat content and increased muscle mass (3–5), decreased atherosclerosis (6,7), and improved hyperinsulinemia in prediabetic rats (8), all of which further heightened interest in these compounds.

The richest natural dietary sources of CLA are the meat and milk of ruminants and products made from them. In these materials the predominant isomer is *cis*-9,*trans*-11-18:2, which constitutes over 75% of the total CLA (3,9). CLA can also be synthesized by alkali isomerization from linoleic acid (all-*cis*), resulting in either two (9,11- and 10,12-18:2) or four (8,10-, 9,11-, 10,12-, and 11,13-18:2) *cis/trans* positional isomers depending on the temperature of the reaction (10). In the predominantly two-isomer mixture, each isomer constituted about 40–42% of the product (11,12), while a commercially available four-isomer mixture contained the four positional *cis/trans* isomers at about 20% each, with smaller amounts of the corresponding *trans,trans* and *cis,cis* forms (12).

Not all isomers of CLA have comparable physiological effects, and much remains to be determined about the activities of the individual isomers. The *cis*-9,*trans*-11-18:2 isomer is believed to be the principal isomer responsible for the anticarcinogenic effects of CLA mixtures (13). A CLA isomer other than *cis*-9,*trans*-11-18:2 was shown recently to suppress liver stearyl-CoA desaturase mRNA expression (14), whereas the *trans*-10,*cis*-12 isomer has very recently been shown to regu-

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Abbreviations: Ag^+–HPLC, silver-ion–high-performance liquid chromatography; CLA, conjugated linoleic acid; DPG, diphosphatidylglycerol; FAME, fatty acid methyl ester; FFA, free fatty acid; GC, gas chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

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late body composition in mice (15) and to influence serum lipid composition and body fat content in hamsters (16). It has not yet been established which CLA isomers are responsible for the antidiabetic and antiatherogenic effects reported for this family of molecules. Furthermore, the CLA isomers have been shown to be incorporated differently into tissue phospholipids of pigs fed a CLA mixture (17). The *cis*-11,*trans*-13-18:2 isomer was found preferentially incorporated into all heart lipids of the animals, particularly diphosphatidylglycerol (DPG). On the other hand, *cis*-9,*trans*-11-18:2 was preferentially incorporated into all liver lipids, with the exception of DPG, which incorporated a higher amount of the 11,13-18:2 isomer. DPG is a unique component of the inner mitochondrial membrane. These observations raise questions as to the effect of the 11,13 CLA isomer on the enzymes of oxidative phosphorylation. These studies indicate the need for evaluating each individual CLA isomer, which requires the preparation of larger amounts of enriched or purified CLA isomers.

To prepare sufficient amounts of some isomers for dietary supplementation, synthesis from methyl 12-hydroxyoctadec-*cis*-9-enoate (ricinoleate) was proposed (18). However, large-scale implementation of this method may be difficult. Thus, there is interest in the further development of methods for the fractionation of CLA mixtures.

Lipases (EC 3.1.1.3) are enzymes that hydrolyze or synthesize fatty acid esters and related compounds, the direction of the reaction being largely dependent on the water activity of the system. Depending on biological origin, lipases exhibit varying degrees of selectivity with regard to the alcohols or fatty acids upon which they will react. Such selectivities have been used for the selective enrichment of eicosapentaenoic (20:5) and docosahexaenoic (22:6) acids (19,20) and γ-linolenic acid (21). Enzyme-catalyzed fatty acid interchange reactions to alter the fatty acid content of glycerides have been conducted at the industrial scale for years (22), thus establishing the feasibility of large-scale enzymatic modification of fats and oils.

Lipases have recently been used to enrich triglycerides with CLA obtained by alkali isomerization of linoleic acid (23,24). Up to 70% of the CLA was incorporated into mono-, di-, and triacylglycerols (23). The isomeric content of the CLA used in those experiments was determined by a gas chromatographic method (2) incapable of resolving all CLA isomers (12,17). It is therefore not known if the synthetic mixture was a two- or a four-positional isomer mixture, and if the lipases used showed any selectivity toward any isomer. It would appear that the lipases investigated showed little selectivity, since the reported degree of CLA esterification exceeded the *cis*-9 content of either a typical two-isomer (41%) or four-isomer (29%) CLA mixture (12). If CLA-enriched lipid products are intended for experimental or general food use, it could be advantageous to enrich them with biologically efficacious CLA isomers.

Substrate-selective lipases are known, and among the premier examples of these are lipases produced by some fungi of the genus *Geotrichum*. Depending on the isolate and isozyme examined, these enzymes display various degrees of selectivity for fatty acids possessing a *cis*-9 double bond (25–29). We have examined the abilities of preparations of *G. candidum* lipase to distinguish between the various CLA isomers. Both the enzymatic esterification of mixtures of CLA as free fatty acids (FFA) with alcohols to form simple esters and the hydrolysis of mixed CLA esters were investigated in this study.

MATERIALS AND METHODS

Reagents and enzymes. The free fatty acid (#UC-59-A) and methyl ester (#UC-59-M) forms of mixed CLA isomers were obtained from Nu-Chek-Prep (Elysian, MN). Olive oil and polyvinylpyrrolidone were obtained from Sigma (St. Louis, MO). Linoleic acid (>95% purity) was obtained from Roche Products (Basel, Switzerland). *Geotrichum candidum* lipase preparation GC-4 was obtained from Amano Pharmaceutical (Nagoya, Japan). A lipase preparation obtained by expressing the highly *cis*-9 selective B lipase gene of *G. candidum* CMICC 335426 (29) in a *Pichia pastoris* expression system (30) was graciously provided by Drs. R. Schmid and U. Bornscheuer of the Institut für Technische Biochemie, University of Stuttgart (Stuttgart, Germany). Solvents and alcohols used in enzymatic reactions were dried over molecular sieves prior to use. All solvents were distilled-in-glass quality. A solution of (trimethylsilyl)diazomethane in hexane (2 M) was obtained from Aldrich Chemical Co. Inc. (Milwaukee, WI).

Laboratory preparation of CLA. The method was based on the procedure described by Ip *et al.* (31) as follows: 50 g of >95% pure linoleic acid was added to a solution of 15 g NaOH and 290 g ethylene glycol. The mixture was heated for 2 h under nitrogen at 180°C. The reaction mixture was cooled and after neutralization with HCl the fatty acids were extracted twice with hexane. The hexane was subsequently removed by rotary evaporation. The product contained 92% CLA of which 49.7% was the *cis*-9,*trans*-11 isomer and 50.3% was the *trans*-10,*cis*-12 isomer according to gas chromatography (GC) analysis. Traces of other CLA isomers were observed on gas chromatograms, but owing to their low concentrations were not considered for the purposes of this study.

Enzyme characterization and immobilization. Lipase activities were measured titrimetrically at 35°C using a continuous titrating pH meter and 10 mM NaOH to determine the rate of fatty acid release from emulsified olive oil in aqueous solution (32). Activities were determined at the pH values of maximal activity: pH 7.0 for *G. candidum* GC-4 (manufacturer's recommendation), and pH 8.5 for the lipase B preparation (30). One Olive Oil Unit of activity released 1 meq of FFA from emulsified olive oil per minute. The *G. candidum* B lipase preparation contained 1583 Olive Oil U/g, and the GC-4 preparation had an activity of 3556 Olive Oil U/g.

For use in organic solvents, *G. candidum* lipases (4000 U of lipase B or 9000 U of GC-4) were dissolved in 26 mL of 0.05 M Tris-HCl (pH 8.5 for lipase B, pH 7.2 for GC-4) and deposited by evaporation onto 10 g of Celite 545 (Fisher Scientific, Fair Lawn, NJ) (33).

Lipase-catalyzed hydrolysis of CLA methyl ester mixtures. Nonimmobilized *G. candidum* lipase B (4.5 U) or GC-4 (10.0 U) lipase was incubated under nitrogen with stirring at 35°C in 4 mL of emulsified substrate consisting of commercial CLA methyl ester (190 mM), $CaCl_2$ (15 mM) and polyvinylpyrrolidone (0.5%, wt/vol) in 0.1 M Tris-HCl, pH 8.5. At selected times, 0.5-mL aliquots were removed and extracted with 2.5 mL of hexane/isopropanol/acetic acid (57:38:5, by vol). To calculate degrees of hydrolysis, the amounts of FFA and fatty acid methyl ester (FAME) in the organic phase were determined by high-performance liquid chromatography (HPLC) using a modification of the method of El-Hamdy and Christie (34) that employed a Chrompack (Raritan, NJ) Spherisorb CN column (3 mm diameter × 10 cm, 5-mm particle size) connected to an evaporative light scattering detector (ELSD IIA; Alltech Associates, Inc., Deerfield, IL) and developed isocratically with hexane/methyl *tert*-butyl ether (98:2, vol/vol). Peak areas were quantitated by reference to standard curves for linoleic acid or methyl linoleate, as appropriate. To speciate the CLA isomers, the products of lipase action were isolated, the FFA fraction was methylated, and the isomers in each fraction were identified as described below in the section on separation and derivatization of products of lipolysis.

Lipase-catalyzed esterification of CLA FFA mixtures. Seventy milligrams (0.25 mmol) of commercial mixed CLA FFA, 0.25 mmol of methanol or ethanol, and 15 mg of immobilized *G. candidum* lipase were added to 1 mL organic solvent containing 0.02% water (vol/vol). The mixture was capped and incubated with mixing for 22.5 h at 30°C. The solvent was removed under N_2, the residue was resuspended in 4 mL hexane/isopropanol/acetic acid (57:38:5, by vol), and enzyme was removed by filtration through polytetrafluoroethylene filters. The FFA and FAME fractions were separated, the FFA fraction was methylated, and CLA isomer contents were determined as described in the next parargaph.

Separation and derivatization of the products of lipolysis. The lipase reaction product in the organic phase was concentrated with a stream of N_2 and separated into its FFA and FAME fractions by thin-layer chromatography (TLC) on Silica Gel G plates (20 × 20 cm, 250 µm thickness; Analtech, Newark, DE) developed with hexane/diethyl ether/acetic acid (80:20:1, by vol). The FFA and FAME bands were visualized under ultraviolet light after spraying the TLC plates with a solution of 2′,7′-dichlorofluorescein in methanol. The acid and ester bands were scraped off the plate and separately eluted with chloroform. The FFA samples were dried under a stream of N_2 and methylated in a screw-cap test tube with a Teflon liner by adding 2 mL of benzene/methanol (4:1, vol/vol) and 1 mL of (trimethylsilyl)diazomethane (35). The reaction was carried out at room temperature for 15 min, during which time the test tube was shaken occasionally. Unreacted (trimethylsilyl)diazomethane was destroyed by adding acetic acid until the yellow color disappeared. After addition of 1.6 mL of methanol and 0.1 mL of water, the FAME were extracted with 2 mL of hexane. The FAME fraction and the methylated FFA fraction were analyzed by silver-ion–HPLC (Ag^+–HPLC) as described below, and by GC as previously described (17).

Selective esterification of laboratory-prepared CLA with octanol using Amano GC-4 lipase preparation. Approximately 2.8 g of 1-octanol was mixed with 6 g of laboratory-prepared CLA isomers to give a mole ratio of 1:1. To this was added a solution of 60 mg of Amano GC-4 lipase powder in 18 mL of distilled water. No organic solvents were used. The reaction mixture was agitated magnetically at 25°C. Samples of the oil phase were periodically removed, and the course of the reaction monitored by titration of the FFA with dilute HCl. Octyl esters were separated from unreacted fatty acids by TLC as described above, and the CLA composition determined by GC as described below.

Analysis of laboratory-prepared CLA by GC. FFA were converted to methyl esters by reaction with methanolic HCl. Up to 100 mg of sample was transferred into a 15-mL screw-capped glass vial and dissolved in 4–5 mL of 5% (vol/vol) HCl in methanol and reacted on a hot block at 70°C for 2 min to minimize artifact formation. After cooling, 2 mL of isooctane and 5 mL of water were added. The upper isooctane layer was removed, dried over anhydrous sodium sulfate, and the concentration adjusted for GC analysis.

Octyl esters were transesterified to methyl esters by reaction with sodium methoxide/methanol. The sample (10–20 mg) was transferred to a 15-mL screw-capped glass vial. Two milliliters of 0.5 N sodium methoxide in methanol reagent was added and the solution reacted for 5 min at 70°C.

Methyl ester derivatives of octyl CLA were analyzed on a 50 m, 0.25 mm i.d., 0.2 µm film, Chrompack CP-Sil 88 capillary GC column using helium as the carrier gas. The column oven was held at 120°C for 1 min, then raised to 160°C at 20°C/min, 160–180°C at 0.5°C/min, and finally 180–230°C at 3°C/min. A Perkin Elmer (Norwalk, CT) 8420 GC system fitted with programmable temperature vaporizing (PTV) injection in split injection mode, an AS8300 autosampler, and a flame-ionization detector (FID) was used. The PTV injector was held at 50°C for 0.1 min during injection, then raised to 260°C for 3.5 min for transfer of the sample into the column oven zone. Injection volumes (0.5 µL) of 10–25 mg/mL solutions were used, with split ratios adjusted for optimal column loading. The FID detector temperature was set at 260°C. The total run time was 60 min.

Ag^+–HPLC. The identity and content of CLA isomers in the fatty acid ester and FFA (after conversion to FAME) fractions were determined by HPLC using three ChromSpher 5 Lipids (4.6 mm i.d. × 25 cm, 5-mm particle size) Ag^+–HPLC columns (Chrompack) in series (36). The columns were operated isocratically at a flow rate of 1.1 mL/min with a mobile phase of 0.1% (vol/vol) acetonitrile in hexane, freshly prepared daily. CLA esters were detected by monitoring absorbance at 233 nm, where conjugated dienes absorb strongly. The identities of the various CLA isomers were established by combined GC–electron impact mass spectrometry and GC–Fourier transform infrared spectrometry, and by comparison to CLA standards (12).

RESULTS AND DISCUSSION

Hydrolysis of CLA esters by G. candidum *lipase B*. The commercial methyl ester preparation of CLA was resolved into 12 CLA peaks using three Ag+–HPLC columns in series (Fig. 1A). These peaks consisted of four positional CLA isomers of each of the geometric *trans,trans*, *cis/trans,* and *cis,cis* isomers. The *cis/trans* geometric isomers of commercial CLA mixtures were previously identified, in the order of their elution under the HPLC conditions used, as *cis*-11,*trans*-13-18:2, *trans*-10,*cis*-12-18:2, *cis*-9,*trans*-11-18:2, and *trans*-8,*cis*-10-18:2 (12,36). In using three Ag+–HPLC columns in series as described here, it was not possible to cleanly resolve the opposite *cis/trans* isomers (e.g., *cis*-8,*trans*-10, *trans*-9,*cis*-11, etc.), given the vast excesses of their partner isomers. These opposite isomers have previously been shown to constitute but a very minor fraction of the commercial CLA employed here (12,36). On the basis of relative peak areas of the detector chromatogram, the *cis*-9,*trans*-11 CLA isomer constituted 34% of the total CLA content.

Representative Ag+–HPLC separations of the CLA methyl ester fraction remaining after 30 min and after 4 h of hydrolysis by *G. candidum* lipase B are shown in Figures 1B and 1C. A progressive reduction of the proportion of *cis*-9,*trans*-11-18:2 isomer to about 20% after 30 min, and 8% after 4 h, occurred (Table 1). The overall extent of hydrolysis after 4 h of reaction was 20%. There were no significant reductions in the normalized peak areas of any of the other components of the mixed CLA isomer ester mixture during incubation with lipase. The resultant FFA fractions contained 95% *cis*-9,*trans*-11-18:2 FFA after 30 min and 94% after 4 to 6 h (Table 1). The Ag+–HPLC separation of the FFA fraction produced during hydrolysis is shown in Figure 2A. Besides the major *cis*-9,*trans*-11-18:2 isomer, there were small amounts of *cis*-9,*cis*-11-18:2 and *trans*-10,*cis*-12-18:2, and trace amounts of *trans*-9,*trans*-11-18:2, *cis*-11,*trans*-13-18:2, and *trans*-8,*cis*-10:18:2 (Fig. 2A). By optimization of reaction conditions it may be possible to further reduce the levels of *cis*-9-CLA isomers in the ester mixture.

It was of interest to determine if *G. candidum* lipase B removed primarily the *cis*-9,*trans*-11-18:2 from the commercial

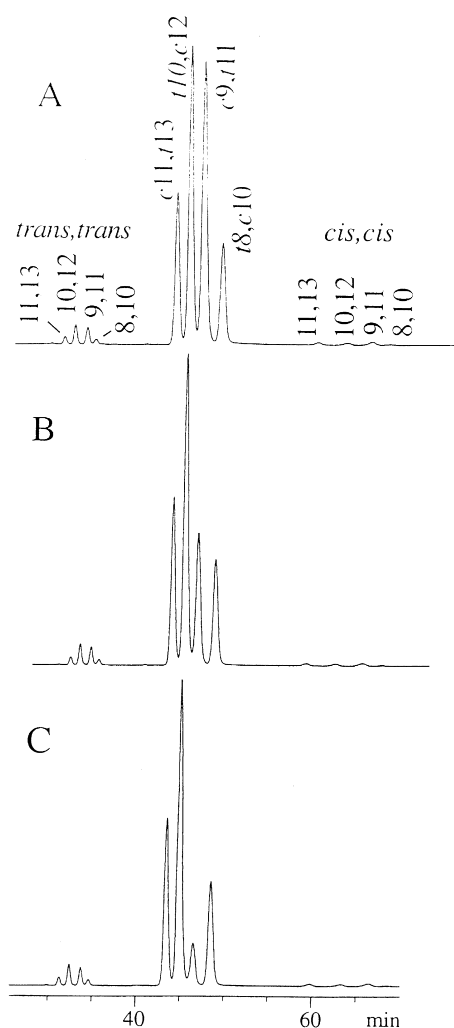

FIG. 1. Selective hydrolysis of a commercial mixture of conjugated linoleic acid (CLA) methyl esters by *Geotrichum candidum* lipase B, analyzed by silver ion–high-performance liquid chromatography (Ag+–HPLC) using three columns in series. (A) Composition of the CLA methyl ester mixture before incubation with lipase. Peak identities are indicated. (B) Composition of the methyl ester fraction following 30 min incubation. (C) Composition of the methyl ester fraction after 4 h incubation.

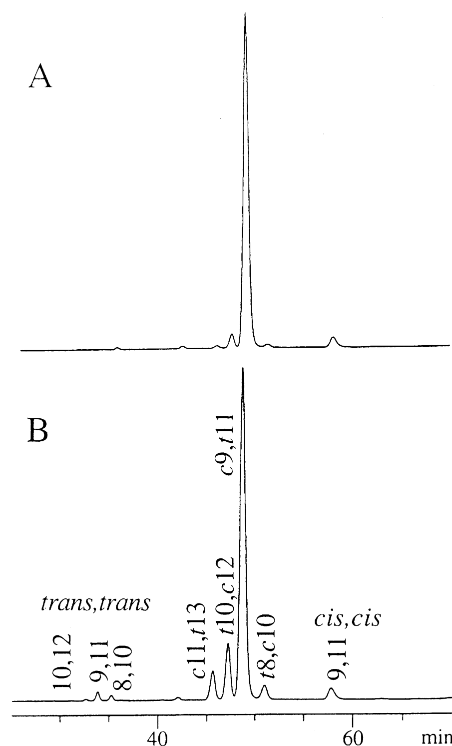

FIG. 2. Ag+–HPLC analysis, using three Ag+–HPLC columns in series, of free fatty acid (FFA) fractions generated by hydrolysis of mixed CLA isomer methyl esters (as shown in Fig. 1A) with (A) *G. candidum* lipase B, or (B) *G. candidum* GC-4 lipase preparation. Peak identities are indicated. For abbreviations see Figure 1.

TABLE 1
Levels (%) of *cis*-9,*trans*-11-18:2 in the Ester and Free Fatty Acid Fractions Following Hydrolysis of a Mixed Isomer CLA Methyl Ester Preparation by *Geotrichum candidum* Lipases, as Determined by Ag^+–HPLC

	Ester fraction			Free fatty acid fraction		
Incubation time (h)	Lipase GC-4	Lipase B[a]	Lipase B[a]	Lipase GC-4	Lipase B[a]	Lipase B[a]
0	34	34	34	0	0	0
0.5	25	19	21	82	95	96
4	17	7	9	77	94	94
6	N.D.[b]	N.D.[b]	8	N.D.[b]	N.D.[b]	94

[a]Results of representative independent experiments.
[b]N.D., not determined; CLA, conjugated linoleic acid; Ag^+–HPLC, silver-ion–high-performance liquid chromatography.

CLA FAME mixture in 4 h, leaving the ester of the opposite geometric isomer *(trans*-9,*cis*-11-18:2) unhydrolyzed, or if some *trans*-9,*cis*-11-18:2 ester was also hydrolyzed by the enzyme. GC is able to resolve the *cis/trans*-9,11 isomers of CLA. The results of GC analysis of the ester and FFA fractions obtained after CLA FAME hydrolysis by *G. candidum* lipase B are shown in Figure 3. The resolution, obtained using a 100 m CP-Sil 88 (Chrompack) fused-silica capillary column, was similar to that described previously (17). GC did not resolve the *cis/trans* CLA isomers *cis*-9,*trans*-11 and *trans*-8,*cis*-10. However, the *trans*-9,*cis*-11, *cis*-11,*trans*-13, and *trans*-10,*cis*-12 isomers were separated. All four positional *cis,cis* isomers were separated in the order 8,10-, 9,11-, 10,12-, and 11,13-18:2. The four positional *trans,trans* isomers were separated as two peaks: 11,13-18:2 followed by an unresolved mixture of 8,10-, 9,11-, and 10,12-18:2. By GC, only a trace peak was found corresponding to *trans*-9,*cis*-11-18:2 in the original CLA FAME mixture (Fig. 3A), and this peak remained in the ester fraction following incubation with lipase (Fig. 3B). None of this CLA isomer was detected in the FFA fraction generated during a 4 h incubation with *G. candidum* lipase B (Fig. 3C).

Further evidence for the lack of hydrolytic activity against the ester of *trans*-9,*cis*-11-18:2 was provided by Ag^+–HPLC. While a single ChromSpher 5 Lipids column did not resolve the geometric pair of 9,11 *cis/trans* CLA isomers (12), three Ag^+–HPLC columns in series just barely separated this pair (36), and six columns in series resulted in clear resolution (37). Analysis of the remaining FAME of the commercial CLA mixture after 4 h of incubation with *G. candidum* lipase B using six columns in series showed that the 9,11-18:2 peak was residual *cis*-9,*trans*-11-18:2 isomer (Fig. 4). There was no evidence of the opposite isomer. Therefore, by a combination of GC and Ag^+–HPLC it was possible to identify the CLA isomers by their relative elution order, and to characterize the specificity of the *G. candidum* lipase. The trace components were not confirmed by GC–mass spectrometry in this study.

Upon inspection of Figures 1 and 2 it is tempting to speculate that the *cis*-9,*cis*-11 isomer is also relatively enriched in the FFA fraction during hydrolysis by the *G. candidum* lipases. This would be expected on the basis of the known selectivity of the enzymes. However, owing to the very low levels of this isomer in the starting material and product, it is not possible to provide quantitative data.

The prominent enrichment of the *cis*-9,*trans*-11 isomer in the FFA fraction indicates that *G. candidum* lipase B exhibited high selectivity for *cis*-9 unsaturates in the hydrolysis of the methyl esters of mixed CLA isomers. The activity and degree of selectivity for *cis*-9,*trans*-11 CLA during hydrolysis were reproducible, as illustrated by the similar contents of this

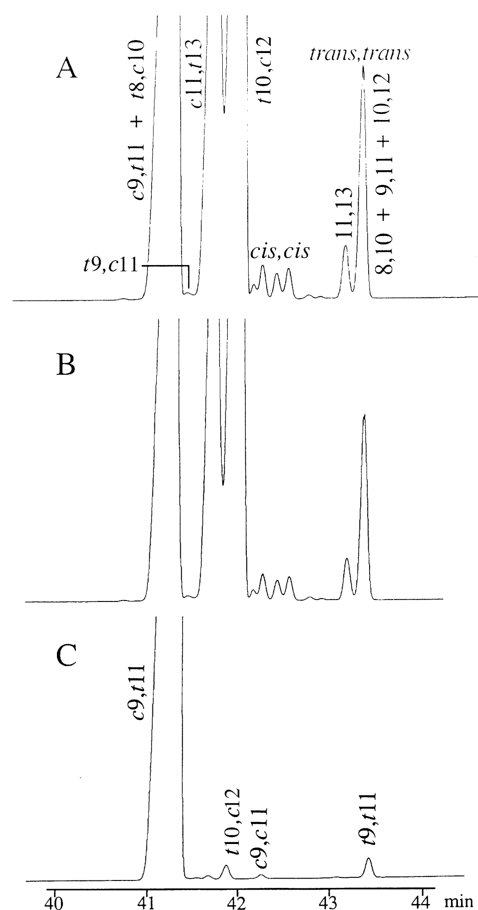

FIG. 3. Partial gas chromatograms of CLA methyl ester hydrolysis by *G. candidum* lipase B using a 100-m CP Sil 88 column. (A) Composition of the CLA methyl ester mixture before incubation with lipase. Composition of the remaining CLA methyl esters (B) and the FFA released by the lipase (C) after 4 h incubation. Peak identities are indicated. For abbreviations see Figures 1 and 2.

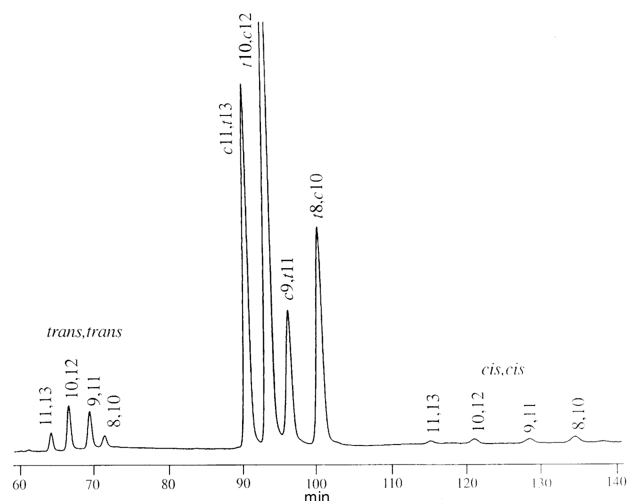

FIG. 4. Ag^+–HPLC separation, using six Ag^+–HPLC columns in series, of the fatty acid esters remaining after a 4 h incubation of a commercial mixture of CLA methyl esters with *G. candidum* lipase B. The *cis/trans* pairs of the 9,11- and 11,13-CLA isomers separate under these conditions (37). The 9,11-18:2 CLA isomer peak of this remaining ester fraction was *cis*-9,*trans*-11-18:2, with no detectable *trans*-9,*cis*-11-18:2. For abbreviations see Figures 1 and 2.

isomer in replicate reactions using the lipase B preparation (Table 1).

Hydrolysis of CLA esters by the Amano GC-4 preparation of G. candidum *lipase*. Similar, though less absolute, selectivity was exhibited by the Amano GC-4 preparation of *G. candidum* lipase in the hydrolysis of commercial mixed CLA methyl esters. The degree of overall hydrolysis during a 4-h incubation was 31%. The resulting FFA fraction contained 77% *cis*-9,*trans*-11 CLA (Table 1; Fig. 2B), significant amounts of the other three *cis/trans* positional isomers (8,10-18:2, 10,12-18:2, and 11,13-18:2), and trace amounts of several *trans,trans* and *cis,cis* CLA isomers (Fig. 2B).

Methylation of commercial CLA FFA isomers by G. candidum *lipase B*. The activity and selectivity of the *G. candidum* lipases when catalyzing the esterification of commercial mixed isomer mixtures of CLA FFA were also investigated. Preliminary experiments demonstrated that in the esterification of CLA with methanol, immobilized *G. candidum* lipase B was active in 3-heptanone but not in hexane or isooctane. Thus 3-heptanone was employed in esterification reactions with this enzyme when the alcohol was methanol. Water was added to the solvent to a concentration of 0.02% (vol/vol) since this increased activity by 10%. GC-4 lipase was not active in any of these solvents. However, when ethanol was the esterifying alcohol, both *G. candidum* lipase preparations were active in all three solvents at water levels of 0.02% (vol/vol). Activities in hexane and isooctane under these conditions were approximately twice that obtained with 3-heptanone.

Incubation of a commercial preparation of CLA FFA containing at least 12 different isomers (Fig. 5A) with methanol and *G. candidum* lipase B in 3-heptanone resulted in the selective esterification of the *cis*-9,*trans*-11 isomer. The proportion of this isomer in the FFA fraction was reduced from 34% to about 25% during a 22.5 h incubation (Table 2), with no apparent reductions in the amounts of other CLA isomers in the FFA fraction (Fig. 5B). Extents of esterification ranged from 8 to 13%. The fatty acid ester product of this reaction was highly enriched in *cis*-9,*trans*-11 CLA, which constituted more than 98% of this fraction (Fig. 5C). Only trace amounts of the other *cis/trans* isomers and of *trans*-9,*trans*-11-18:2 were observed (Fig. 5C). Thus, high specificity for the *cis*-9 isomer is displayed by *G. candidum* lipase B in the esterification of CLA FFA. However, as generally observed, enzyme activity in organic solvents (esterification) was considerably reduced compared to that seen in water (hydrolytic reactions).

Ethylation of CLA FFA isomers by G. candidum *lipase B.* Similarly high substrate selectivity was demonstrated during lipase B-catalyzed synthesis of the ethyl, rather than methyl, esters of CLA. In this case, the proportion of the *cis*-9,*trans*-11 CLA isomer in a mixture of FFA isomers of CLA was reduced from 34% (Fig. 6A) to as low as 16% (Fig. 6B, Table 2). Compared to isooctane, the enzyme exhibited higher activity, but no greater selectivity, in hexane (Table 2). The ethyl ester fraction produced was 96% *cis*-9,*trans*-11-CLA (Table 2). The *trans*-10,*cis*-12-18:2 isomer appeared to constitute the bulk of the contaminants (Fig. 6C).

Ethylation of CLA FFA by the Amano GC-4 preparation of G. candidum *lipase.* GC-4 lipase showed much less activity than did lipase B in the esterification of CLA isomers, esterifying 1.1% of the substrate during a 22.5 h incubation. The specificity of this enzyme for *cis*-9,*trans*-11 CLA was also lower than that of lipase B. The ethyl ester fraction generated by incubation of a commercial mixed CLA FFA preparation with GC-4 lipase consisted of 80% *cis*-9,*trans*-11-CLA (Fig. 6D, Table 2). The *cis*-11,*trans*-13-, *trans*-10,*cis*-12-, and *trans*-8,*cis*-10-18:2 isomers constituted the bulk of the remainder of the ethyl ester fraction, plus trace amounts of *trans,trans* and unidentified *cis/trans* isomers (Fig. 6D).

Selective esterification of laboratory-prepared CLA isomers using an aqueous solution of G. candidum *lipase.* The esterification of laboratory-prepared CLA with octanol was catalyzed using an aqueous solution of Amano GC-4 lipase as described in the Materials and Methods section. The course of the reaction was followed by measuring the decrease in FFA concentration. After 72 h of reaction, a conversion of 35% (i.e., a decrease in the FFA concentration of 35%) was attained. A sample of the oil was fractionated by TLC, and the fatty acid composition of the unreacted FFA and the octyl esters was determined by GC. The octyl ester fraction was composed of 97.6% of the *cis*-9,*trans*-11 isomer while the unreacted fatty acids were correspondingly depleted in this isomer, with a concentration of 70.7% of the *trans*-10,*cis*-12 isomer. Clearly the lipase had shown a strong preference for the *cis*-9,*trans*-11 isomer with a very low relative reaction rate toward the *trans*-10,*cis*-12 isomer.

In another experiment, the reaction was allowed to proceed for 96 h, resulting in a conversion of 53%. Analysis of the re-

TABLE 2
Levels of *cis*-9, *trans*-11-18:2 in the Ester and Free Fatty Acid Fractions Following Esterification of a Mixed Isomer CLA Free Fatty Acid Preparation by *G. candidum* Lipases, as Determined by Ag^+–HPLC

				cis-9,*trans*-11-18:2 (%)			
			Degree of completion	Free fatty acid fraction		Ester fraction	
Lipase	Solvent[a]	Alcohol	(%)	Initial	Final	Initial	Final
B[b]	3-Heptanone	Methanol	8.0	34	27	0	>98
B[b]	3-Heptanone	Methanol	13.4	34	23	0	>98
GC-4	Isooctane	Ethanol	1.1	34	33	0	80
B	Isooctane	Ethanol	11.8	34	26	0	96
B	Hexane	Ethanol	22	34	16	0	96

[a]All solvents contained 0.02% water (vol/vol).
[b]Results of representative independent experiments. For abbreviations see Table 1.

action products revealed that the octyl ester fraction contained 81% of the *cis*-9,*trans*-11 isomer, and the FFA fraction contained 85% of the *trans*-10,*cis*-12 isomer. At the higher degree of conversion, a lesser enrichment of the *cis*-9,*trans*-11 isomer was obtained owing to competition from the gradually increasing relative concentration of the unreacted *trans*-10,*cis*-12 isomer remaining in the FFA fraction. However, the higher conversion resulted in a high degree of enrichment of the *trans*-10,*cis*-12 isomer (85%). By careful control of the reaction conditions both the *cis*-9,*trans*-11 and the *trans*-10,*cis*-12 isomers can be enriched using this reaction system.

During the preparation of this manuscript, a brief report by Chen and Sih (38) demonstrated selectivities similar to those reported here for commercial enzyme preparations from *G. candidum* and *Aspergillus niger.* The substrate used was reported to be a two-isomer CLA mixture containing *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2, not the more complex multiple-isomer mixture used in parts of the work reported here. Owing to differences in methods, the results of Chen and Sih (38) are not directly comparable to those reported here. However, it appears that the enzymes investi-

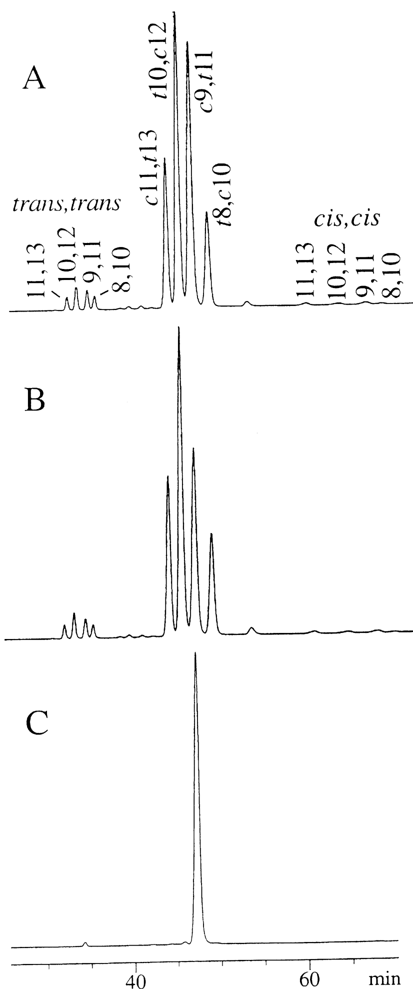

FIG. 5. Lipase-mediated methylation of a commercial preparation of mixed CLA FFA by *G. candidum* lipase B, analyzed using three Ag^+–HPLC columns in series. (A) CLA FFA substrate prior to addition of lipase. Peak identities are indicated. (B) FFA fraction following 22.5 h of incubation with lipase. (C) Fatty acid ester fraction generated during 22.5 h of reaction. For abbreviations see Figures 1 and 2.

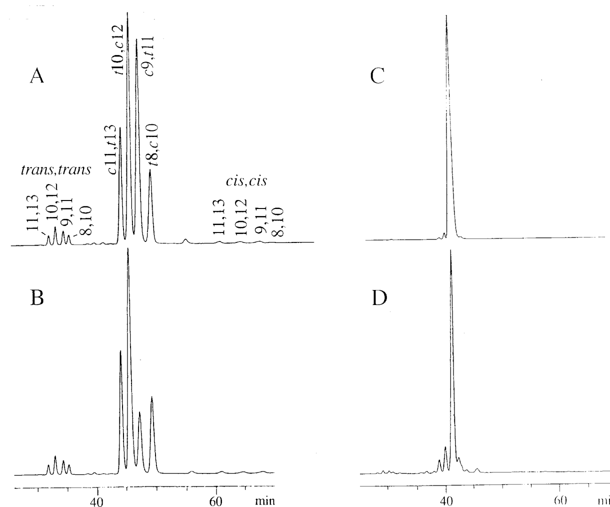

FIG. 6. Selective synthesis of CLA ethyl esters from mixed CLA FFA by *G. candidum* lipases, analyzed using three Ag^+–HPLC columns in series. (A) Starting CLA FFA material. (B) FFA fraction following 22.5 h of incubation with *G. candidum* lipase B. (C) Fatty acid ethyl ester fraction generated during 22.5 h of reaction with *G. candidum* lipase B. (D) Fatty acid ethyl ester fraction generated during 22.5 h of reaction with *G. candidum* GC-4 lipase preparation. For abbreviations see Figures 1 and 2.

gated here display *cis*-9,*trans*-11-18:2 selectivities that are similar to or greater than those of the enzymes studied by Chen and Sih (38). Furthermore, a better resolution of the products of enzyme reaction, by the use of three to six Ag^+–HPLC columns as in the present study, is advisable in order to identify minor CLA isomers in the starting material and products.

CONCLUSION

In conclusion, two different preparations of *G. candidum* lipase were shown to be selective for *cis*-9,*trans*-11-18:2. The B lipase isoenzyme showed high selectivity for *cis*-9,*trans*-11-18:2 in both hydrolysis and esterification reactions. During aqueous hydrolysis, and also during esterification in a nonaqueous system, the GC-4 preparation displayed selectivity for *cis*-9,*trans*-11-CLA isomers, though it was less stringent, as well as less active, than the B lipase preparation. An aqueous esterification system using octanol with GC-4 resulted in higher selectivity, comparable to that displayed by lipase B.

Selective lipase-catalyzed fractionation has the potential of being scaled up to produce both *cis*-9,*trans*-11-18:2 and *trans*-10,*cis*-12-18:2 of considerable purity. Both may have use in determining the biochemical and physiological effects of these molecules, and as nutritional supplements. The enzyme-catalyzed esterification process would be more practical than the hydrolysis reaction, since the industrial preparation of CLA by alkali isomerization of linoleic acid produces FFA (10).

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Enhancement of Both Reaction Yield and Rate of Synthesis of Structured Triacylglycerol Containing Eicosapentaenoic Acid Under Vacuum with Water Activity Control

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ABSTRACT: Production of structured triacylglycerols (sTAG) containing eicosapentaenoic acid (EPA) at the *sn*-1 (or 3) position using Lipozyme in a solvent-free system was studied. Optimal water activity (a_w) for the synthesis of the sTAG was investigated. Vacuum was applied to shift reaction equilibrium toward the synthesis reaction by removing by-products. During vacuum application, the water level of the reaction system was controlled at the optimal level by addition of a suitable amount of water at a predetermined interval. Intermittent periodic addition of a suitable amount of water into the reaction mixture made the reaction rate faster than that without adding water. A molar yield of 89.7% of the targeted sTAG was obtained after 16 h reaction with a_w control during the vacuum application as compared with the yield of 87.0% after 24 h of reaction without a_w control during the vacuum application.

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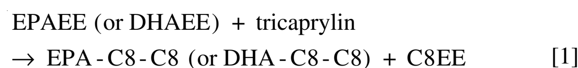
n-3 Polyunsaturated fatty acids (PUFA), such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), have several health benefits toward cardiovascular disease, immune disorders and inflammation, renal disorders, allergies, diabetes, and cancer (1). These fatty acids also may be essential for brain and retina development in humans. For PUFA-based therapy, structured triacylglycerols (sTAG) containing PUFA are effective (2). In particular, sTAG containing one molecule of PUFA and two medium-chain fatty acids (MCFA) are notable (3). Several studies have been carried out on the synthesis of sTAG containing PUFA at a specific site(s) on the glycerol backbone (3–5).

The absorption of PUFA into the body may be dependent upon the position of PUFA on the glycerol backbone, i.e., at the *sn*-1 (or 3) or the *sn*-2 position (6–8). TAG containing PUFA at the *sn*-2 position and MCFA at the *sn*-1 and 3 positions can be hydrolyzed into 2-monoacylglycerol containing PUFA and fatty acids by pancreatic lipase and efficiently absorbed into intestinal mucosa cells in normal adults (8). Therefore, for dietary supplementation for adult health, sTAG containing PUFA at the *sn*-2 position and MCFA at the *sn*-1 and 3 positions may be suitable. On the other hand, owing to the antiatherogenic, antineoplastic, and anti-inflammatory effects of n-3 PUFA, their intake is important from an early age for eicosanoid synthesis as well as for normal neonatal brain and nerve development and cell membrane structure (9). Although PUFA are essential to the neonate for both normal growth and metabolism, neonatal intestinal function is immature, resulting in reduced levels of pancreatic lipase and bile salts (10). In addition, pancreatic lipase does not hydrolyze ester bonds containing long-chain n-3 PUFA (10). Therefore, in the case of the newborn, PUFA absorption by pancreatic lipase is not feasible. However, for neonatal absorption of PUFA, there is an alternative mechanism. PUFA are released from gastric digestion, and gastric lipase exhibits stereospecificity for the position of TAG and hydrolyzes the *sn*-3 position twice as fast as the *sn*-1 position (6,10). Therefore, for PUFA therapy for the neonate, sTAG containing PUFA at the *sn*-1 (or 3) position and MCFA at the other sites might be suitable. TAG containing two or more PUFA resist hydrolysis by lipase owing to their bulky structure (11). Hence, TAG containing one molecule of PUFA at the *sn*-1 (or 3) position and two MCFA at the remaining positions of the glycerol backbone are the target product in this study.

We reported lipase-catalyzed incorporation of EPA or DHA onto tricaprylin by a transesterification between tricaprylin and EPA ethyl ester (EPAEE) or DHA ethyl ester (DHAEE) in a solvent-free system as follows (12,13):



$$\text{EPAEE (or DHAEE)} + \text{tricaprylin} \rightarrow \text{EPA-C8-C8 (or DHA-C8-C8)} + \text{C8EE} \quad [1]$$

where EPA-C8-C8 is 1-eicosapentaenoyl-2,3-dicaprylin and/or 1,2-dicapryl-3-eicosapentaenoin, DHA-C8-C8 is 1-docosahexaenoyl-2,3-dicaprylin and/or 1,2-dicapryl-3-docosahexaenoin, and C8EE is caprylic acid ethylester.

From previous results, we recognized that Lipozyme was very stereospecific for EPA or DHA incorporation into the *sn*-1 or 3 position. Therefore, Lipozyme may be useful for the

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Abbreviations: C8EE, caprylic acid ethylester; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; EPA-C8-C8, 1-eicosapentaenoyl-2,3-dicaprylin and/or 1,2-dicapryl-3-eicosapentaenoin; EPAEE, EPA ethyl ester; MCFA, medium-chain fatty acids; PUFA, polyunsaturated fatty acids; sTAG, structured triacylglycerols; TAG, triacylglycerols.

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production of sTAG containing PUFA at the *sn*-1 or 3 position, which might be effective for newborns.

In this work, we report effective production of sTAG containing one EPA at the *sn*-1 or 3 position using Lipozyme in a solvent-free system as a model reaction for the synthesis of sTAG containing one PUFA. Vacuum application was performed to shift reaction equilibrium toward synthesis reaction by removing by-product, C8EE.

Enzyme activity in organic media critically depends on the water level in the reaction system (14–16). Therefore, maintaining the optimal water level in the reaction system is very important for maximal enzyme activity (17,18). However, use of a vacuum may also remove essential water of both reaction medium and enzyme. Hence, in this study, we attempted to control optimally the water level of the reaction system during the vacuum application for the production of our target sTAG.

MATERIALS AND METHODS

Materials. Tricaprylin (1,2,3-trioctanoylglycerol) was from Wako Pure Chemicals Co. (Osaka, Japan). Dicaprylin (1,3-dioctanoylglycerol) was purchased from Funakoshi Co. (Tokyo, Japan). Pure EPAEE (*cis*-5,8,11,14,17-eicosapentaenoic acid ethyl ester) was kindly donated by Nippon Suisan Co. (Tokyo, Japan). All chemicals used were reagent grade, and the solvents used were high-performance liquid chromatography grade and purchased from Wako Pure Chemicals.

Lipozyme (Novo Nordisk Bioindustry, Chiba, Japan) was used as a catalyst. The initial water content of the Lipozyme used in this work was 2.4 ± 0.3% as determined with a Karl Fischer moisture meter (MKS-I, Kyoto Electronics Co. Ltd., Kyoto, Japan).

Determination of water sorption isotherm of Lipozyme. Lipozyme samples were preequilibrated in desiccators containing saturated salt solutions for 7 d at 25°C. The salts used were (a_w shown in parentheses): LiCl (0.12), $MgCl_2$ (0.33), $Mg(NO_3)_2$ (0.55), LiAc (0.67), and KCl (0.86). The water contents of the preequilibrated Lipozyme samples were analyzed by the Karl Fischer moisture meter.

Determination of water sorption isotherm of Lipozyme for adsorption from substrate solution and reaction medium. Reportedly, deactivating Lipozyme by prolonged exposure to over 100°C does not significantly affect water retention characteristics of Lipozyme (19). Heat-deactivated Lipozyme is also needed to prevent enzymatic reaction during investigation of the sorption curve. Therefore, heat-deactivated anhydrous Lipozyme was used to investigate the water sorption curve of Lipozyme from the reaction medium. Lipozyme samples were heat-deactivated during 1 wk at 105°C. Substrate solution of tricaprylin and EPAEE (molar ratio = 3:1) was mixed and made anhydrous by adding molecular sieve. The resultant reaction mixture obtained from a transesterification reaction between tricaprylin and EPAEE (molar ratio = 3:1) after 9 h was centrifuged at 10,000 rpm for 5 min to remove the enzyme. Molecular sieves were added to remove water from the supernatant. Heat-deactivated anhydrous Lipozyme samples (50 mg) were put into each 1.5-mL microtube, and 580 mg of the anhydrous substrate solution or the 9-h reaction mixture was added into each microtube. An amount of water (2, 4, 6, 8, 10, or 12 µL) was added into each microtube, and the tubes were vigorously vortexed, maintained for 1 h at room temperature, and then centrifuged for 5 min at 10,000 rpm. Water contents of the supernatants were checked on the Karl Fischer titrator.

Determination of dewatering rate of substrate solution by vacuum. Water (580 µL) was added to the mixture of 17.4 g of substrate solution (molar ratio of tricaprylin and EPAEE = 3:1) and 1.5 g of heat-deactivated anhydrous Lipozyme. Vacuum (3 mm Hg) was applied to determine the dewatering rate at 40°C. Samples were withdrawn at predetermined time intervals, and the water contents of the samples were checked on the Karl Fischer titrator.

Transesterification reaction. In order to determine optimal a_w for the synthesis of the sTAG, 300 mg of Lipozyme and 6 mmol of tricaprylin were preequilibrated separately in desiccators containing saturated salt solutions for 7 d at 25°C. For the investigation of the effect of a_w on the synthesis of the sTAG, the a_w of enzyme and all the substrates should be adjusted to the same value. However, since the equilibration process takes time, the result may be complicated by oxidation of EPAEE. Water solubility in EPAEE is low enough to be ignored in comparison with that in tricaprylin and Lipozyme. Therefore, in the present investigation, only a_w of lipase and of tricaprylin were adjusted. The salts used were the same as described in the section of determination of sorption isotherm of Lipozyme. The preequilibrated tricaprylin at each a_w and EPAEE were mixed by a magnetic stirrer in a closed vial, and each vial was placed into a water bath at 40°C. The reaction was started by adding lipase preequilibrated at each a_w at 400 rpm.

Molar ratios of tricaprylin/EPAEE of 5:1, 6:2, 4:2, and 2:2 were investigated to study the effect of molar ratio on the incorporation of EPAEE with tricaprylin at 40°C. Unless otherwise stated, transesterification was performed at a molar ratio of 30 mmol tricaprylin to 10 mmol EPAEE at 40°C using 1.5 g of Lipozyme.

Intermittent addition of a suitable amount of water to the reaction mixture was intended to maintain the optimal a_w of the reaction system. From the polynomial data of the water sorption isotherms of reaction medium and Lipozyme was calculated the range of water content of reaction medium corresponding to the target range of a_w. From the polynomial data of the dewatering rate by vacuum from the mixture of substrate solution (molar ratio of tricaprylin and EPAEE = 3:1) and Lipozyme, the time interval of adding water and the necessary amount of water to be added were determined. The calculated amount of water was added into the reaction medium intermittently at the determined time interval (*ca.* 10 min). Yield was calculated according to the following equation:

$$\text{yield (\%)} = 100 \times \text{mole of EPA-C8-C8/mole of EPAEE used as a substrate} \quad [2]$$

Chromatographic analysis. For time-course analysis, aliquots were taken from the vial at predetermined intervals and diluted to 1% solutions in chloroform. Solid materials were removed by filtration through a polytetrafluoroethylene membrane filter. Eluates (1 μL) were analyzed by our previously reported method (12) using a gas chromatograph (Shimadzu GC14A, Shimadzu Co., Kyoto, Japan) equipped with an OCI-14 capillary on-column injector. The column used was Supelco SGE HT5 aluminum-clad fused-silica capillary column (6 m long, 0.53 mm i.d., 0.15 μm film thickness; Supelco Inc., Bellefonte, PA). *n*-Eicosane was used as an internal standard. The oven temperature was programmed with an initial temperature of 50°C, held for 0.5 min, then heated to 110°C at 30°C /min, then to 140°C at 10°C/min, then to 250°C at 20°C/min, then to 330°C at 10°C/min, and then to 360°C at 5°C/min. The detector was maintained at 393°C. On-column injection was used for sample transfer onto the analytical column. The on-column injector was programmed with an initial temperature of 55°C held for 0.01 min, and then heated to 370°C at 40°C/min and held for 18 min. Helium was used as carrier gas at 15 mL/min.

To check isomeric purity of the synthesized sTAG containing one EPA, a silver ion–high-performance liquid chromatography method developed in our laboratory was used (12,13). Each sample was made into 1% solution in *n*-hexane/isopropanol (350:100, vol/vol) solution; 5 μL of the sample was injected, and analyzed by silver ion–high-performance liquid chromatography. All analyses were performed on a Chrompack 5 Lipid column (250 × 4.6 mm; Chrompack, Middelburg, The Netherlands) with ultraviolet detection at 206 nm. JASCO (Japan Spectroscopic Co. Ltd., Tokyo, Japan) model 880-DU solvent delivery was used, together with JASCO model UVDEC 100 detector. The solvent gradient program was as follows. Two solvent reservoirs contained (A) hexane/isopropanol/acetonitrile (350:100:2.75, by vol) and (B) hexane/isopropanol/acetonitrile (350:100:10, by vol). The gradient program was started with 100% A over 3 min, then gradient of A to 100% B over 10 min, and held for 20 min. The flow rate was 0.7 mL/min.

RESULTS AND DISCUSSION

As we reported previously (12), during the reaction, the targeted TAG containing one EPA was formed as a main product, and a small amount of TAG containing two EPA was formed. One molecule of EPA was likely incorporated into tricaprylin relatively easily, but incorporation of the second EPA molecule was much slower. Our previous results showed that Lipozyme was very stereospecific to the *sn*-1 or *sn*-3 position for EPA incorporation into tricaprylin (12). Therefore, production of the sTAG containing one EPA molecule at the *sn*-1 or 3 position was performed using Lipozyme.

Effect of molar ratio of tricaprylin to EPAEE for the synthesis of sTAG containing one EPA. Time-course changes of the conversion based on EPAEE at various molar ratios of tricaprylin to EPAEE are shown in Figure 1. The conversion increased as the molar ratio of tricaprylin to EPAEE increased from 1:1 to 3:1, but it did not increase much at molar ratios higher than 3:1 of tricaprylin to EPAEE. Therefore, subsequent experiments were done at a molar ratio of 3:1 tricaprylin to EPAEE.

Effect of vacuum on the conversion of sTAG synthesis. The reaction equilibrium may be shifted toward the synthesis reaction by removal of by-products. In our study, high vacuum was applied for removing by-product, i.e., C8EE. The effect of vacuum on the conversion based on EPAEE is shown in Figure 2. When vacuum was applied from the initial stage of the reaction, the reaction rate was very slow, which may be due to the removal of water necessary for optimal enzyme activity (data not shown). When a reaction was run at normal pressure for 9 h and then vacuum was applied, C8EE was slowly removed and the yield was gradually increased to 87.0% after 24 h. In this condition EPAEE was not removed (data not shown).

Effect of a_w *on the synthesis of sTAG.* Enzyme reactions in organic media critically depend on the level of a_w in the reaction system (14–16). Therefore, maintaining optimal a_w of the reaction system is very important for maximal production of the targeted product. However, in this study, vacuum was used for removing C8EE, and it may also remove water from the reaction medium and the enzyme, resulting in a decrease in the water necessary for optimal enzyme activity. Although

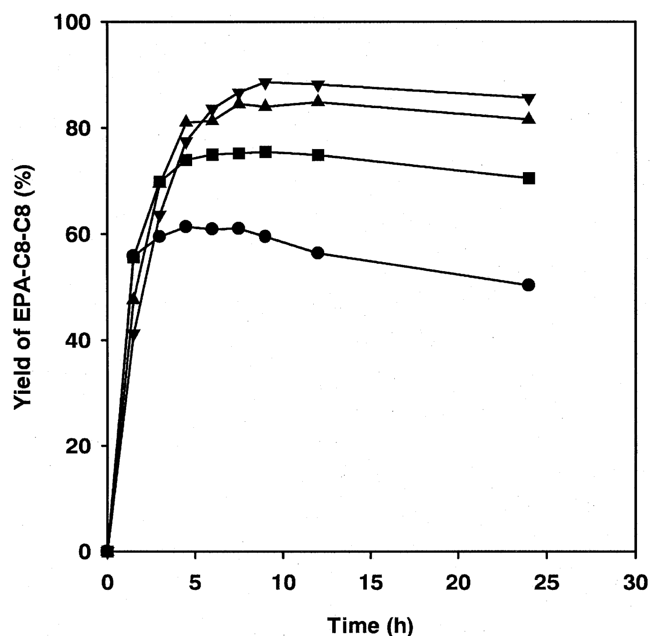

FIG. 1. Effect of molar ratio of substrates on molar yield of dicaprylleicosapentaenoin (EPA-C8-C8). Ratios of tricaprylin to eicosapentaenoic acid ethyl ester (EPAEE) are 2 mmol to 2 mmol (●), 4 to 2 (■), 6 to 2 (▲), and 5 to 1 (▼). The reaction was performed with 300 mg Lipozyme at 40°C and 400 rpm stirring speed.

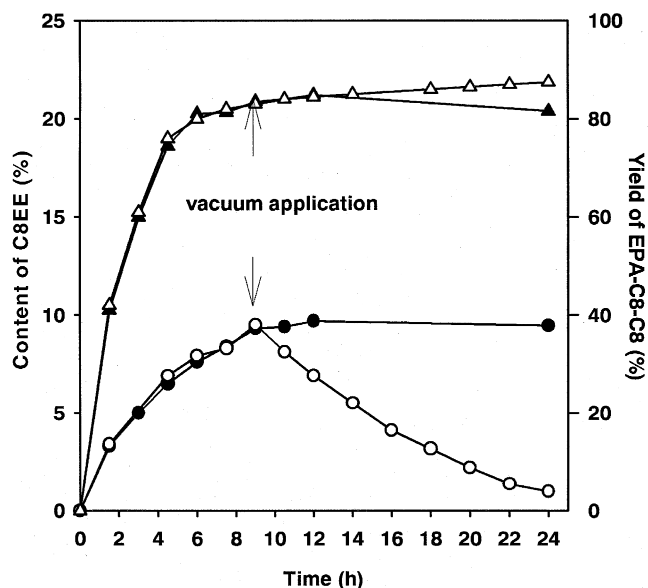

FIG. 2. Effect of vacuum on molar yield of EPA-C8-C8. The reaction was performed with 30 mmol tricaprylin, 10 mmol EPAEE, and 1.5 g Lipozyme at 40°C and 400 rpm stirring speed. The reaction was performed without vacuum at normal pressure in a closed vessel [▲, molar yield of EPA-C8-C8; ●, content of caprylic acid ethylester (C8EE)], followed by reaction under 3 mm Hg vacuum applied at 9 h thereafter (△, molar yield of EPA-C8-C8; ○, content of C8EE). For abbreviations see Figure 1.

it is often desirable to reduce the water content as much as possible so as to minimize unfavorable enzyme-catalyzed side reactions (i.e., hydrolysis), water also acts as a lubricant or plasticizer and allows enzymes to have more conformational mobility, required for optimal catalysis (20). Several reports have shown that water content has a profound influence on both yield and rate of reactions, and the suitable water content in the reaction medium is critical for optimal enzymatic activity (14–17).

The optimal water level must be determined empirically for each reaction. It would be desirable to be able to make some predictions. Unfortunately, this is hard to do in terms of the water content by weight or volume. Problems often arise in enzyme reactions in organic media because these reaction systems contain at least two distinct phases, and the water is distributed between them. Some of the water will be dissolved in the bulk organic liquid phase, and some will be in the more polar phase, sometimes as liquid water, sometimes bound to the solid-state enzyme (19). Water activity is preferable to moisture content for the characterization of all equilibria. The thermodynamic activity of water a_w is conventionally given relative to pure water at the same temperature and pressure, as follows:

$$a_w = P_{p,H_2O} / P_{p,H_2O,ref} \text{ (in equilibrium)} \quad [3]$$

where P_{p,H_20} is the vapor pressure of water in equilibrium with the system used and $P_{p,H_20,ref}$ is the vapor pressure at the same temperature above pure water. Like other activities, a_w is by definition equal in all phases at equilibrium. Hence, an organic reaction mixture may be characterized by a single a_w value, instead of water concentrations or contents in each phase (21–23).

Figure 3 shows the effect of initial a_w of substrate and lipase on the synthesis of the sTAG. Among the investigated a_w, synthetic rate of the targeted product was highest at 0.12, which means that optimal a_w of the reaction system for the product synthesis is near 0.12. Therefore, it was suggested that controlling a_w of the reaction system near 0.12 might maintain the highest reaction rate. Usually, however, water content of reaction medium can be checked easily during the reaction rather than a_w value. To this end, the relationship between the water content and a_w of the reaction medium is needed to know what level of water content of reaction medium corresponded to a certain value of a_w.

Sorption isotherm. The water sorption profile may be different as the result of a different partition of water in the medium if the polarity of the medium is different. That is, water content of a reaction medium at the same a_w may be different according to the polarity of the medium. In that case, estimating a_w by monitoring water content is very difficult. Isotherms for water sorption onto Lipozyme at two different conditions were investigated. In the first, water was adsorbed from a substrate mixture (molar ratio of tricaprylin to EPAEE = 3:1), whereas in the second case water was adsorbed from its 9-h reaction mixture. As shown in Figure 4A, the sorption isotherm curves had a similar profile, indicating that the polarity of medium was little changed. From the data fitting of

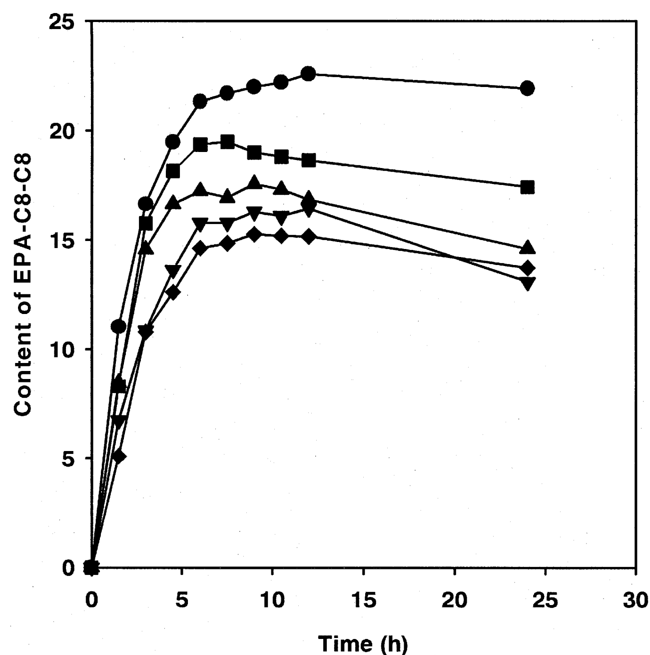

FIG. 3. Effect of initial water activity (a_w) on the synthesis of EPA-C8-C8. a_w = 0.12 (●); a_w = 0.33 (■); a_w = 0.55 (▲); a_w = 0.67 (▼); and a_w = 0.87 (◆). The reaction was performed with 6 mmol tricaprylin, 2 mmol EPAEE, and 300 mg Lipozyme at 40°C and 400 rpm stirring speed. For abbreviations see Figure 1.

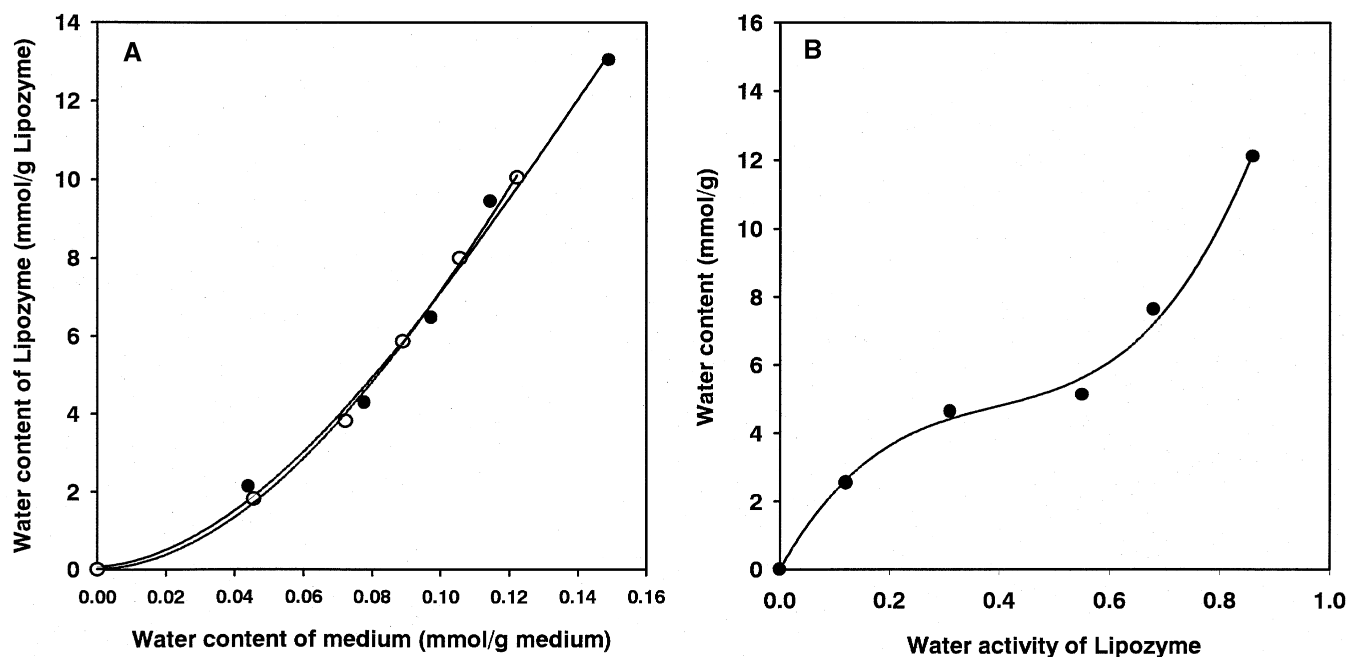

FIG. 4. Water sorption curves of substrate solution and its 9-h reaction mixture onto Lipozyme. (A) Water sorption curve of substrate solution (molar ratio of tricaprylin and EPAEE = 3:1) (○); water sorption curve of its 9-h reaction mixture (●). (B) Water sorption isotherm of Lipozyme. For abbreviation see Figure 1.

the isotherm of 9-h reaction mixture of water sorption onto Lipozyme, Equation 4 was obtained:

$$q_w = 5.30\, q_m + 851.40\, q_m^2 - 1990.25\, q_m^3 \quad [4]$$

where q_w is the water content of Lipozyme and q_m is the water content of the reaction mixture. From Figure 4A, one can know what level of water in reaction mixture corresponds to what level of water content of Lipozyme. The corresponding a_w of Lipozyme can be also estimated from the water sorption isotherm onto Lipozyme in Figure 4B, which shows the relationship between water content and a_w. From Figure 4B, the following polynomial equation was obtained:

$$q_w = 28.67\, a_w - 63.25\, a_w^2 + 53.97\, a_w^3 \quad [5]$$

where q_w is water content of Lipozyme and a_w is water activity of Lipozyme.

Since a_w is an equilibrium concept, it is equal in all phases at equilibrium. The a_w of Lipozyme may be also considered as the a_w of the reaction system under the assumption that the reaction system is in equilibrium state. The a_w of the reaction system could be inferred by monitoring water content of the reaction system during reaction.

Strategy for controlling a_w *of reaction medium during vacuum application.* As mentioned before, a lipase-catalyzed reaction is greatly influenced by a_w. In this study, vacuum application was intended to increase the reaction conversion by removing the by-product, i.e., C8EE. However, vacuum application also removes water from the reaction medium that is necessary for optimal enzyme activity. From the previous result of the effect of a_w on the sTAG synthesis, it was desirable to maintain the water level of the reaction system near 0.12 a_w during the reaction for the optimal production of the sTAG. In this study, intermittent addition of a suitable amount of water to the reaction mixture was intended to maintain the a_w of the reaction system near 0.12 during the vacuum application.

Our goal was to control a_w within the range of 0.07–0.17. From the polynomial data of sorption isotherms of Figure 4A and 4B, the range of water content of reaction medium corresponding to the target a_w range was calculated as 0.04–0.07 mmol/g reaction medium. To determine the time interval of adding water and the necessary amount of water to be added during the vacuum, the dewatering rate by vacuum from the mixture of substrate solution (molar ratio of tricaprylin and EPAEE = 3:1) and Lipozyme was investigated. The profile of dewatering rate was nonlinear as shown in Figure 5. The dewatering rate was high at higher water content, but it decreased as water content decreased. From the nonlinear regression of the data of dewatering profile, Equation 6 was obtained:

$$q_r = 0.0005 + 0.0026 \exp(-0.1883 t) \quad [6]$$

where q_r is water content of the reaction medium (mmol/g), and t is dewatering time (min).

From the nonlinear regression data of the dewatering rate by vacuum from the mixture of substrate solution (molar ratio of tricaprylin and EPAEE = 3:1) and Lipozyme, the time interval of adding water and the necessary amount of water to be added were determined. It took about 10 min for the water

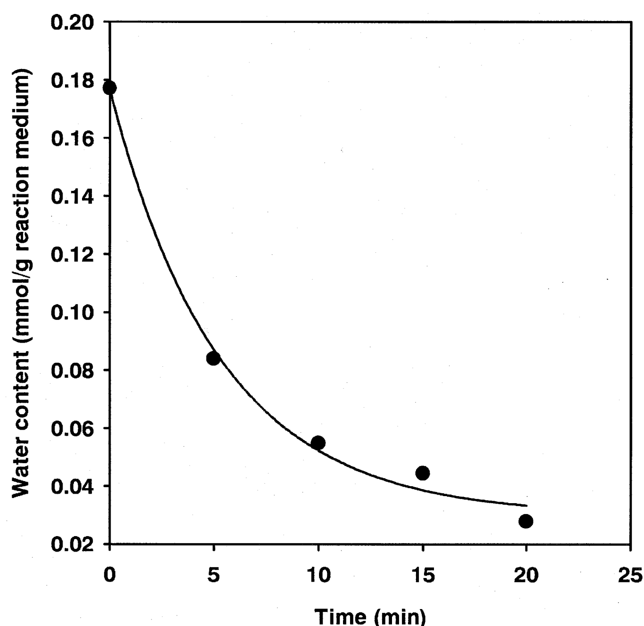

FIG. 5. Profile of dewatering rate by vacuum from the mixture of substrate solution and Lipozyme.

content of reaction medium to be decreased from 0.07 mmol/g reaction medium to 0.04 mmol/g reaction medium. The calculated amount of water (amount of water needed to replace the water evaporated from reaction medium and Lipozyme during vacuum application, *ca.* 2.46 μL/g reaction

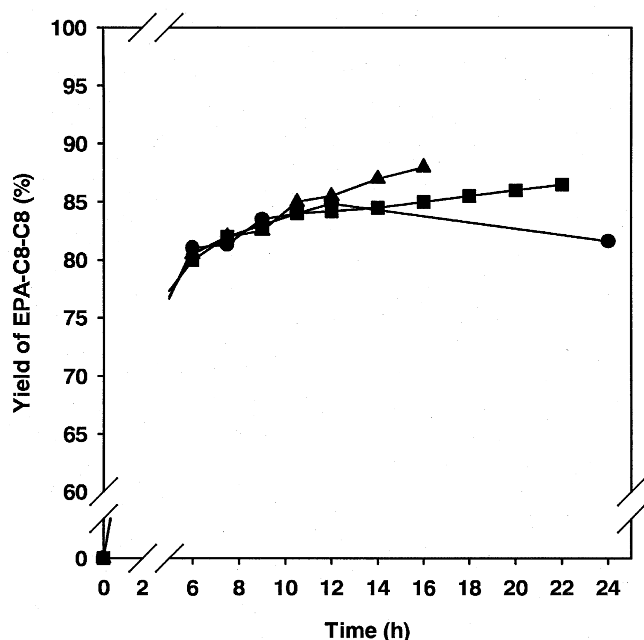

FIG. 6. Effect of a_w control during vacuum application on molar yield of EPA-C8-C8. Reaction performed without vacuum at normal pressure in a closed vessel (●); reaction under 3 mm Hg vacuum applied at 9 h thereafter without a_w control (■); reaction under 3 mm Hg vacuum applied at 9 h thereafter with a_w control at 0.07–0.17 (▲). The calculated amount of water was added about every 10 min. For the rest of the reaction conditions, see Figure 2. For abbreviations see Figure 1.

mixture) was added into the reaction medium intermittently at the determined time interval (*ca.* 10 min) during the vacuum application.

Figure 6 shows the time course of the sTAG synthesis reaction with or without maintaining a_w during the vacuum. When a suitable amount of water was added to the reaction medium at the predetermined time interval (*ca.* 10 min), the reaction rate was faster than that without adding water. A molar yield of 87.3% of EPA-C8-C8 from EPAEE was obtained after 16 h reaction with a_w control during the vacuum application as compared with 87.0% after 24 h reaction without a_w control. The converted EPAEE were incorporated as 91.5 mol% EPA-C8-C8, 3.1 mol% dieicosapentaenoyloctanoyl glycerol, and 5.4 mol% eicosapentaenoyloctanoyl glycerol, respectively.

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Dietary Conjugated Linoleic Acid Reciprocally Modifies Ketogenesis and Lipid Secretion by the Rat Liver

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ABSTRACT: The effects of dietary conjugated linoleic acid (CLA) and linoleic acid (LA) on ketone body production and lipid secretion were compared in isolated perfused rat liver. After feeding the 1% CLA diet for 2 wk, the concentration of post-perfused liver cholesterol was significantly reduced by CLA feeding, whereas that of triacylglycerol remained unchanged. Livers from CLA-fed rats produced significantly more ketone bodies; and the ratio of β-hydroxybutyrate to acetoacetate, an index of mitochondrial redox potential, tended to be consistently higher in the liver perfusate. Conversely, cumulative secretions of triacylglycerol and cholesterol were consistently lower in the livers of rats fed CLA, and the reduction in the latter was statistically significant. Thus dietary CLA appeared to exert its hypolipidemic effect at least in part through an enhanced β-oxidation of fatty acids at the expense of esterification of fatty acid in the liver.

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Conjugated linoleic acid (CLA) is a mixture of geometrical and positional isomers of linoleic acid (*cis*,*cis*-9,12-octadecadienoic acid, LA). CLA occurs exclusively in ruminant meats and dairy products, although its concentration is as low as 5 mg/g fat (1). In addition to the diverse positive influence of CLA on carcinogenesis and atherogenesis (2–6), Park *et al.* found that dietary CLA reduces fat accumulation in adipose tissues (7). They suggested that this was due to increased oxidation of fatty acids as reflected by enhanced activity of carnitine palmitoyl transferase in fat pad and skeletal muscle. Belury *et al.* also reported that dietary CLA causes an increase in mRNA levels of acyl CoA oxidase, fatty acid-binding protein, and cytochrome P450, an indication of peroxisomal proliferation in the liver of mice fed CLA-containing diets for 6 wk (8). Thus, CLA may cause peroxisomes to proliferate, thus enhancing fatty acid oxidation in the liver. These observations suggest that beneficial effects of dietary CLA on lipid metabolism are attributable at least partly to enhanced fatty acid oxidation in the liver.

In the present study, we examined whether CLA added at a 1% level to the diet could influence lipid secretion and ketone body production by perfused rat liver.

MATERIALS AND METHODS

Male Sprague-Dawley rats (Kyudo Co., Kumamoto, Japan) weighing 140–150 g were housed individually at constant temperature (22–24°C) with lights turned on from 0700 to 1900. Rats were acclimated to the conditions for 5–9 d, and then divided into two groups with equal body weight. Each group was fed a CLA or LA diet formulated according to the AIN76^{TM} formula (9) (in wt%): 5, corn oil; 20, casein; 15, cornstarch; 5, cellulose; 3.5, mineral mixture (AIN76^{TM}), 1, vitamin mixture (AIN76^{TM}), 0.3, DL-methionine; 0.2, choline bitartrate; either 1 LA or CLA (supplied by Rinoru Oil Mills, Nagoya, Japan), and sucrose to 100. The fatty acid composition of dietary LA and CLA preparations is shown in Table 1. CLA prepared by alkaline isomerization of linoleic acid in safflower oil was provided by Rinoru Oil Mills. These diets were given *ad libitum* for 14 d. Food intake and body weight were recorded every other day.

On the day of the perfusion experiments, rats were given intraperitoneal injection of pentobarbital Na (5 mg/100 g body weight) around 0830. The liver was then isolated and perfused with 120 mL of recirculating Krebs-Henseleit perfusion medium (pH 7.4), which contained 25 mM glucose, 1.5% (wt/vol) bovine serum albumin (Fraction V, Boehringer Mannheim, Mannheim, Germany), and 25% (vol/vol) washed bovine erythrocytes, at the rate of 20 mL/min at 37°C, in the presence of exogenous oleic acid. Five milliliters of 20 mM potassium oleate (100 μmol) was added at the beginning of recirculation, and the same solution was continuously infused at the rate of 4.5 mL/h (90 μmol oleate/h). Every hour, 20 mL of the perfusate was removed for analyses of ketone bodies and lipids, and the same amount of the fresh perfusion medium was added to maintain 120 mL of recirculating volume. The perfusions were continued for 4 h, as described in detail previously (10–12).

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Abbreviations: CLA, conjugated linoleic acid; LA, linoleic acid.

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TABLE 1
Fatty Acid Compositions of Linoleic Acid (LA) and Conjugated Linoleic Acid (CLA) Preparations

	LA	CLA
16:0	7.2	6.9
18:0	2.6	2.5
18:1	14.8	15.3
18:2	73.3	0.8
18:2 (CLA)	—	73.8[a]

[a]CLA was composed of 34.6, 35.9, 1.7, and 1.6% of 9*c*,11*t*/9*t*,11*c*-, 10*t*, 12*c*-, 9*c*,11*c*/10*c*,12*c*-, and 9*t*,11*t*/10*t*,12*t*-octadecadienoic acids, respectively.

TABLE 2
Effect of CLA Feeding on Body Weight, Liver Weight, and Postperfused Liver Lipids[a]

	Dietary group	
	LA	CLA
Body weight (g)	289 ± 2	296 ± 6
Liver weight (g)	15.9 ± 0.5	17.6 ± 0.8
Liver lipids (μmol/g liver)		
Total cholesterol	6.04 ± 0.38	4.25 ± 0.53[b]
Free cholesterol	3.13 ± 0.15	2.56 ± 0.12[b]
Triacylglycerol	22.7 ± 1.5	21.4 ± 2.6
Phospholipid	27.7 ± 2.2	28.8 ± 1.1

[a]Mean ± SE of 5 or 6 rats. Liver weight was measured at the end of perfusion.
[b]Significantly different from LA group at $P < 0.05$. Rats weighing 180–200 g were fed the diets supplemented with either 1% of LA or CLA preparation for 14 d. The livers were then isolated and perfused in the presence of an oleic acid substrate. For abbreviations see Table 1.

In another experiment, CLA and LA were added directly to the perfusion medium: 5 mL of 1% potassium salt solutions was added at the beginning of recirculation, and the same solution was continuously infused at the rate of 4.5 mL/h, to compare the direct effect of these fatty acids *per se* on ketone body production and lipid secretion in the perfused liver. Male Wistar rats (260–270 g) fed commercial rat chow served as liver donors in this experiment. All aspects of the experiment were approved by the Miyazaki University Animal Policy and Welfare Committee.

The methods employed for the lipid and ketone body analyses in perfusates and postperfused livers were described previously (10–13). Data were analyzed by Student's *t*-test (14) to evaluate significant differences of the means.

RESULTS

Dietary CLA did not influence food intake, growth, or relative liver weight of rats (Table 2). The concentration of postperfused liver triacylglycerol and phospholipid was not different between the groups, whereas that of total and free cholesterol was significantly lower in CLA-fed rats than in LA-fed rats. The amount of oleic acid taken up by the perfused livers was calculated to be constant at 1-h intervals, and the amounts for a total of 4 h were comparable; 427 ± 7 μmol/liver in the LA group, and 420 ± 8 μmol/liver in the CLA group. Therefore, the following observations on the diverse responses in ketone body production and lipid secretion by the livers were attributed in part to the influence of dietary fatty acids on intracellular fatty acid metabolism.

The livers of rats fed the CLA diet produced significantly more ketone bodies than those of rats fed the LA diet (Table 3). The ratio of β-hydroxybutyrate to acetoacetate tended to increase after feeding CLA. On the other hand, dietary CLA consistently lowered the secretion of triacylglycerol and cholesterol by the perfused liver, as compared to dietary LA, and the difference in cholesterol secretion after 2 h of perfusion was significant. Therefore, the relationship between ketogenesis and lipid secretion was inverse. Although this relationship was inconsistent with the observation with other chemi-

TABLE 3
Effect of CLA Feeding on Ketone Body Production and on Secretion of Triacylglycerol and Cholesterol by the Perfused Liver[a]

	Perfusion period (h)			
	1	2	3	4
Ketone body production (μmol/liver)				
LA	239 ± 11	296 ± 9	368 ± 16	434 ± 22
CLA	282 ± 10[b]	380 ± 13[b]	489 ± 17[b]	598 ± 21[b]
β-Hydroxybutyrate/ acetoacetate ratio				
LA	0.56 ± 0.05	0.66 ± 0.12	0.75 ± 0.17	0.90 ± 0.20
CLA	0.96 ± 0.06[b]	0.95 ± 0.17	1.04 ± 0.19	1.19 ± 0.21
Triacylglycerol secretion (μmol/liver)				
LA	15.4 ± 1.8	22.0 ± 3.0	32.0 ± 3.0	44.6 ± 6.3
CLA	12.8 ± 0.7	19.6 ± 0.9	25.0 ± 2.2	32.2 ± 3.3
Cholesterol secretion (μmol/liver)				
LA	4.76 ± 0.46	7.36 ± 0.71	9.51 ± 0.84	12.5 ± 0.6
CLA	3.74 ± 0.26	5.74 ± 0.14[b]	7.46 ± 0.21[b]	9.67 ± 0.33[b]

[a]Mean ± SE of 5 or 6 rats.
[b]Significantly different from LA group at $P < 0.05$. For abbreviations see Table 1.

TABLE 4
Effect of Addition of CLA to the Perfusion Medium on Ketone Body Production and on Secretion of Triacylglycerol and Cholesterol by the Perfused Liver[a]

	Perfusion period (h)			
	1	2	3	4
Ketone body production (μmol/liver)				
LA	264 ± 10	376 ± 36	459 ± 44	574 ± 53
CLA	247 ± 31	368 ± 54	467 ± 68	558 ± 72
β-Hydroxybutyrate/ acetoacetate ratio				
LA	0.69 ± 0.12	0.72 ± 0.05	0.70 ± 0.02	0.88 ± 0.07
CLA	0.74 ± 0.10	0.71 ± 0.06	0.72 ± 0.08	0.72 ± 0.06
Triacylglycerol secretion (μmol/liver)				
LA	3.69 ± 0.54	6.38 ± 0.74	9.43 ± 0.51	12.7 ± 0.8
CLA	3.24 ± 0.49	6.02 ± 0.80	9.14 ± 0.98	11.7 ± 1.0
Cholesterol secretion (μmol/liver)				
LA	2.53 ± 0.37	4.39 ± 0.51	6.47 ± 0.35	8.69 ± 0.55
CLA	2.22 ± 0.33	4.14 ± 0.55	6.28 ± 0.67	8.07 ± 0.70

[a]Mean ± SE of 4 rats. For abbreviations see Table 1.

cals reported previously (10,15), the significant reduction in the secretion of cholesterol was characteristic for CLA.

The addition of CLA to the perfusion medium did not influence hepatic ketogenesis and lipid secretion when compared with the addition of LA (Table 4). These results indicate that the adaptive changes of fatty acid metabolism ocurred only when rats were fed CLA.

DISCUSSION

The present study demonstrated that dietary CLA enhanced hepatic fatty acid oxidation as reflected by a stimulated ketone body production, while CLA itself did not exert a direct influence on fatty acid oxidation. In accordance with the observation of Park *et al.* (7), that CLA increased mitochondrial activity of carnitine palmitoyl transferase of epididymal adipose tissues and muscles, we can suggest that CLA influences fatty acid metabolism not only of liver, but also of extrahepatic tissues.

On the other hand, feeding of CLA to mice resulted in proliferation of peroxisomes, another site in the liver of fatty acid oxidation (8). However, peroxisomal proliferators such as fibrate and drugs related to it commonly lead to an enlarged liver, accompanied by a marked increase in hepatic phospholipid and a decrease in the ratio of β-hydroxybutyrate to acetoacetate even when hepatic ketogenesis is stimulated (10,16). These changes were not confirmed in the present study as shown in Tables 2 and 3. Thus, stimulated hepatic ketogenesis with a concomitant elevation of the ratio of β-hydroxybutyrate to acetoacetate observed in the liver of rats fed CLA may be attributed to the mitochondrial process, but not to the response of peroxisomal oxidation, since the paralleled increase in these two parameters is mostly associated with an enhancement of fatty acid oxidation in mitochondrial compartments (10,12).

Stimulated ketogenesis was inversely related to the reduced secretion of triacylglycerol by the livers of rats fed CLA, a reciprocal response in these metabolic pathways. In this context, Yotsumoto *el al.* (17) recently reported that addition of 10*t*,12*c*-octadecadienoic acid, one of the major CLA constituents, to HepG2 cells resulted in an impairment of the synthesis and secretion of apolipoprotein B-containing lipoproteins. The inverse relationship between fatty acid oxidation and triacylglycerol secretion has been observed in livers of rats under various nutritional and physiological conditions (10–12,15). Therefore, dietary CLA seemed to be a potent stimulator for fatty acid oxidation in the liver.

CLA decreased secretion of cholesterol by the liver. Thus, the hypocholesterolemic effect of dietary CLA as observed in hamsters (6) may in part be attributed to this reduction. Lee *et al.* (5) reported that supplementation of 0.5 g/d CLA resulted in the reduction of serum low density lipoprotein cholesterol and triacylglycerol in rabbits fed the cholesterol-enriched diet. In these animal models, the extent of atherosclerosis of arterial walls was less marked (5,6).

In considering these reports as well as the present work, dietary CLA may exert the hypocholesterolemic effect at least in part through a decreased hepatic secretion of very low density lipoprotein-cholesterol. In addition, dietary CLA may reduce the biosynthesis of cholesterol in the liver, since CLA reduced liver cholesterol level.

Accumulation of CLA in rat liver lipids was also checked in the feeding experiment. The level of CLA was about 0.6% of the fatty acids in liver triacylglycerol of rats fed CLA. We detected no CLA peaks in phospholipids and cholesteryl ester fractions. The low incorporation of CLA into liver tissue may be attributed to either its oxidation during perfusion or the length of feeding period. However, it was observed that feeding CLA for as little as 2 wk induces fatty acid oxidation.

In conclusion, a decreased secretion of triacylglycerol-rich

lipoproteins by the liver from CLA-fed rats is a consequence of an increase in ketone body production. This specific fatty acid may also exert its hypocholesterolemic effect by decreasing hepatic cholesterol level. However, no such responses were observed when the liver from rats fed the diet free of CLA was perfused with CLA.

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ARTICLE

On the Specificity of Allene Oxide Cyclase

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ABSTRACT: Jasmonic acid is a carbocyclic fatty acid that is biosynthesized from α-linolenic acid in several steps. The formation of the ring structure of jasmonic acid is catalyzed by the enzyme allene oxide cyclase (EC 5.3.99.6) and involves the cyclization of an unstable allene oxide into the cyclopentenone 12-oxo-10,15(*Z*)-phytodienoic acid. In this study, a number of allene oxides were generated, and their enzymatic and nonenzymatic cyclization into cyclopentenones was investigated. Nonenzymatic cyclization was observed with allene oxides having one pair of conjugated double bonds and an additional isolated double bond in the β,γ position relative to the epoxide group, i.e., the partial structure 4,5-epoxy-1,3,7-octatriene. Enzymatic cyclization took place provided that this structural element was inserted in the fatty acid chain with its epoxide group in the n-6,7 position and the isolated double bond in the n-3 position. A number of oxygenated fatty acids having structural features in common with the natural allene oxides were tested as inhibitors of allene oxide cyclase. Fatty acids having an allene oxide structure in the n-6,7 position but lacking the double bond in the n-3 position, as well as fatty acids having a saturated epoxide group in the n-6,7 position, served as competitive inhibitors of the enzyme. Data on the substrate specificity of allene oxide synthase (EC 4.2.1.92) from corn seeds indicated that fatty acid hydroperoxides with a double bond at n-3 and with the hydroperoxide function at n-6 exhibit the highest affinity but the slowest reaction velocity.

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Abbreviations and trivial names: 9(*S*),10-EOD, 9(*S*),10-epoxy-10,12(*Z*)-octadecadienoic acid; 9(*S*),10-EOT, 9(*S*),10-epoxy-10,12(*Z*),15(*Z*)-octadecatrienoic acid; 10,11(*S*)-EHT, 10,11(*S*)-epoxy-7(*Z*),9,13(*Z*)-hexadecatrienoic acid; 12,13(*S*)-EOD, 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid; 12,13(*S*)-EOT, 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid; 14,15(*S*)-EETE, 14,15(*S*)-epoxy-5(*Z*),8(*Z*),11(*Z*),13-eicosatetraenoic acid; 9(*S*)-HPOD, 9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid; 9(*S*)-HPOT, 9(*S*)-hydroperoxy-10(*E*),12(*Z*),15(*Z*)-octadecatrienoic acid; 11(*S*)-HPHT, 11(*S*)-hydroperoxy-7(*Z*),9(*E*),13(*Z*)-hexadecatrienoic acid; 13(*S*)-HPOD, 13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid; 13(*S*)-HPOT, 13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid; 15(*S*)-HPEP, 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*),17(*Z*)-eicosapentaenoic acid; 15(*S*)-HPET, 15(*S*)-hydroperoxy-11(*Z*),13(*E*),17(*Z*)-eicosatrienoic acid; 15(*S*)-HPETE, 15(*S*)-hydroperoxy-5(*Z*),8(*Z*),11(*Z*),13(*E*)-eicosatetraenoic acid; 17(*S*)-HPDP, 17(*S*)-hydroperoxy-7(*Z*),10(*Z*),13(*Z*),15(*E*),19(*Z*)-docosapentaenoic acid; γ-9(*S*),10-EOT, 9(*S*),10-epoxy-6(*Z*),10,12(*Z*)-octadecatrienoic acid; γ-9(*S*)-HPOT, 9(*S*)-hydroperoxy-6(*Z*),10(*E*),12(*Z*)-octadecatrienoic acid;12-oxo-PDA, 12-oxo-10,15(*Z*)-phytodienoic acid; AOC, allene oxide cyclase; AOS, allene oxide synthase; GC–MS, gas–liquid chromatography–mass spectrometry; GLC, gas–liquid chromatography, JA, jasmonic acid [used as a collective name for (−)-jasmonic acid and (+)-7-*iso*-jasmonic acid]; α-ketol, 12-oxo-13-hydroxy-9(*Z*),15(*Z*)-octadecadienoic acid; γ-ketol, 12-oxo-9-hydroxy-10(*E*),15(*Z*)-octadecadienoic acid; MC, (−)-menthoxycarbonyl; RP-HPLC, reversed-phase high-performance chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

The cyclopentanones jasmonic acid (JA) and its methyl ester (MJ) are involved as signal molecules in plant defense reactions against herbivores and phytopathogens (1) as well as in mechanical responses such as tendril coiling (2). Additionally, they affect plant growth and developmental processes such as seed germination, leaf senescence, tuberization (3) and pollen development (4).

The biosynthesis of JA proceeds *via* an oxylipin pathway (5–7). Fatty acid hydroperoxides produced by lipoxygenase-catalyzed oxygenation of polyunsaturated fatty acids serve as important intermediates in this pathway. Hydroperoxide-metabolizing enzymes include lyases, which catalyze chain cleavage of hydroperoxides into short-chain aldehydes and ω-oxo-carboxylic acids (8,9), and divinyl ether synthases, which catalyze the dehydration of hydroperoxides into regio- and stereoisomeric divinyl ether derivatives (10–12). Fatty acid hydroperoxides can also be used as cosubstrates for plant peroxygenases in the epoxidation of unsaturated fatty acids (13,14). One specific hydroperoxide, i.e., α-linolenic acid 13(*S*)-hydroperoxide [13(*S*)-HPOT] serves as the precursor of the jasmonate family of compounds (15). The first step of the transformation of 13(*S*)-HPOT into jasmonates is catalyzed by allene oxide synthase (AOS) and results in the formation of an unstable allene oxide derivative, 12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid (16–18). This allene oxide is chemically degraded in aqueous media with a half life of 26 s at 0°C and pH 6.7 (16) mainly to α-ketol [12-oxo-13-hydroxy-9(*Z*),15(*Z*)octadecadienoic acid] and γ-ketol [12-oxo-9-hydroxy-10(*E*),15(*Z*)octadecadienoic acid]. Additionally, a fraction of the allene oxide (10–15%) undergoes cyclization to produce the cyclopentenone 12-oxo-10,15(*Z*)-phytodienoic acid (12-oxo-PDA) in racemic form (Scheme 1). In contrast to the chemical degradation, the allene oxide is converted by the next enzyme of jasmonate biosynthesis, allene oxide cyclase (AOC), to optically pure 9(*S*),13(*S*)-12-oxo-PDA (16,19,20). This compound is further

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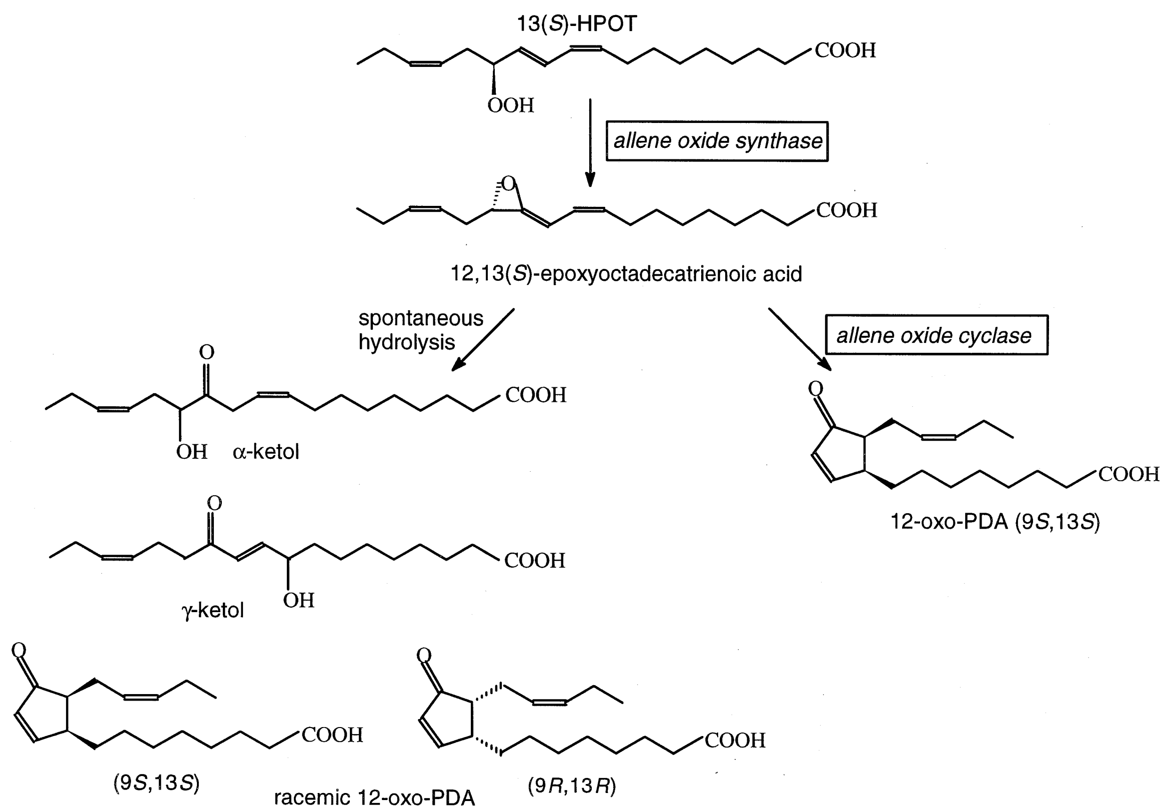


SCHEME 1

converted into JA by enzymatic reduction of the ring double bond followed by three steps of β-oxidation (15). Whereas lipoxygenase- and AOS-derived products can be diverged into pathways different from JA biosynthesis, the AOC-catalyzed reaction provides the first intermediate which, according to the current knowledge, ultimately leads to JA. It is therefore conceivable that this enzyme may represent an important regulatory point in the biosynthesis of this plant signal compound. AOC from corn kernels was characterized as a soluble enzyme having a molecular mass of about 45 kD (20). The enzyme was recently purified to homogeneity and characterized with respect to molecular properties, pH optimum, responsiveness to divalent ions, and feedback inhibition (21). The present work is concerned with the substrate specificity of AOC and with its inhibition by substrate analogs.

EXPERIMENTAL PROCEDURES

Fatty acids. For the preparation of 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acid, potato leaves were cut and homogenized in chloroform/methanol (2:1, vol/vol) containing 10 ppm of 2,6-di-*tert*-butyl-4-methylphenol. The oil was saponified, and the fatty acid fraction was esterified by treatment with diazomethane. Methyl 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoate was obtained following preparative reversed-phase high-performance liquid chromatography (RP-HPLC). Saponification followed by final purification by means of open-column SiO_2 chromatography afforded pure 7(*Z*),10(*Z*),13(*Z*)-hexadecatrienoic acid as a colorless oil. 9(*Z*),12(*E*)-Octadecadienoic acid was prepared by deoxygenation of methyl 12(*S*),13(*R*)-epoxy-9(*Z*)-octadecenoate (22). Briefly, a sample of the epoxyester was refluxed with glacial acetic acid for 3 h and the product saponified. Crystallization from ethyl acetate afforded pure *threo*-12,13-dihydroxy-9(*Z*)-octadecenoic acid having an m.p. of 50–52°C. A sample (500 mg) was methyl-esterified and refluxed with 1,1′-thiocarbonyldiimidazole (500 mg) in toluene (25 mL) for 3 h. The cyclic thionocarbonate derivative was purified by SiO_2 chromatography and subsequently refluxed with trimethylphosphite (20 mL) for 15 h under argon. The product (410 mg) was saponified and purified by SiO_2 chromatography to yield 9(*Z*),12(*E*)-octadecadienoic acid (220 mg; purity in excess of 97%). 9(*Z*),11(*E*)-Octadecadienoic acid and 10(*E*),12(*Z*)-octadecadienoic acid were purchased from Larodan AB (Malmö, Sweden). The remaining fatty acids were obtained from Nu-Chek-Prep (Elysian, MN).

Epoxy fatty acids. (±)-*cis*-9,10-Epoxy-12(*Z*)-octadecenoic acid and (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid were prepared by monoepoxidation of methyl linoleate followed by separation by thin-layer chromatography (TLC), saponification, and crystallization (20). In a similar way, monoepoxidation of methyl 9(*Z*),12(*E*)-octadecadienoate and methyl 11(*Z*),14(*Z*)-eicosadienoate afforded (±)-*trans*-12,13-epoxy-9(*Z*)-octadecenoic acid and (±)-*cis*-14,15-epoxy-11(*Z*)-

eicosenoic acid, respectively. Methyl 12(*S*),13(*R*)-epoxy-9(*Z*)-octadecenoate was isolated from seeds of *Euphorbia lagascae* (23) by Soxhlet extraction, transmethylation, and purification by SiO_2 chromatography. The ester had $[\alpha]_D$ + 2.4° (c 5.5, hexane), thus confirming the 12(*S*),13(*R*) configuration of the epoxide group (24). Saponification under mild conditions followed by crystallization from hexane yielded 12(*S*),13(*R*)-epoxy-9(*Z*)-octadecenoic acid [(+)-vernolic acid] having a purity of 99%. Methyl 12(*R*),13(*S*)-epoxy-9(*Z*)-octadecenoate was isolated from seeds of *Malope trifida purpureae* (25) by Soxhlet extraction, transmethylation, and SiO_2 chromatography. The optical rotation measured on the ester corresponded to $[\alpha]_D$ −2.6° (c 4.7, hexane), thus demonstrating that the epoxide group had the *R*,*S* configuration (24). Saponification followed by crystallization from hexane yielded 12(*R*),13(*S*)-epoxy-9(*Z*)-octadecenoic acid [(−)-vernolic acid] having a purity of 99%.

Methyl 12-hydroxy-dinor-phytonoate (side chain trans *isomer*). The methyl ester of (±)-JA was hydrogenated and reduced with sodium borohydride as was earlier described for (+)-7-*iso*-JA (26). Part of the acetoxy acid obtained following saponification and acetylation (256 mg, 1 mmol) was subjected to anodic coupling with methyl hydrogen adipate (3 mmol) in methanol (15 mL) containing sodium methoxide (0.2 mmol). The reaction product was saponified, methyl-esterifed, and purified by open-column SiO_2 chromatography. Epimers A (R_f = 0.44) and B (R_f = 0.53) of methyl (±)-12-hydroxy-dinor-phytonoate (side chain *trans* isomer) were obtained in a 1:1 ratio by preparative TLC (solvent system: ethyl acetate/hexane, 25:75, vol/vol). On the basis of the data for the corresponding epimers of 18-carbon chain length (27), epimer A was identified as a racemic mixture of the two enantiomers methyl 12(*R*)-hydroxy-9(*S*),13(*R*)-dinor-phytonoate and methyl 12(*S*)-hydroxy-9(*R*),13(*S*)-dinor-phytonoate. In the same way, epimer B was due to a racemic mixture of methyl 12(*R*)-hydroxy-9(*R*),13(*S*)-dinor-phytonoate and methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-dinor-phytonoate. The mass spectra of the two epimers were virtually identical and showed prominent ions at *m/z* 266 (M^+ – 18; loss of H_2O), 228, 209 [M^+ – (57 + 18); loss of $(CH_2)_3CH_3$ + H_2O], 195 [M^+ – (71 + 18); loss of $(CH_2)_4CH_3$ + H_2O], 164, and 137 [M^+ – (129 + 18); loss of $(CH_2)_5COOCH_3$ + H_2O]. Derivatization of epimer B with (−)-menthoxycarbonyl (MC) chloride followed by purification by TLC afforded two diastereomeric MC derivatives which were separable by gas–liquid chromatography (GLC) [diastereomer 1, MC derivative of methyl 12(*R*)-hydroxy-9(*R*),13(*S*)-dinor-phytonoate; retention time, 17.4 min; diastereomer 2, MC derivative of methyl 12(*S*)-hydroxy-9(*S*),13(*R*)-dinor-phytonoate; retention time, 18.0 min; methyl silicone capillary column; column temperature raised from 220 to 280°C at a rate of 5°C/min]. The mass spectra of MC-derivatives 1 and 2 were virtually identical and showed prominent ions at *m/z* 266 (M^+ – 200; loss of MC-OH), 235 [M^+ – (200 + 31); loss of MC-OH plus OCH_3], 138 ($C_{10}H_{18}$; menthyl – H), and 83.

Fatty acid hydroperoxides. The n-6 hydroperoxides were prepared by incubation of the corresponding fatty acids with soybean lipoxygenase (Sigma Chemical Co., St. Louis, MO) (28), whereas the n-10 hydroperoxides were obtained by incubation of the appropriate precursor acids with tomato fruit homogenates (29).

Enzyme preparations. The membrane fraction of corn seed homogenates sedimenting at 105,000 × *g* was used as source of AOS. As earlier shown, this preparation is devoid of AOC activity (20). The AOC preparation from corn consisted of the 50-fold enriched AOC activity after Octyl-Sepharose chromatography (21). The experiments with potato AOC were performed with a 20-fold-enriched AOC preparation from potato tubers. To obtain this, potato tubers were cut into small pieces and homogenized in 50 mM potassium phosphate buffer pH 6.7 containing 1 mM β-mercaptoethanol, 0.5 mM mercaptobenzothiazole (tissue to buffer ratio 1:2, wt/vol) with an Ultraturrax (Janke & Kunkel, Staufen, Germany). The homogenate was filtered through four layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 10,000 × g for 10 min. The supernatant was recentrifuged at 100,000 × *g* for 60 min, and the soluble fraction was subjected to acetone precipitation. The pellet containing the AOC activity obtained between 40 and 60% acetone saturation was dissolved in 0.75 M ammonium sulfate in 20 mM Tris-HCl pH 7.5 and loaded on an Octyl-Sepharose column (35 mL) equilibrated in 1 M ammonium sulfate 20 mM Tris-HCl pH 7.5. AOC activity was eluted with a linear-decreasing ammonium sulfate gradient over 350 mL at a flow rate of 2.5 mL/min.

Enzyme assays. AOS activity was measured in 50 mM potassium phosphate pH 6.7 in a total volume of 1 mL. The reaction was initiated by the addition of the fatty acid hydroperoxide, and the decrease in the absorbance at 235 nm was recorded. For the determination of the kinetic parameters of corn AOS, the initial reaction velocity was determined at 14 concentrations in the range of 5 to 90 μM for each substrate. The K_m and v_{max} values were calculated directly using the Michaelis-Menten equation and by plotting the data according to Eadie-Hofstee. Both methods gave similar results. AOC activity was determined by a method based on RP-radio-HPLC as described (21).

Analysis of cyclization products of allene oxides. Fatty acid hydroperoxides (150 μM) were stirred at 0°C for 10 min with 0.5 mL of a suspension of corn seed membranes [0.5 mg of protein; 28 nkat using 13(*S*)-HPOT as the substrate] in 0.1 M potassium phosphate buffer pH 6.7, and 2.5 mL of the AOC preparation in ammonium sulfate/20 mM Tris-Cl buffer pH 7.5 (total assay volume 5 mL). The activities of the corn and potato AOC added to these assays were equivalent to 178 nmol PDA and 156 nmol PDA, respectively, measured by the method based on RP-radio-HPLC. Control incubations, in which the AOC solution was replaced by potassium phosphate buffer, were run in parallel. The incubations were terminated by the addition of 10 mL of methanol, and the reaction products were extracted with diethyl ether, methylated, and subjected to TLC (solvent system: ethyl acetate/toluene

(15:85, vol/vol). Bands were visualized under ultraviolet (UV) light after spraying with 2′,6′-dichlorofluorescein, and the R_f value of the band due to cyclopentenone (the methyl ester of 12-oxo-PDA and its homologs and analogs) was recorded. A broad zone of silica gel containing bands due to the methyl esters of cyclopentenone, hydroxy acid, and α-ketol was scraped off and eluted with ethyl acetate. An aliquot of the material was trimethylsilylated and subjected to product analysis by GLC and gas–liquid chromatography–mass spectromety (GC–MS). The remaining part of the material was used for steric analysis of cyclopentenones (20,26). Briefly, cyclopentenones were treated with 0.1 M NaOH in 90% aqueous methanol in order to effect *cis* → *trans* isomerization of the side chain attached α to the ring carbonyl group. Following reesterification and purification by TLC (solvent system: ethyl acetate/hexane. 20:80, vol/vol), the material was treated with sodium borohydride and hydrogenated. The resulting epimers of methyl 12-hydroxyphytonoate and its homologs were separated by TLC (solvent system: ethyl acetate/hexane, 20:80, vol/vol) and separately derivatized with MC chloride. The MC derivatives were purified by TLC (solvent system: ethyl acetate/hexane, 7:93, vol/vol) and subsequently analyzed by GC–MS. Most analyses were performed in the selected-ion-monitoring (SIM) mode using the ion formed by elimination of MCOH ($M^+ - 200$). Authentic reference compounds having 16 or 18 carbon atoms chain length were synthesized (present work) or were available from earlier work (26), respectively.

Chromatographic and instrumental methods. Analytical RP-radio-HPLC was performed with a column of Nucleosil 100-5 C_{18} (250 × 4.6 mm) purchased from Macherey-Nagel (Düren, Germany). The solvent system used consisted of acetonitrile/water/acetic acid (55:45:0.02, by vol) and the flow rate was 1 mL/min. The absorbance (221 nm) and radioactivity of HPLC effluents were determined on-line using a Spectromonitor III UV detector (Laboratory Data Control, Riviera Beach, FL) and a liquid scintillation counter (IN/US Systems, Tampa, FL), respectively. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methyl silicone capillary column (length, 25 m; film thickness, 0.33 µM). Helium at a flow rate of 25 cm/s was used as the carrier gas. Retention times found on GLC were converted into C-values as described (30). GC–MS was carried out with a Hewlett-Packard model 5970B mass selective detector connected to a Hewlett-Packard model 5890 gas chromatograph. The scan mode was used for identification of reaction products, whereas the selected ion monitoring mode was used for accurate analysis of diastereomeric MC derivatives. Optical rotation was measured using a Schmitt & Haensch polarimeter (Berlin, Germany) model NH8. Hexane was used as solvent, and the path length was 1 dm.

RESULTS

Ten allene oxides were used to test the substrate specificity of AOC (Scheme 2). Based on the most commonly used linolenic acid derived substrate, 12,13(*S*)-EOT, we chose allene oxides derived from the n-3 or n-6 series of polyunsaturated fatty acids and differing with respect to the position of the epoxide group (n-6,7 or n-9,10), chain length (16 to 22 carbons) and number of double bonds at the carboxyl terminus.

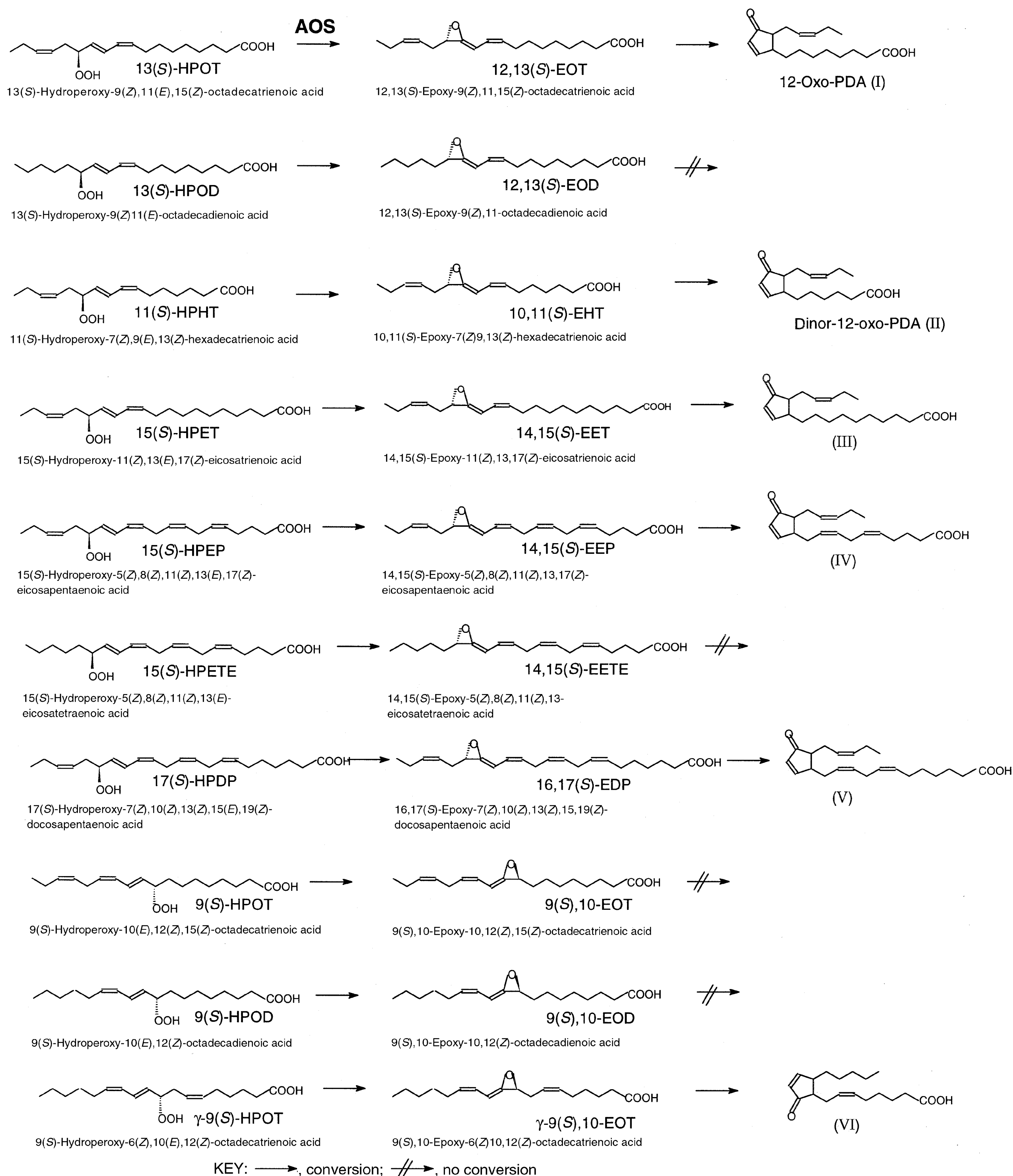
Substrate specificity of AOS. The AOS-catalyzed generation of allene oxides from hydroperoxides is a prerequisite to perform the AOC assay. It was therefore necessary to check whether all the hydroperoxides were substrates for AOS. For this reason, we determined the K_m and v_{max} values for each hydroperoxide using the 100,000 × *g* membrane pellet from the corn seed homogenate as AOS preparation. As shown in Table 1, all hydroperoxides were converted by AOS, but with rather different kinetics. Roughly, one can sort the substrates into three different groups: group I with a high K_m (30–40 µM) and high v_{max} (above 100 nkat mg^{-1}), such as γ-9(*S*)-HPOT, 9(*S*)-HPOD and 13(*S*)-HPOD; group II with a rather high affinity (K_m value below 25 µM) and a lower reaction velocity [v_{max} between 15 and 60 nkat mg^{-1}), like 15(*S*)-HPET, 13(*S*)-HPOT, 11(*S*)-HPHT, 15(*S*)-HPETE]; and group III with the highest affinity (K_m below 15 µM) and a very low v_{max} of less than 15 nkat mg^{-1}, such as 17(*S*)-HPDP and 15(*S*)-HPEP. 9(*S*)-HPOT represents one exception in this ranking since it has a low affinity but a medium reaction velocity.

Nonenzymatic cyclization of allene oxides. Ten unstable allene oxides (Scheme 2) were generated by incubation of the appropriate hydroperoxide precursors with AOS and subsequently allowed to decompose in the absence of AOC. The material isolated by extraction with diethyl ether was methylesterified, purified by TLC, and analyzed by GC–MS following trimethylsilylation. All 10 allene oxides produced α-ketols which were readily identified by their C-values and mass spectra. Six allene oxides, i.e., those generated from 13(*S*)-HPOT, 11(*S*)-HPHT, 15(*S*)-HPET, 15(*S*)-HPEP, 17(*S*)-HPDP, and γ-9(*S*)-HPOT, produced cyclopentenones (Scheme 2, Table 2). The mass spectra of the six cyclopentenones all showed prominent ions due to M^+ and $M^+ - 31$ (loss of OCH_3). Cyclopentenones derived from the five first-men-

TABLE 1
Substrate Specificity of Allene Oxide Synthase[a,b]

Substrate[c]	K_m (µM)	v_{max} (nkat mg^{-1})
γ-9(*S*)-HPOT	37.1 ± 2.8	158.9 ± 17.4
9(*S*)-HPOD	32.0 ± 1.9	143.7 ± 6.7
13(*S*)-HPOD	37.4 ± 6.5	139.5 ± 32.8
9(*S*)-HPOT	38.9 ± 7.7	72.6 ± 6.7
15(*S*)-HPET	20.9 ± 6.2	51.6 ± 9.6
13(*S*)-HPOT	11.1 ± 1.5	33.9 ± 9.1
11(*S*)-HPHT	19.2 ± 5.3	24.7 ± 0.4
15(*S*)-HPETE	11.6 ± 1.1	19.4 ± 1.6
17(*S*)-HPDP	10.0 ± 2.0	12.4 ± 0.9
15(*S*)-HPEP	4.6 ± 1.3	7.4 ± 2.8

[a]Maximal initial velocity (v_{max}) and Michaelis constant (K_m) were determined as described in detail in the Materials and Methods section.
[b]Data represent the mean value ± SD of at least four replicates.
[c]Abbreviations and structures of substrates are given in Scheme 2.

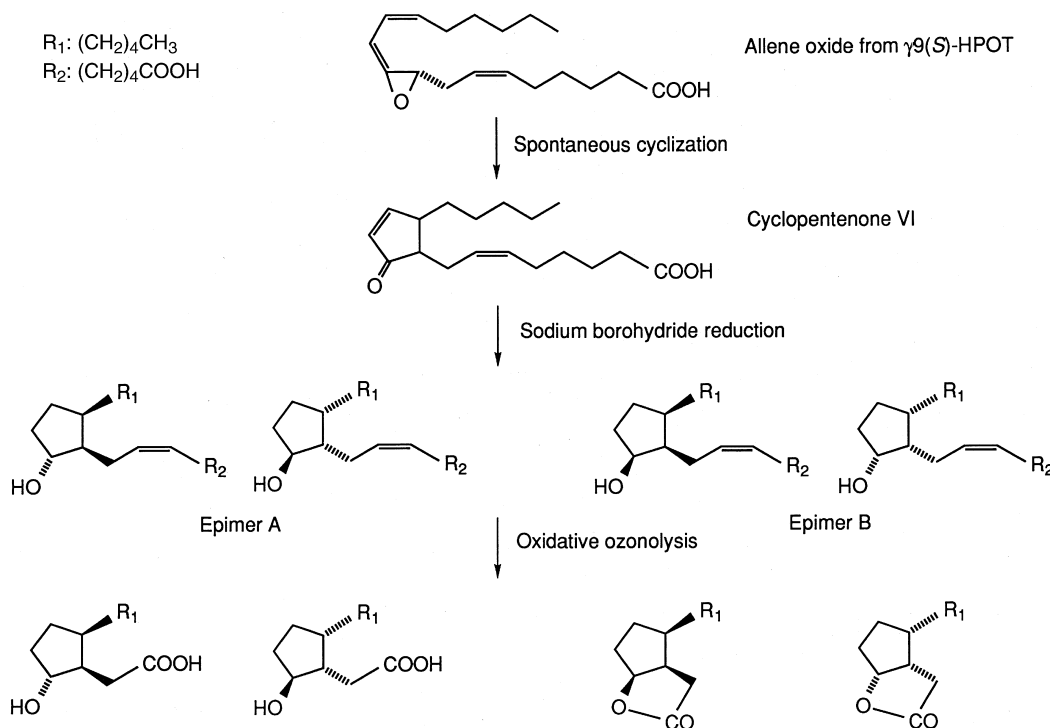


SCHEME 2

tioned hydroperoxides showed an ion due to $M^+ - 68$ (loss of C_5H_8, i.e., the pentenyl side chain minus one hydrogen by cleavage β to the ring carbonyl). The three first-mentioned hydroperoxides afforded cyclopentenones that had a saturated carboxylic ester side chain. During electron impact, cleavage occurred α, β, or γ to the ring-carbon to which the carboxyl

chain was attached resulting in prominent ions at *m/z* 149, 163, and 177, respectively. Cyclopentenones produced from 15(*S*)-HPEP and 17(*S*)-HPDP possessed two double bonds in the carboxylic acid chain. In these cases, fragmentation took place mainly by α-cleavage at the carboxylic side chain-bearing carbon with elimination of the carboxylic side chain minus one hydrogen (*m/z* 150). Ions due to the charged carboxylic side chain (*m/z* 181 and 209, respectively) were also observed. The mass spectrum of the cyclopentenone formed from the allene oxide generated from γ9(*S*)-HPOT showed a diagnostic ion at *m/z* 152 (M^+ – 154) due to β-cleavage and loss of C_7H_{11}-$COOCH_3$, i.e., the carboxyl side chain minus one hydrogen. To determine the arrangement and stereochemistry of the side chains, a larger amount (1 mg) of the methyl ester of this cyclopentenone was prepared by scaling up the incubation protocol. The UV spectrum showed an absorption band with λ_{max} (EtOH) = 221 nm (ε 14,000). An aliquot of the material was reduced by treatment with sodium borohydride to afford two cyclopentanol epimers separable by TLC (solvent: ethyl acetate/hexane, 25:75, vol/vol), i.e., epimer A (minor; R_f = 0.42) and epimer B (major; R_f = 0.60) (Scheme 3). The mass spectra of the trimethylsilylated epimers showed prominent ions at *m/z* 382 (M^+), 367 (M^+ – 15; loss of CH_3), 311 (M^+ – 71; loss of C_5H_{11}), and 292 (M^+ – 90; loss of trimethylsilanol). The relative stereochemistry of the ring hydroxyl group and the carboxyl side chain of epimers A and B was determined by subjecting the acetylated epimers to oxidative ozonolysis. The product was saponified, acidified to pH 1, and extracted with 2 vol of diethyl ether. GC–MS analysis of the methyl-esterified oxidation product produced from epimer A demonstrated the presence of the methyl ester of a C_{12} cyclopentol fatty acid formed by cleavage of the side-chain double bond (Scheme 3). In the case of epimer B, the oxidation product was mainly due to the γ-lactone form of a C_{12} cyclopentanol fatty acid. These results demonstrated that the stereochemical relationship between the ring hydroxyl group and the carboxyl side chain was *trans* in epimer A and *cis* in epimer B (Scheme 3).

Aliquots of the six cyclopentenones obtained by nonenzymatic cyclization of allene oxides were subjected to steric analysis and found to be racemic or nearly so (Table 2). As seen in Table 2 and Scheme 2, cyclization of allene oxides lacking a double bond in the β,γ position relative to the epoxide group could not be detected. In the case of 12,13(*S*)-EOD, i.e., the allene oxide generated from 13(*S*)-HPOD, a specific search for its putative cyclization product (15,16-dihydro-12-oxo-PDA) was performed. In these experiments, 13(*S*)-HPOD (150 μM) was incubated with corn AOS in the presence or in the absence of AOC, and the product was treated with sodium hydroxide in order to isomerize any 15,16-dihydro-12-oxo-PDA into its side chain *trans* isomer, of which an authentic reference sample was available (27). By selected monitoring of the ion *m/z* 308 (M^+), the amount of the 15,16-dihydro-12-oxo-PDA derivative was found to correspond to a yield from the incubated 13(*S*)-HPOD of 0.2% or less. This yield is *ca.* 100 times lower than that of 12-oxo-PDA formed from 13(*S*)-HPOT in the absence of AOC. As expected, when bovine serum albumin (15 mg mL^{-1}) was added to such incubations, formation of the 15,16-dihydro-12-oxo-PDA isomer



SCHEME 3

TABLE 2
Substrate Specificity of Allene Oxide Cyclase

Substrate[b]	Cyclopentenone[b]	R_f-value	C-value	Enantiomeric composition (% *S/S*)[a]		
				No AOC	Corn AOC	Potato AOC
12,13(*S*)-EOT	I	0.45	20.10	50	89	95
12,13(*S*)-EOD	—	—	—	—	—	—
10,11(*S*)-EHT	II	0.35	18.09	49	90	96
14,15(*S*)-EET	III	0.50	22.18	50	89	96
14,15(*S*)-EEP	IV	0.48	21.52	49	94	N.D.
14,15(*S*)-EETE	—	—	—	—	—	N.D.
16,17(*S*)-EDP	V	0.53	23.55	50	94	N.D.
9,10(*S*)-EOT	—	—	—	—	N.D.	
9,10(*S*)-EOD	—	—	—	—	N.D.	
γ-9,10(*S*)-EOT	VI	0.43	19.87	48	49	51

[a]% *S/S* represents the amount of PDA in *S/S* form compared to the total amount of PDA.
[b]Structures of substrates and cyclopentenones are given in Scheme 2. N.D., not determined; AOC, allene oxide cyclase.

(side chain *trans* isomer; C-value, 19.71) was readily observed (27).

Enzymatic cyclization of allene oxides. Corn or potato AOC enhanced the formation of cyclopentenones from allene oxides generated from the three homologous trienoic acid hydroperoxides 11(*S*)-HPHT, 13(*S*)-HPOT, and 15(*S*)-HPET, as well as from the pentaenoic acid hydroperoxides 15(*S*)-HPEP and 17(*S*)-HPDP. Steric analysis demonstrated that these cyclopentenones were mainly due to the natural *S,S* isomers (Table 2, Scheme 2). The enantiomeric excess of cyclopentenones, which directly reflects the extent of enzyme catalysis in their formation, varied between 78 and 92%. As described above, the structural requirement for nonenzymatic cyclization of allene oxides to take place was found to be the presence of an isolated double bond located in the β,γ position with respect to the epoxide group. This structural element was present in all of the five allene oxides that served as substrates for AOC. Interestingly, the allene oxide γ-9(*S*),10-EOT, which also possessed such a double bond, but in which compound the epoxide group was located in the n-9,10 position rather than that at n-6,7, did not undergo enzymatic cyclization in the presence of corn or potato AOC (Table 2, Scheme 2).

Inhibition of AOC. As mentioned above, allene oxides lacking a double bond in the β,γ position relative to the epoxide group did not undergo chemical or enzymatic cyclization into cyclopentenones. It seemed conceivable that allene oxides belonging to this group might serve as competitive inhibitors of AOC. In order to test this possibility, allene oxides were generated by preincubation of the corresponding hydroperoxides (100 μM) with AOS in the presence of AOC. After 10 s, [1-^{14}C]13(*S*)-HPOT (40 μM) was added and the mixture was stirred for an additional 10 min. Analysis of the yield of ^{14}C-labeled 12-oxo-PDA relative to α-ketol by RP-radio-HPLC demonstrated a significant inhibition of AOC-catalyzed formation of 12-oxo-PDA in the presence of nonsubstrate allene oxides containing the epoxide group in the n-6,7 position [12,13(*S*)-EOD and 14,15(*S*)-EETE] (Fig. 1). Allene oxides having the epoxide group in the n-9,10 position [9(*S*),10-EOD, 9(*S*),10-EOT, γ-9(*S*),10-EOT] had no inhibitory effect. The inhibition by 12,13(*S*)-EOD and 14,15(*S*)-EETE was concentration-dependent and reversible with higher substrate concentrations (data not shown), indicating a competitive inhibition.

In another series of experiments, we explored the inhibition of AOC by chemically stable fatty acids incorporating one or more structural elements in common to the natural al-

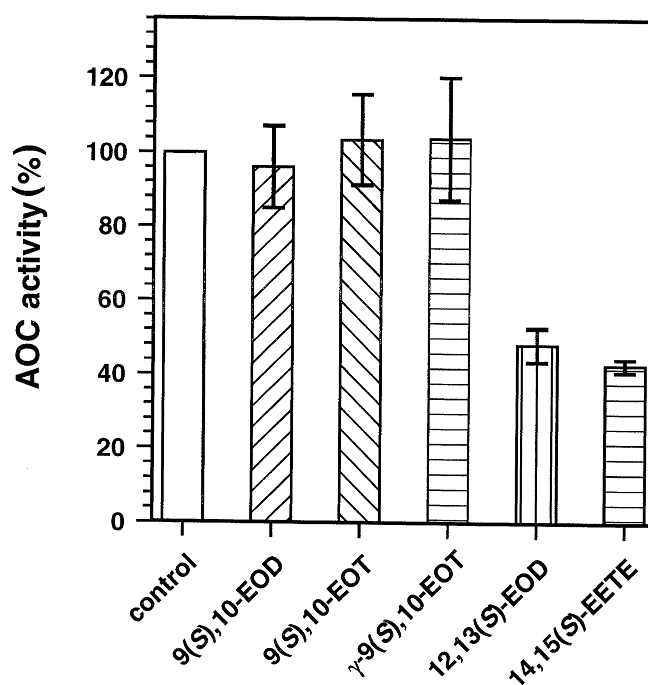


FIG. 1. Effect of nonsubstrate allene oxides on allene oxide cyclase (AOC) activity. The allene oxides were generated from their corresponding hydroperoxide precursors (100 μM). The substrate for AOC in all incubations consisted of 12,13(*S*)-EOT [12,13(*S*)-epoxy-9(*Z*),11,15(*Z*)-octadecatrienoic acid] generated from 13(*S*)-HPOT [13(*S*)-hydroperoxy-9(*Z*),11(*E*),15(*Z*)-octadecatrienoic acid]. Error bars indicate SD (n = 3). Other abbreviations: 9(*S*),10-EOD, 9(*S*),10-epoxy-10,12(*Z*)-octadecadienoic acid; 9(*S*), 10-EOT, 9(*S*),10-epoxy-10,12(*Z*),15(*Z*)-octadecatrienoic acid; γ-9(*S*),10-EOT, 9(*S*),10-epoxy-6(*Z*),10,12(*Z*)-octadecatrienoic acid; 12,13(*S*)-EOD, 12,13(*S*)-epoxy-9(*Z*),11-octadecadienoic acid; 14,15(*S*)-EETE, 14,15(*S*)-epoxy-5(*Z*),11(*Z*),13-eicosatetraenoic acid.

lene oxide substrates (Scheme 4; abbreviations in parentheses in this scheme correspond to Fig. 2). As seen in Figure 2, AOC activity was strongly inhibited by a number of n-6,7 epoxy fatty acids, i.e., (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid, (±)-*trans*-12,13-epoxy-9(*Z*)-octadecenoic acid, and (±)-*cis*-14,15-epoxy-11(*Z*)-eicosenoic acid. The two enantiomers of the first-mentioned epoxy acid were prepared and individually tested for inhibitory potency. The (−) form caused a significantly stronger inhibition compared to the (±)-epoxide, whereas the (+)-form was a weaker inhibitor than the (±)-epoxide (Figs. 2 and 3). Interestingly, the (−)-form [12(*R*),13(*S*)-epoxy-9(*Z*)-octadecenoic acid] has the same stereochemistry at C-13 as the natural substrate 12,13(*S*)-EOT. The strongest inhibition observed in this series of compounds was that of (±)-*trans*-12,13-epoxy-9(*Z*)-octadecenoic acid and (−)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid (IC_{50}, 10–20 μM). The inhibitory effect of the n-6,7 epoxy acids was reversible with higher substrate concentration, indicating a competitive inhibition (data not shown). The methyl esters of the n-6,7 epoxy acids lacked the inhibitory effect. Furthermore, no inhibition was observed with an n-9,10-epoxy acid [(±)-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid].

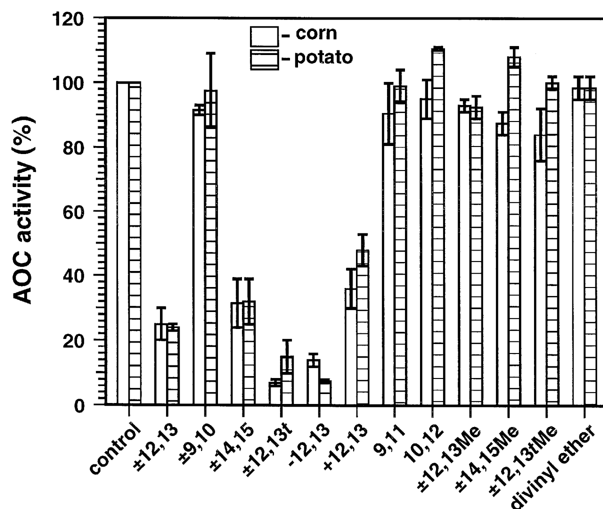
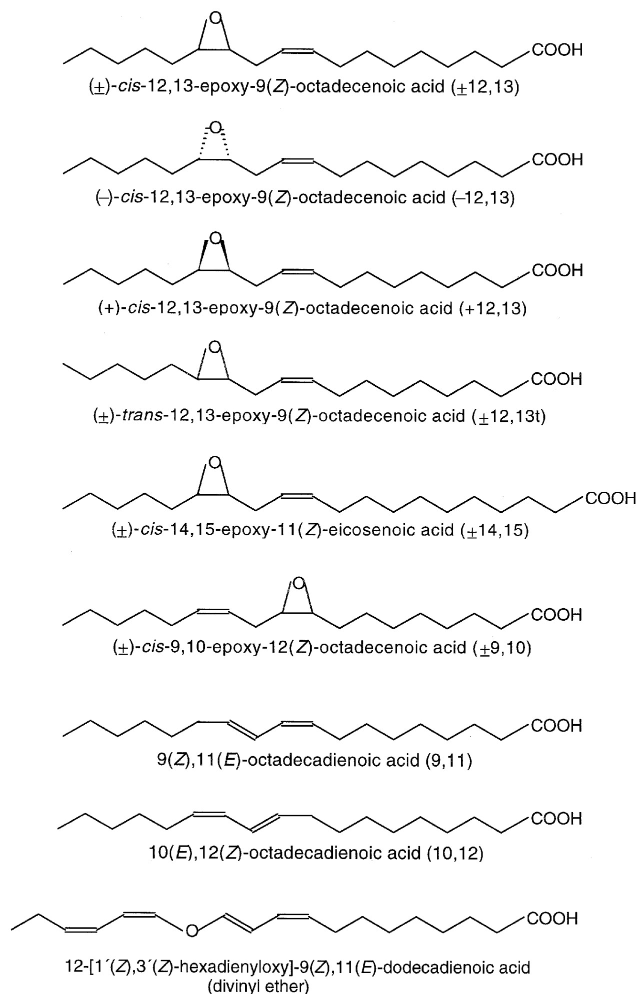


FIG. 2. Effect of substrate analogs on AOC activity at a final concentration of 100 μM each. Me = methyl ester of the respective compound. Error bars indicate SD (*n* = 3). For abbreviations see Figure 1 and Scheme 4.



SCHEME 4

Besides the importance of the position and stereochemistry of the epoxide group, we also tested whether the arrangement of double bonds in a carbon chain solely might exert an influence on AOC activity. As shown in Figure 2, neither the 10,12 nor the 9,11 unsaturated fatty acids have any effect on the enzyme, although the position of double bonds in the latter compound is identical to the inhibitory allene oxide 12,13(*S*)-EOD.

To examine whether the oxygen in position 12,13 has to be present as an epoxide function, we included a divinyl ether fatty acid (12-[1′(*Z*),3′(*Z*)-hexadienyloxy]-9(*Z*),11(*E*)-dodecadienoic acid, see Ref. 12) in our study that bears the oxygen as an ether-bridge but also at position 12. This ether was not able to inhibit the AOC activity up to a concentration of 100 μM.

It may be concluded that the presence of an epoxide group (allene oxide or saturated epoxide) located in the n-6,7 position of the fatty acid chain is a critical structural feature of compounds serving as competitive inhibitors of AOC. Another important structural element is the carboxyl group, which apparently must exist in its nonesterified form.

DISCUSSION

The kinetic parameters of AOS from corn presented here for different substrates show that the enzyme tends to convert the n-10 hydroperoxides with a lower affinity, but with a higher reaction velocity than the n-6 hydroperoxides. Additional double bonds near the carboxyl terminus attribute to a further decrease in the K_m and v_{max} values, whereas the chain length does not show a pronounced effect. Furthermore, the presence of a double bond at position n-3 significantly increases the affinity and lowers the v_{max} of AOC. The substrate specificities shown here for the corn enzyme are similar to those of the recombinant AOS from barley leaves (Maucher, H.,

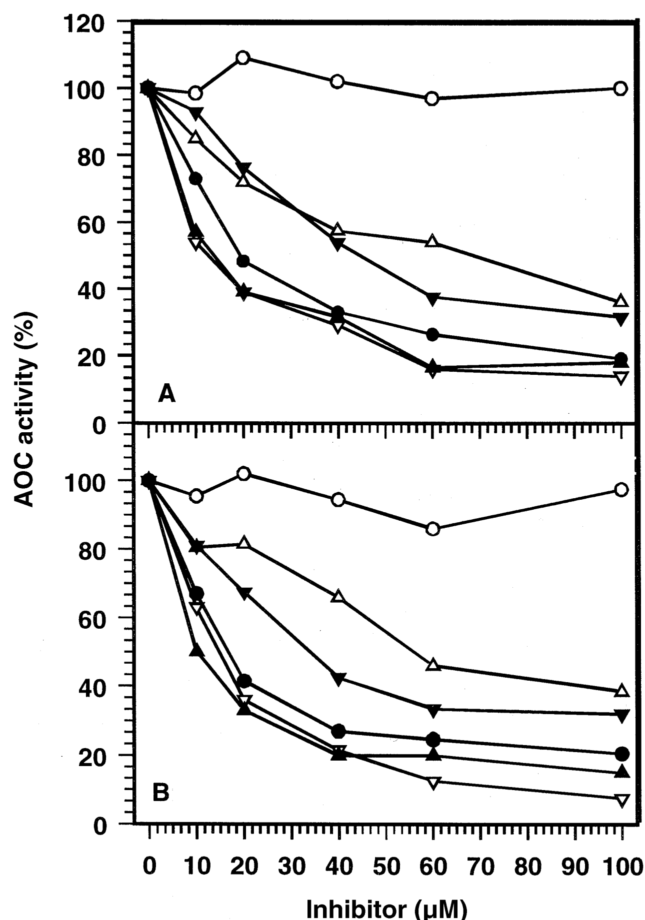


FIG 3. Dose-dependent effect of substrate analogs on AOC activity of (A) corn and (B) potato. (●), (±)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid; (△), (+)-*cis*-12,13-epoxy-9(*Z*)-octadececenoic acid; (▽), (−)-*cis*-12,13-epoxy-9(*Z*)-octadecenoic acid; (▲), (±)-*trans*-12,13-epoxy-9(*Z*)-octadecenoic acid; (▼), (±)-*cis*-14,15-epoxy-11(*Z*)-eicosenoic acid; (○), (±)-*cis*-9,10-epoxy-12(*Z*)-octadecenoic acid. The mean values of three replicates are shown.

Hause, B., Feussner, I., Ziegler, J., and Wasternack, C., in preparation) and guayule (31), where a steady decrease in K_m and v_{max} in the order 13(*S*)-HPOD > 13(*S*)-HPOT > 15(*S*)-HPETE was also observed. The AOS from flax showed only minor differences in affinity toward 13(*S*)-HPOD, 9(*S*)-HPOD, and 9(*S*)-HPOT but, in contrast to AOS from corn and barley, converted the 13-hydroperoxide at a rate almost 40-fold higher than that of the 9-hydroperoxides (32).

Interestingly, the affinity of the corn AOS is generally higher toward substrates that give rise to allene oxides that can later be cyclized enzymatically. Regarding the "naturally" occurring substrates present in plants, it is worth mentioning that those hydroperoxides, which are precursors of physiologically active cyclopentenones, such as 12-oxo-PDA from 13(*S*)-HPOT and dinor-12-oxo-PDA from 11(*S*)-HPHT, possess a higher affinity to the AOS from corn and barley than 9(*S*)-HPOD, 9(*S*)-HPOT, or 13(*S*)-HPOD, whose allene oxides are not metabolized to physiologically active compounds known so far.

The occurrence and identification of cyclopentenones after incubation of isomeric fatty acid hydroperoxides with AOS either in the absence or presence of AOC was determined by GC–MS after TLC purification. The method chosen to distinguish between nonenzymatic and AOC-catalyzed cyclization of allene oxides was based on steric analysis of cyclopentenones. That the enantiomeric excess, i.e., the difference between percentage of 9(*S*),13(*S*)-cyclopentenone and of 9(*R*),13(*R*)-cyclopentenone, can be taken as a direct measure of the extent of enzymatic cyclization has been confirmed previously (20).

Nonenzymatic cyclization into cyclopentenones of the 12-oxo-PDA type was documented for six allene oxides, all of which had an isolated double bond in the β,γ position relative to the epoxide group (Table 2, Scheme 2). A zwitterionic species is formed as an intermediate in chemical cyclizations of allene oxides (33), and it has been suggested that a double bond in the β,γ position will stabilize such an intermediate by partial π-electron overlap and hence promote cyclization (34). The importance of this double bond for cyclization was pointed out in 1979, at a time when allene oxides and AOC were unknown in biochemistry (35).

The possibility of spontaneous, chemical cyclization of allene oxides raised the question whether 12-oxo-PDA in plant tissue is formed, partially or wholly, by a nonenzymatic reaction independent of AOC. Although JA isolated from plant tissues is optically pure and has a configuration corresponding to that of 12-oxo-PDA formed enzymatically *in vitro*, in theory this might be attributed to the stereospecificity of enzyme(s) catalyzing further conversion of 12-oxo-PDA to JA rather than to the stereospecificity of the cyclization step. Recent studies concerned with the absolute configuration and optical purity of 12-oxo-PDA in intact plant tissue are not in agreement with such a hypothesis and reaffirm the importance of AOC. Thus, 12-oxo-PDA extracted from plant leaves is the pure 9(*S*),13(*S*) stereoisomer (36). Furthermore, it was recently found that constitutive, as well as wound-induced levels of 12-oxo-PDA in tomato leaves, are exclusively due to the 9(*S*),13(*S*) enantiomer (Hamberg, M., in preparation).

For enzymatic cyclization to take place, it was found that the structural requirements for the chemical cyclization had to be fulfilled, with the added requirement that the allene oxide be located with its epoxide group in the n-6,7 position of the fatty acid chain (Table 2, Scheme 2). This is exemplified by γ-9(*S*),10-EOT, which only cyclizes chemically and is not a substrate for AOC from potato or corn. In flaxseed extracts, the conversion of γ-9(*S*)-HPOT into the respective cyclopentenone had been observed earlier (37), but at that time, the existence of allene oxides and the mechanisms of chemical and enzymatic cyclization were not known. Flaxseed does not contain AOC (38), and in retrospect it seems likely that the conversion of γ-9(*S*)-HPOT into a cyclopentenone observed in flaxseed extracts (37) was due to chemical cyclization of γ-9(*S*),10-EOT.

These two requirements will limit the AOC-catalyzed formation of cyclopentenones to allene oxides generated from

polyunsaturated fatty acids of the n-3 type. The biosynthesis of 15,16-dihydro-12-oxo-PDA from linoleic acid, an n-6 fatty acid, has been suggested (39); and minute amounts of 9,10-dihydro-JA have been isolated from plant tissue (40). In previous work (20,34,35), the formation of the putative 15,16-dihydro-12-oxo-PDA by enzymatic or nonenzymatic cyclization of the allene oxide derived from 13(*S*)-HPOD could not be detected. In the present study, the specific search for the putative 15,16-dihydro-12-oxo-PDA, made by mass spectrometry performed in the sensitive selected-ion-monitoring mode, showed that the extent of cyclization of the linoleic acid-derived allene oxide was 1% or less compared to that of the α-linolenic acid-derived allene oxide. Serum albumin of several mammalian species stabilizes allene oxides and also induces formation of a nearly racemic 15,16-dihydro-12-oxo-PDA derivative, which has, however, its two side chains arranged in the *trans* orientation instead of the *cis* for the natural 12-oxo-PDA (27). This also means that the mechanism of albumin-induced cyclization differs from that of chemical and AOC-catalyzed cyclization. Bovine serum albumin-induced cyclization of allene oxides was recently confirmed by co-incubation with 13(*S*)-HPOD with flaxseed AOS (41); however, the relevance of this finding for the biosynthesis of the putative 15,16-dihydro-12-oxo-PDA in plant tissues is unclear. It should be noted in this context that the albumin-promoted cyclization is not a general "protein effect" but requires that the allene oxide structure be bound to hydrophobic binding sites of the albumin molecule (27).

With respect to the substrate specificities of lipoxygenase and AOS, AOC seems to be the first (and probably only) enzyme of the jasmonate cascade that shows an absolute requirement for an n-3 fatty acid-derived substrate. In comparing the specificity of chemical and enzyme-catalyzed cyclization, it is likely that both types proceed by the same mechanism, which involves anchimeric assistance by π-electron overlap. This would identify a specific function for the n-3 double bond of α-linolenic acid, which can be described in purely chemical terms (4).

It was noteworthy that 10,11(*S*)-EHT could be cyclized in the presence of AOC from corn and potato into optically active dinor-12-oxo-PDA. This supports the suggestion that the biosynthesis of this cylopentenone, which was recently detected in *Arabidopsis thaliana* (42), occurs by the same steps as those in the formation of 12-oxo-PDA.

AOC was inhibited by allene oxides having the epoxide group in the n-6,7 position but lacking the n-3 double bond. Likewise, various stable n-6,7 epoxy fatty acids served as potent competitive inhibitors of AOC. The two enantiomers of one such epoxy acid were individually tested as inhibitors. The epoxy acid with the same absolute configuration at n-6 as the natural allene oxide substrates, i.e. "*S*," was a significantly better inhibitor compared with the racemic epoxide, whereas the n-6(*R*) epoxide was a weaker inhibitor than the racemic epoxide. Analogs with the epoxy group in n-9,10 or that carry the oxygen as an ether function, as well as nonoxidized unsaturated fatty acids, were ineffective.

One can assume therefore that recognition and binding of substrates proceed by the epoxide group in the n-6,7 position. In addition, the substrates seem to enter the active site of AOC *via* their ω-terminal end, since cyclization of allene oxides or inhibition by fatty acid derivatives is not affected by the number of carbons at their carboxyl terminus, whereas the position of the epoxide group in n-6,7 is critical. But the presence of a nonesterified carboxyl group also is required to ensure binding to AOC since methylation of substrate analogs abolished their inhibitory effect on the enzyme activity. Once recombinant AOC becomes available, it will be possible to broaden the current knowledge by mutational analysis of AOC to learn more about the biochemistry of this important step in the biosynthesis of jasmonic acid.

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Effects of Conjugated Linoleic Acid on Oxygen Diffusion-Concentration Product and Depletion in Membranes by Using Electron Spin Resonance Spin-Label Oximetry

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ABSTRACT: The effect of conjugated linoleic acid (CLA) on the relation between structure and function of membranes is described in this paper. Electron spin resonance (ESR) spin-label oximetry was used in the present study to evaluate if oxygen transport and oxygen depletion were affected by incorporation of CLA instead of linoleic acid into membrane phospholipids. Specifically, 1-stearoyl-2-(9*cis*,11*trans*-octadecadienoyl)-phosphorylcholine (SCLAPC) was incorporated into soy plant phosphatidylcholine (soy PC) or egg yolk PC (EYPC) bilayers. The use of spin labels attached to different carbons along the fatty acid chain makes it possible to carry out structural and oximetric determinations with the same test sample. For example, the incorporation of 5 mol% SCLAPC increased the oxygen diffusion-concentration product in soy PC or EYPC liposomes at 37°C, slightly decreased the ordering of the hydrocarbon chains at the C10 and C12 positions (in the region of the conjugated double bonds), and increased the rate of oxygen depletion from the aqueous medium. Similar results were not obtained by incorporating 5 mol% of 1-stearoyl-2-linoleoyl-PC (SLPC). In our model system, free-radical generation was initiated by extended incubation of the liposomes, by induction by 2,2′-azobis(2-amidinopropane)hydrochloride, or by ultraviolet irradiation of H_2O_2. The rate of consumption of molecular oxygen was studied by monitoring the oxygen concentration in the aqueous phases of the liposomes. The effect of 5 mol% SCLAPC in soy PC was significantly larger than 5 mol% SLPC in soy PC; the response patterns with soy PC and EYPC were similar. Furthermore, 5 mol% SCLAPC in 1-palmitoyl-2-linoleoyl-PC showed similar oxygen consumption to that observed with 5 mol% SCLAPC in EYPC. On the other hand, 5 mol% SCLAPC in synthetic PC membranes containing saturated or monounsaturated fatty acids showed low oxygen depletion rates. The perturbation of membrane structure and the increase of the relative oxygen diffusion-concentration products provided a potential mechanism by which CLA incorporated into membrane lipids could affect oxidative stress.

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Conjugated linoleic acid (CLA) has received unprecedented interest because of its reported anticarcinogenic (1,2), antiatherogenic (3,4), and fat repartitioning (5,6) effects. It also increases bone mass (7) and normalizes diabetic (8) effects in experimental animal models. At first, the anticarcinogenic response was attributed to the antioxidant activity of CLA (1,2). However, further investigation showed that CLA functions as a prooxidant (9,10), and its effectiveness is different depending on its chemical form, i.e., as free fatty acids, methyl esters, or as a constituent in triacylglycerols or phospholipids (9–11). In biological systems, CLA is incorporated into phospholipids (2,12,13), which are the major structural components of cell membranes. Visonneau *et al*. (14) reported that feeding 1% CLA in the diet inhibits local tumor growth of human breast adenocarcinoma cells inoculated subcutaneously into severe combined immunodeficient mice. Furthermore, CLA completely prevented the spread of breast cancer cells to lungs, peripheral blood, and bone marrow, whereas metastatic spread of transplantable mammary tumors was observed in control animals. This inhibition of metastasis suggests that CLA may provide protective biological effects.

Several matrices were previously used to evaluate the mechanism by which CLA functions in biological systems; however, each one of these experiments was conducted under nonphysiological conditions and/or had limitations. Ha *et al*. (1) used a phosphate buffer/ethanol mixture with free CLA. Ip *et al*. (2) used the thiobarbituric acid-reactive substances assay known to measure only selected oxidation products. Van den Berg *et al*. (9) prepared model phospholipid membranes consisting of 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)hydrochloride; CLA, conjugated linoleic acid; CTPO, 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl; DMPC, L-α-dimyristoylphosphorylcholine; ESR, electron spin resonance; EYPC, egg yolk phosphatidylcholine; n-PCSL, 1-palmitoyl-2-stearoyl-(n-doxyl)-*sn*-glycero-3-phosphorylcholine; PLPC, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphorylcholine; SCLAPC, 1-stearoyl-2-(9*cis*,11*trans*-octadecadienoyl)-phosphorylcholine; SLPC, 1-stearoyl-2-linoleoyl-PC; SOPC, 1-stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine; soy PC, soy plant phosphatidylcholine; TEMPO, 4 (*N*,*N*-dimethyl-*N*-(2-hydroxylethyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl; UV, ultraviolet.

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phosphorylcholine (PLPC), but added CLA as the free fatty acid. Chen *et al*. (10) prepared mixtures of CLA (as free acid, methyl ester, or triacylglycerol) with canola oil in hexane and measured the decrease of oxygen in the headspace after heating the solutions at 90°C for 1–2 d. In the present study, we used 1-stearoyl-2-(9*cis*,11*trans*-octadecadienoyl)-phosphorylcholine (SCLAPC), prepared by custom synthesis, and compared it to other synthetic and natural phosphatidylcholines in membrane vesicles. The electron spin resonance (ESR) spin-label oximetry method was used to measure oxygen uptake and to study membrane structure and dynamic properties in the presence or absence of CLA containing phospholipids. There is much controversy on the anti- or prooxidation activity of CLA in membranes. In this work, we studied the effects of CLA on membrane structure and fluidity, on the collision rate between oxygen and lipids at different locations of the membrane, and on the oxygen depletion rates during lipid peroxidation in membranes.

Oxygen is essential to provide normal conditions for intact cell functions. Variation in oxygen availability often has a profound impact on physiological and homeostatic processes within living organisms. Because changes in oxygen concentration in living cells reflect the rate of oxygen consumption and degree of oxygen permeability of the plasma membrane, it is important to measure the effect of CLA on collision rate between oxygen and lipids in membranes to determine the structure–activity relationship of membranes. It is known that lipid peroxidation is a free radical-mediated chain reaction that consists of initiation, propagation, and termination (15–17). However, possible molecular mechanisms that specifically address the effect of CLA on oxidative stress in membranes are still under investigation (1,2,9–11).

Lipid peroxidation and biological defense mechanisms have been studied intensively (18–20). The highly sensitive, noninvasive ESR oximetry method enables one to study early stages of lipid peroxidation by measuring oxygen uptake (21–23). Ongoing conceptual and methodological advancements concerning measurements of oxygen concentration, diffusion, transport, and related issues, e.g., oxygen-mediated reactions, underscore the fundamental importance of this burgeoning field of studies pertaining to health and disease (24,25). ESR oximetry methods are based on bimolecular collisions of O_2 with spin labels, which effectively shorten the spin-lattice relaxation time and broaden the line width of the ESR signal. Possible methods for observing these collisions, classified as T_1- or T_2-sensitive, are outlined elsewhere (26,27). Among them, the T_2-sensitive method (or line width method) is the more widely used for ESR oximetry, for both *in vivo* and *in vitro* studies (28–32).

The ESR spin-labeling and ESR oximetry method have been successfully employed to investigate membrane structure and functions in the studies of fumonisin–membrane interactions (33–35). We found that there are apparent correlations between the perturbation effect of fumonisin (a mycotoxin) on membrane structure (on membrane surface) (33), enhancement on the relative oxygen diffusion-concentration production on membrane surface (34), increasing membrane leakage to small ions as well as hydrophilic solutes, and increasing lipid peroxidation rate in membranes under oxidative stress (35).

In this work, we have carried out systematic studies on the influence of 5 mol% SCLAPC on the structure and dynamic properties of phosphatidylcholine membranes. This percentage was selected based on the observed maximal effectiveness of 1% CLA in the diet in cancer prevention in animal models (2,14); this is equivalent to 5% CLA in the fat of the average human diet which consists of 20% fat. Multilamellar liposomes consisting of soy plant phosphatidylcholine (soy PC) or egg yolk PC (EYPC) were used, in addition to several synthetic phosphorylcholines: 14:0,14:0-PC (DMPC); 18:0,18:1n-9-PC (SOPC); 18:0,18:2n-6-PC (SLPC); and 16:0,18:2n-6-PC (PLPC). These phospholipids were chosen because they allowed us to compare a conjugated diene system to nonconjugated, monounsaturated, and saturated reference materials with different carbon chain lengths. Six different lipid spin labels were implemented to determine the effect of CLA on membrane structure and dynamics. These spin labels were used to study the influence of CLA on oxygen diffusion-concentration product in membranes by using a T_2-sensitive method. Free-radical generation was initiated either by incubation, induction by 2,2′-azobis(2-amidinopropane)-hydrochloride (AAPH), or ultraviolet (UV) irradiation of H_2O_2. Oxygen consumption during oxidative stress of membranes was studied by monitoring oxygen concentration in the aqueous phase.

MATERIALS AND METHODS

1-Stearoyl-2-octadec-9*cis,*11*trans*-dienoyl-*sn*-glycero-3-phosphorylcholine (18:0,CLA-PC; SCLAPC) and 1-stearoyl-2-octadec-9*cis,*12*cis*-dienoyl-*sn*-glycero-3-phosphorylcholine (18:0,18:2n-6-PC; SLPC) were custom synthesized by Matreya, Inc. (Pleasant Gap, PA). 1-Stearoyl-2-oleoyl-*sn*-glycero-3-phosphorylcholine (18:0,18:1n-9-PC; SOPC); 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphorylcholine (16:0,18:2n-6-PC; PLPC); 1-palmitoyl-2-stearoyl-(n-doxyl)-*sn*-glycero-3-phosphorylcholine (n-PCSL) where n is 5, 7, 10, 12, and 16; 2-dioleoyl-*sn*-glycero-3-phosphotempocholine (TEMPO-PC), EYPC, and soy plant PC (soy PC) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL). The fatty acid compositions of EYPC and soy PC were: 16:0, 33.95%; 16:1, 1.00%; 18:0, 10.52%; 18:1, 31.06%; 18:2, 17.72%; 20:4, 3.10%; other, 1.74%; and 16:0, 17.58%; 18:0, 4.27%; 18:1, 11.05%; 18:2, 61.18%; 18:3, 5.11%; respectively. 3-Carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl (CTPO) and L-α-dimyristoylphosphorylcholine (DMPC) were obtained from Sigma Chemical Co. (St. Louis, MO). AAPH was acquired from Polysciences (Warrington, PA).

The membranes used in this work were a multilamellar dispersion of several phosphatidylcholines containing 0.5 mol% spin labels, n-PCSL or TEMPO-PC. The former was used as a depth probe, whereas the latter was used for

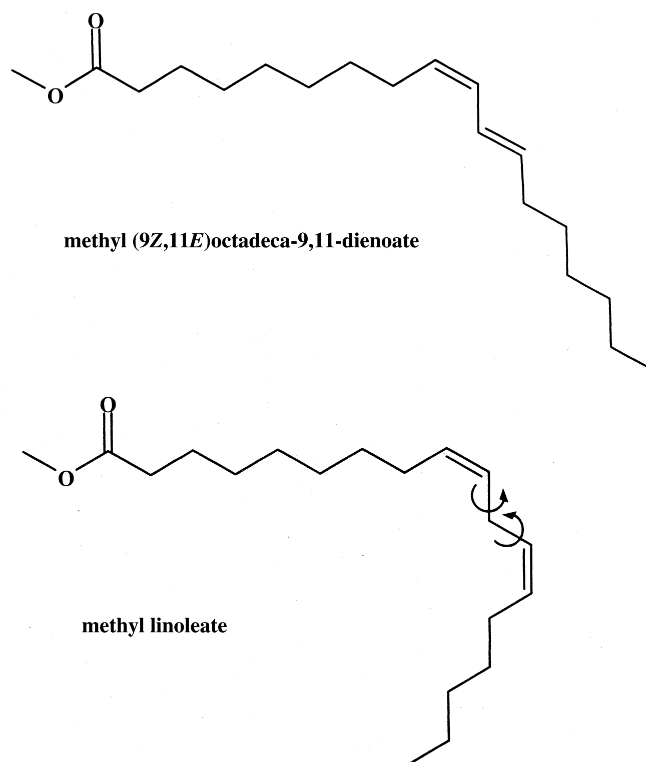
water–lipid interface measurements. Membranes were prepared according to methods of Kusumi *et al*. (36). For oxygen diffusion-concentration product studies, all test samples were run in capillaries made of the methylpentene polymer TPX (0.7 mm i.d). This plastic capillary is permeable to oxygen and nitrogen and is substantially impermeable to water (37). The TPX capillary was placed inside the ESR Dewar insert and equilibrated with a flow of various gases (nitrogen, oxygen, or air) which were also used for temperature control. Fifty-microliter glass capillaries were used for the ESR oximetry studies. CTPO is water soluble and was used at a concentration of 0.14 mM.

Conventional ESR spectra were obtained with a Varian E-109 X-band spectrometer and a variable temperature controller accessory. ESR signals were obtained with 1 mW incident microwave power and 100 kHz field modulation of 1 G (for order parameter), 0.1 G (for line broadening), or 0.05 G (for oxygen consumption) measurements. Spectra were recorded after 5 min sample preparation and temperature equilibration, stored, and manipulated in an IBM computer (program VIKING was obtained from The National Biomedical ESR Center, MCW, Milwaukee, WI). A total of 1024 data points were taken by using a scan width of 100 G (for order parameter) and 5 or 8 G (for line broadening and oxygen consumption). UV photolyses were performed *in situ* at 37°C in liposome suspensions, in 50-μL quartz capillaries placed in the ESR cavity, using a Schoeffel 1000 W xenon lamp coupled with a Schoeffel grating monochromator. The excitation light had a maximum centered at 270 nm. All experiments were performed in duplicate. The data were obtained with error of less than 10%.

RESULTS AND DISCUSSION:

Structural effects. The ESR spectra of n-PCSL (n = 5, 7, 10, 12, 16) incorporated in three membrane systems provided information about the degree of lipid organization. These systems were soy PC, 5 mol% SLPC, and 5 mol% SCLAPC in soy PC. At 37°C, spin labels underwent anisotropic rotational motion. The ESR spectra of 5, 7, 10, and 12-PCSL labeled membranes were characterized by calculating the values of the order parameter S (38,39). Our data show that there was no significant change in order parameters with incorporation of 5 mol% SLPC at all four C5, C7, C10, and C12 positions in the membranes. However, with 5 mol% of SCLAPC, some perturbation effects at C7, C10, and C12 positions in the membranes were observed as indicated by the decrease in the values of the order parameter. At those three positions, the values of S were 0.481 vs. 0.438; 0.291 vs. 0.227; and 0.212 vs. 0.171 in soy PC vs. 5 mol% SCLAPC in soy PC, respectively. The decrease in ordering in the region of the conjugated double bonds indicated that SCLAPC had a perturbation effect on the membrane that may be attributed to the configuration of the conjugated double bond system along the fatty acid chain of CLA. For comparison, the lowest energy configuration of 9*cis*,11*trans*-octadecadienoic (18:2) acid (CLA) and linoleic acid (9*cis*,12*cis*-18:2) are shown in Scheme 1. CLA shows a unique bend in the molecule (C9 to C12) which is not free to rotation, while the bend in linoleic acid (C9 to C13) can rotate about the methylene-interrupted carbon (C11). In the membrane center at C16, the spin labels underwent isotropic rotational motion, and the rotation correlation time τ (21) was used to describe this motion behavior. Our results indicated that there were no significant differences in rotation correlation times in all three different membrane systems investigated (data not shown).

Oxygen diffusion-concentration product. Because bimolecular collisions of molecular oxygen and nitroxide groups induce Heisenberg spin exchange, which yields ESR line broadening (40), the changes in the ESR line width, ΔH[x], are proportional to the oxygen diffusion-concentration product (41). This was used as an experimental parameter to assess the oxygen diffusion-concentration product (or bimolecular collision rate) of fluid phase membranes. The ESR line width changes, which were measured at six different depths in the three membrane systems discussed above, provided profiles (42) of oxygen diffusion-concentration product across membranes, as depicted in Figure 1. These depths (Fig. 1, *x* axis) were at the water-lipid interface (labeled T-PC), and at C5, C7, C10, C12, and C16 of the bilayer membrane. Changes were highest in the values of the line width (Fig. 1, *y* axis) near C7, C10, and C12 of the 5 mol% SCLAPC in soy PC bilayer (open circles) relative to the control (100% soy PC, solid circles). There were no significant changes with 5 mol% SLPC in soy PC (solid triangles) as indicated in Figure 1. The relative changes



SCHEME 1

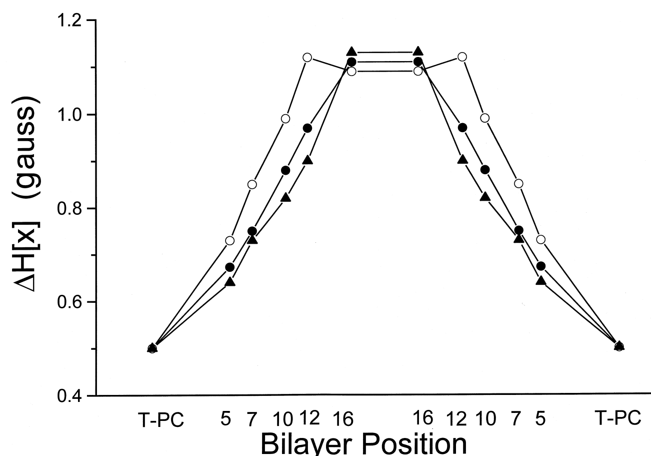


FIG. 1. Profiles of the oxygen electron spin resonance (ESR) line broadening (or relative oxygen permeability) across soy plant phosphatidylcholine (soy PC) membranes (●), with 5 mol% 1-stearoyl-2-(9*cis*,11*trans*-octadecadienoyl)-phosphorylcholine (SCLAPC) (○), and 5 mol% 1-stearoyl-2-linoleoyl-phosphorylcholine (SLPC) (▲) at 37°C. The abscissa indicates the approximate depth in the membrane bilayers at which each spin label is positioned (T-PC represents Tempocholine-PC at the water–lipid interface). $\Delta H[x] = H_{pp}(x, O_2) - H_{pp}(x, N_2)$, where $H_{pp}(x, O_2)$ and $H_{pp}(x, N_2)$ are the peak-to-peak ESR line widths of the first derivative spectrum, measured at the center line position for test samples saturated with oxygen and nitrogen, respectively.

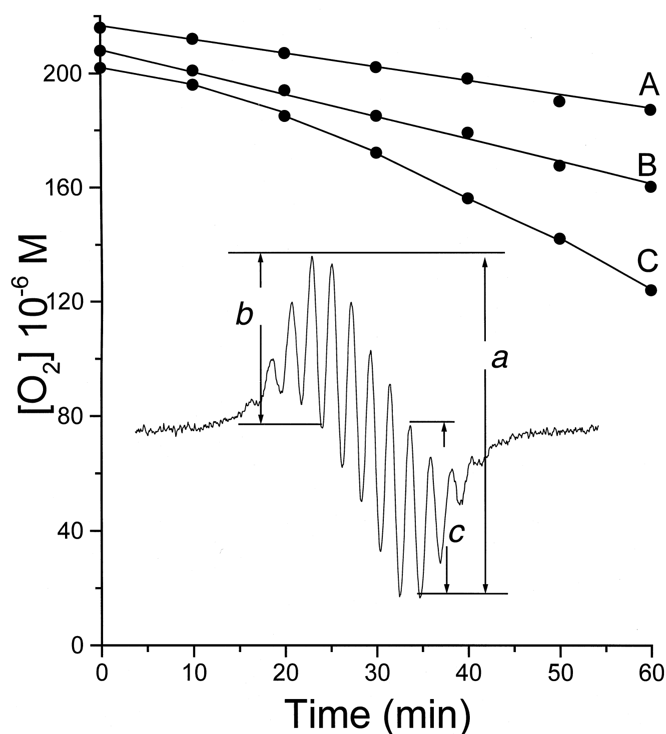


FIG. 2. Oxygen consumption measured in a closed chamber with samples of (A) 100% SLPC, (B) 100% SCLAPC, and (C) 100% soy plant PC (soy PC) membrane suspensions (30 mg/mL) at 37°C in Hepes buffer (pH 7.2) containing 3-carbamoyl-2,2,5,5-tetramethyl-3-pyrroline-1-yloxyl (CTPO) (0.14 mM). Aliquots were taken from (aerated) stock samples that had been incubated at 37°C for 45 min. The ESR spectra were taken 5 min after the temperature equilibration of sealed samples in the cavity. The inset illustrates the superhyperfine structure of the low-field line from ESR spectra of CTPO in nitrogen-saturated aqueous solution. The parameter *K*, which is used in calibration charts to determine the oxygen concentration, was calculated by using the equation $K = (b + c)/2a$, where *a, b,* and *c* are determined as shown in the figure. Spectra were recorded at 37°C, microwave power 1 mW, modulation amplitude 0.05 G, and scan range 5 G. For abbreivations see Figure 1.

in ΔH on the oxygen diffusion-concentration products indicate that SCLAPC enhanced the oxygen diffusion-concentration product in the region of the conjugated double bonds in the membranes.

Oxygen consumption. An oximetry method that requires ESR spin labels and a closed chamber (21,22) was used to measure the oxygen concentrations during lipid peroxidation. ESR spectra of nitroxide spin labels exhibit three lines because of the hyperfine interaction of the unpaired electron with the nitrogen nucleus. Each line is further split into another group of lines because of proton superhyperfine interaction. The resolution of the proton superhyperfine lines depends on the oxygen concentration due to the bimolecular collisions of the spin label with molecular oxygen, a paramagnetic molecule, broadening each line through Heisenberg exchange interactions. Resolution of the superhyperfine structure of the low-field line of the ESR spectrum of CTPO depended on the oxygen concentration of the test solution. Oxygen consumption was thus obtained from the calibration curves relating the *K* parameter (21,22; see Fig. 2) to oxygen concentration.

Loss of molecular oxygen in closed capillaries containing liposomes has been used as a diagnostic indicator of lipid peroxidation (21–23). The rate of oxygen consumption was monitored with the spin label CTPO. The insert in Figure 2 displays typical results when the spin-probe closed-chamber technique is used. The incubation of liposomes containing fatty acids with double bonds on the alkyl chain led to a depletion of oxygen from the medium; liposomes containing DMPC showed no depletion of oxygen (data not shown). The oxygen consumption rate in pure (100%) SLPC and SCLAPC was slower than for soy PC liposomes; EYPC gave similar results to soy PC.

Figure 3 shows the relative rates of oxygen consumption in different systems during extensive incubation. There was very slow depletion of oxygen in DMPC with 5 mol% SCLAPC. The oxygen consumption rates in soy PC with and without 5 mol% SLPC were similar. In marked contrast, enhanced rates of oxygen consumption occurred in soy PC with successively higher concentrations of SCLAPC (Fig. 3). The oxygen consumption of pure SLPC and SCLAPC were less than that of soy PC (data not shown). These results showed a unique effect attributed to a CLA-containing phospholipid as part of a liposome membrane. The effect of CLA-containing phospholipids on oxygen consumption increased with increasing concentration, up to 5 mol% (Fig. 3), a concentration shown previously to result in the greatest beneficial effects based on mammary cancer prevention in laboratory animals (2,14).

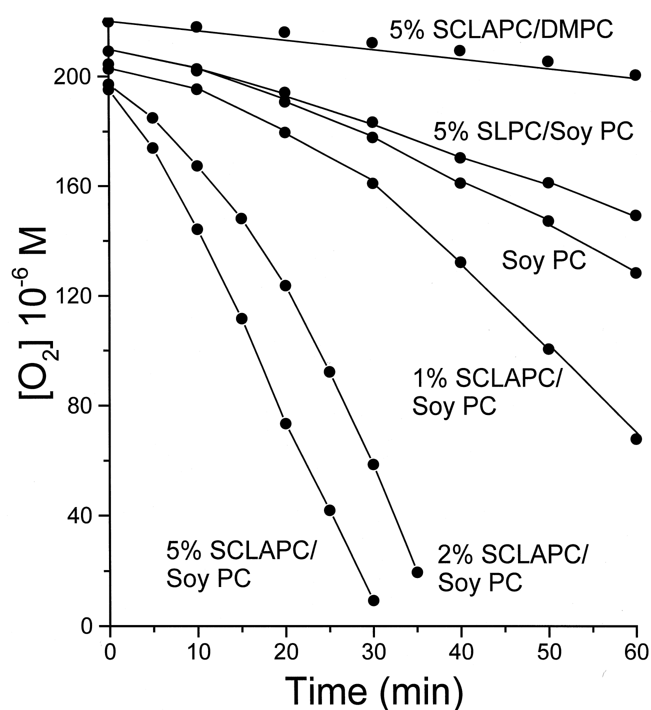


FIG. 3. Oxygen consumption measured in a closed chamber with test samples of 5 mol% SCLAPC in DMPC, 5 mol% SLPC in soy PC, 100% soy PC, and 1, 2, and 5 mol% SCLAPC in soy PC membrane suspensions (30 mg/mL) at 37°C in 25 mM Hepes buffer (pH 7.2) containing CTPO (0.14 mM). Aliquots were taken from (aerated) stock samples that had been incubated at 37°C for 45 min. The ESR spectra were taken 5 min after the temperature equilibration of sealed samples in the cavity. DMPC, L-α-dimyristoylphosphorylcholine; for other abbreviations see Figures 1 and 2.

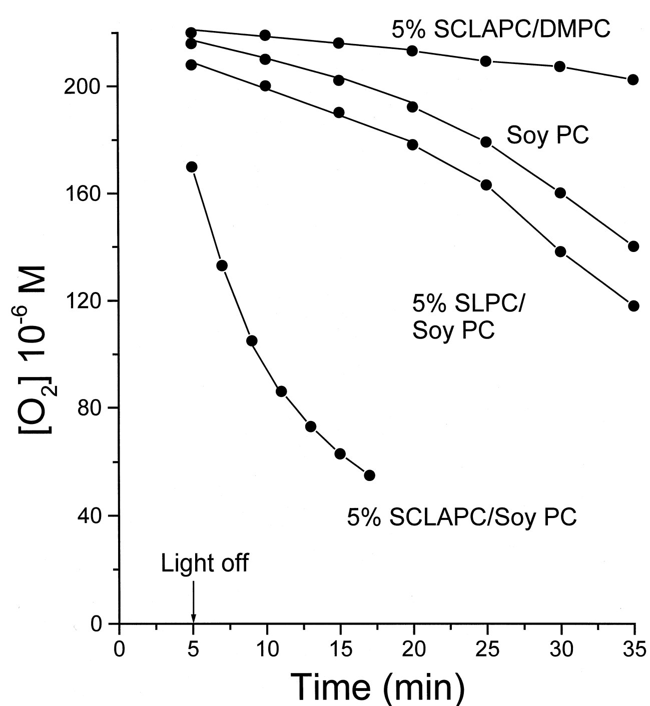


FIG. 4. Oxygen consumption measured in a closed chamber with samples of 5 mol% SCLAPC in DMPC, 5 mol% SLPC in soy PC, 100% soy PC, and 5 mol% SCLAPC in soy PC membrane suspensions (60 mg/mL) at 37°C in 50 mM Tris buffer (pH 7.4) containing CTPO (0.14 mM). Test samples were prepared at 37°C first, then aliquots were mixed with H_2O_2 (0.6%), transferred to the quartz capillary tube, and placed in the ESR cavity. Each test sample was ultraviolet-irradiated (270 nm, power 1 kW, from a distance of 70 cm) for 5 min, and spectra were recorded subsequently. For abbreviations see Figures 1–3.

The effects of 5 mol% SCLAPC or SLPC in soy PC (or EYPC) on oxygen consumption were also tested when membranes were exposed to free radical attack. Several representative experiments, in which hydroxyl radicals were generated by using UV illumination of H_2O_2, are depicted in Figure 4. Each test sample was exposed to UV irradiation for 5 min to initiate free-radical generation. Based on the *K* parameter obtained, test samples containing 5 mol% SCLAPC in soy PC exhibited markedly lower levels of residual oxygen in solution, or increased rates of oxygen consumption, than those consisting of 5 mol% SLPC in soy PC or 100% soy PC (Fig. 4). Our results indicated that the relative rates of oxygen consumption were enhanced by SCLAPC, both during and after UV irradiation. As a control, Figure 4 shows the slow oxygen consumption rate with 5 mol% SCLAPC in DMPC. The rate of oxygen consumption of 5 mol% SCLAPC in SOPC was similar to that in DMPC, whereas that of 5 mol% SCLAPC in PLPC was similar to that in EYPC (data not shown).

AAPH is known to strongly induce lipid peroxidations in cell and reconstituted membranes (43,44). Figure 5 shows the results of oxygen consumption of soy PC liposomes induced by AAPH in the presence of 5 mol% SCLAPC or SLPC. In this experiment, the lipid concentrations were reduced 10 times in order to discriminate between this and another potential pathway of oxygen consumption involving CLA oxidation products such as furans (45). Again, 5 mol% of SCLAPC in soy PC dramatically increased the oxygen consumption in comparison to 5 mol% SLPC in soy PC.

Results of the three different free radical generation experiments (Figs. 3–5) showed the same trends. The oxygen consumption rate in soy PC with incorporation of 5 mol% SCLAPC was always faster than that in soy PC alone or with incorporation of 5 mol% SLPC. In addition, the incorporation of 5 mol% SCLAPC into all the synthetic phosphorylcholine (DMPC, SOPC, and SLPC) resulted in low oxygen depletion rates, except into PLPC. Similar results were found with both EYPC and soy PC, but only the data for soy PC are presented. The apparent role of double-bond conjugation in SCLAPC and mixed chain length in the major PC on oxygen consumption in soy PC, EYPC, or synthetic PC membranes, is not fully understood.

The interaction between CLA and lipid bilayers is probably complex. SCLAPC may slightly disturb membrane structure and enhance oxygen diffusion-concentration product due to the rotational restriction dictated by the presence of a conjugated double bond system (Scheme 1). The increase in oxygen diffusion-concentration product induced by conjugation

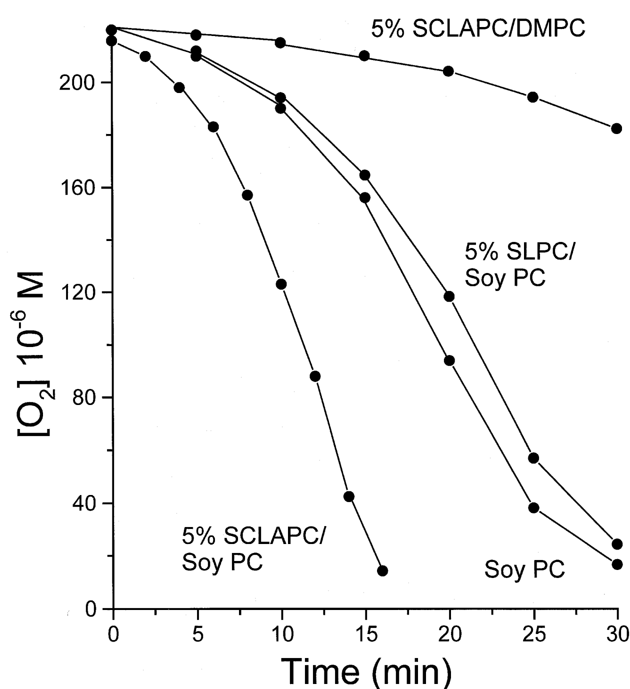


FIG. 5. Oxygen consumption measured in a closed chamber with samples of 5 mol% SCLAPC in DMPC, 5 mol% SLPC in soy PC, 100% soy PC and 5 mol% SCLAPC in soy PC membrane suspensions (3 mg/mL) at 37°C in 50 mM phosphate buffer (pH 7.4) containing CTPO (0.14 mM). Samples were prepared at 37°C first, then aliquots were mixed with 40 mM freshly made AAPH [2,2′-azobis(2-amidinopropane)hydrochloride], transferred to the capillary tube, and placed in the ESR cavity. Spectra were recorded after 5 min equilibration.

correlates with the decrease in the ordering of the membranes. As shown by Hubbell and McConnell (38), the disordering of lipid alkyl chains can be ascribed to an increase in the amplitude of motion of the long molecular axis and to an increase in the probability of gauche conformations, P_g, of carbon–carbon single bonds. The increase in P_g leads to an increase in the formation of alkyl chain "kinks," which provide a molecular mechanism for the associated increase in oxygen diffusion-concentration product. In addition to perturbation caused by double bonds, there is evidence that differences in chain length exacerbate membrane disturbance.

A correlation is apparent between the structural properties of membranes, the enhancement effect of SCLAPC on oxygen diffusion-concentration product, and the susceptibility to oxygen depletion. The results reported in this work indicate that CLA perturbs a complex interrelationship between membrane structure and the susceptibility to oxygen consumption. At physiological temperatures, the membrane, which is in the fluid phase, is altered and displays an increase in oxygen permeability. This leads to increased exposure of membrane components to oxygen and might make lipids more susceptible to attack by free radicals. The combined effects of perturbing membrane structure and increasing oxygen diffusion-concentration products by double bond conjugation imply that CLA molecules probably increase the bimolecular collision frequency of oxygen, and/or reactive oxygen species, with target molecules, thereby accelerating the oxygen consumption. Whether this increase in oxygen consumption is involved in, or contributes to, the observed beneficial physiological or anticancer effects of CLA requires further study.

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Interaction of Curcumin with Human Serum Albumin—A Spectroscopic Study

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ABSTRACT: Curcumin (diferuloyl methane) has a wide range of physiological and pharmacological actions. Curcumin interaction with human serum albumin (HSA) has been followed by fluorescence quenching and circular dichroism (CD) measurements. Based on fluorescence measurements, the equilibrium constant for the interaction is $2.0 \pm 0.2 \times 10^5\ M^{-1}$. Binding of curcumin to HSA induces an extrinsic CD band in the visible region. From the induced CD band measurements, the equilibrium constant has a value of $2.1 \pm 0.3 \times 10^4\ M^{-1}$. Thus, HSA has two kinds of affinity sites for curcumin, one with high affinity and the other with lower affinity. Job's plot indicated a binding stoichiometry of 1:1 for the high-affinity site. The equilibrium constant was invariant with temperature in the range of 15 to 45°C, suggesting the role of hydrophobic interactions in the binding of curcumin to HSA. Curcumin does not change the conformation of the HSA molecule. These measurements have implications in the understanding of the curcumin transport under physiological conditions.

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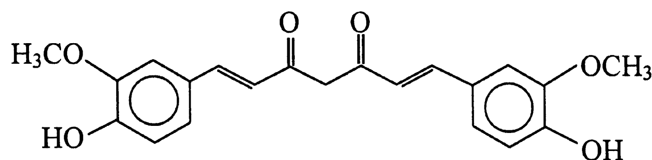
Curcumin is the major coloring pigment present in the spice turmeric (1). Curcumin, a β-diketone, contains two ferulic acid molecules linked *via* a methylene bridge at the carbon atoms of the carboxyl groups (Scheme 1). This phenolic compound exhibits a wide range of physiological and pharmacological effects. Curcumin has antioxidant and antiinflammatory activities (2–4). Dietary curcumin reduces iron-induced hepatic toxicity in rats (5). Curcumin is a good scavenger of reactive oxygen species (6) and also protects hemoglobin from nitrate-induced oxidation (7). The anticarcinogenic properties of curcumin in animals have been demonstrated by its inhibitory role in both tumor initiation induced by benz[α]pyrene and 7,12-dimethyl benz[α]anthracene, and tumor promotion induced by phorbol esters (8,9). Recently, curcumin has been shown to be highly effective in inhibiting type I human immunodeficiency virus long terminal repeat directed gene expression and virus replication (10). It inhibits neutrophil activation, mixed lymphocyte reaction, and proliferation of smooth muscle cells, and it suppresses nitrogen-induced proliferation of blood mononuclear cells (11). Curcumin lowers the formation of inflammatory compounds such as prostaglandins and leukotrienes by inhibiting the arachidonic acid metabolism by lipoxygenase and cyclooxygenase enzyme systems in mouse epidermis (12).



SCHEME 1

Furthermore, curcumin can access both hydrophobic and hydrophilic environments of a microheterogeneous system; the corresponding radical is stable with respect to oxygen and can be repaired by the natural antioxidants vitamins C and E in hydrophilic and hydrophobic environments, respectively (13). Under certain conditions, curcumin accelerates oxygen radical formation by reducing ferric ion to give more active iron(II) (14). Curcumin was shown to lower carrageenan-induced edema in rats and to give relief from rheumatoid arthritis in patients (15,16). These studies indicate that curcumin probably reaches the target tissues to exhibit its diverse activities. However, its mode of transport *in vivo* is not clear.

Ravindranath and Chandrashekar (17) detected the presence of orally administrated curcumin in the portal blood, liver, and kidney of rats. Curcumin is a lipophilic molecule with phenolic groups and conjugated double bonds (1). Many lipophilic molecules, like long-chain fatty acids, are known to be carried by albumin in blood (18). However, it is not known whether albumin can function as a carrier molecule for curcumin. To assess this, the present investigation was undertaken to study the interaction of curcumin with human serum albumin (HSA). The interaction has been followed by fluorescence and induced circular dichroism (CD) measurements. These measurements indicate the existence of two kinds of binding sites for curcumin on the HSA molecule.

MATERIALS AND METHODS

HSA (fatty acid-free), *N*-acetyl tryptophan amide, and Trizma base were purchased from Sigma Chemical Co. (St. Louis, MO). Curcumin (>99% pure) was purchased from M/s

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Abbreviations: CD, circular dichroism; HSA, human serum albumin; Q_{max}, maximal quench; UV, ultraviolet.

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Flavours and Essence Pvt. Ltd. (Mysore, India). Concentration and purity of curcumin were determined by established spectral measurements and by high-performance liquid chromatography (19).

A stock solution of curcumin (1 mM) was prepared in ethyl alcohol and diluted to 10 μM with 0.05 M Tris-HCl buffer (pH 7.0). A working solution of HSA (1.13 μM) was prepared in Tris-HCl buffer (pH 7.0). Intrinsic fluorescence of HSA in the presence and absence of curcumin at different concentrations was followed after 280 nm excitation and at 330 nm emission wavelength in a Shimadzu RF5000 recording spectrofluorometer (Kyoto, Japan) attached with thermostat cuvette holder for constant temperature.

Quenching of relative fluorescence intensity of HSA by curcumin was analyzed in terms of the binding of curcumin to protein using an established method (20), with the assumption that the binding of each molecule of curcumin causes the same degree of fluorescence quenching. The percentage quenching of the fluorescence intensity of proteins by curcumin was corrected empirically for internal absorption and filtration by subtracting the percentage quenching by the same concentration of curcumin of the fluorescence of *N*-acetyltryptophan amide equivalent in absorption to protein at 285 nm. The equilibrium constant, *K,* is given by the following equation:

$$K = \frac{\beta}{(1-\beta)} \cdot \frac{1}{\mathrm{Cf}} \quad [1]$$

where $\beta = Q/Q_{max}$ (where Q_{max} = maximum quench), Cf = $C_T - n\beta T$, and C_T is the total concentration of curcumin added. The value of K is given by the slope of a plot of $\beta/(1-\beta)$ against Cf, the free ligand concentration. Q_{max} was determined by extrapolation of a double reciprocal plot of Q vs. C_T to intercept. The value of n for curcumin–HSA interaction was obtained by Job's method of continuous variation (21). The total concentration of curcumin and protein was held constant at 10 μM and relative proportions were varied.

CD studies. CD measurements were made with a JASCO J20-C automatic recording spectropolarimeter (Tokyo, Japan), calibrated with *d*(+)-10-camphor sulfonic acid. Quartz cells of 1-cm pathlength for the region 250–550 nm and 1 mm for the far-ultraviolet (UV) region were used. Slits were programmed to yield a 10-Å band width at each wavelength. Molar ellipticity values, $[\Theta]_M$, were calculated using a value of 368 for the molecular weight of curcumin.

RESULTS

Fluorescence spectroscopic studies. Fluorescence emission spectra of HSA after being excited at 280 nm were observed at 330 nm. Addition of increasing amounts of curcumin quenched the fluorescence of HSA without significantly affecting the fluorescence maxima (data not shown). The relative fluorescence emission intensities at 330 nm were plotted against curcumin concentration (Fig. 1). Fluorescence emission of HSA decreased by 25% at a curcumin concentration of 5.8 μM. From the corresponding double reciprocal plot of fluorescence quenching data, it is evident that curcumin quenches the fluorescence of HSA with a Q_{max} of 34 ± 2%. The stoichiometry of curcumin binding to HSA was established using Job's method of continuous variation (Fig. 2).

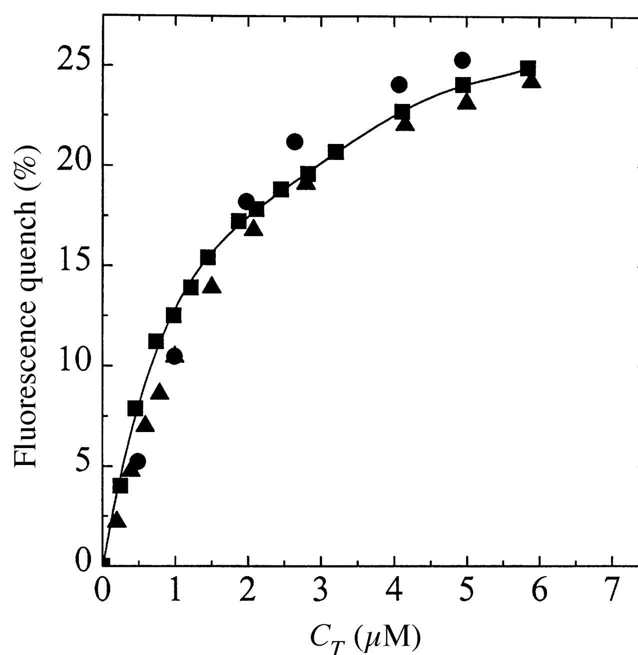

FIG. 1. Fluorescence quenching of human serum albumin (HSA) by curcumin at (■) 15°C , (●) 25°C, and (▲) 45°C. Concentration of HSA was 75 μg/m. Measurements were made in 0.05M Tris HCl, pH 7.0.

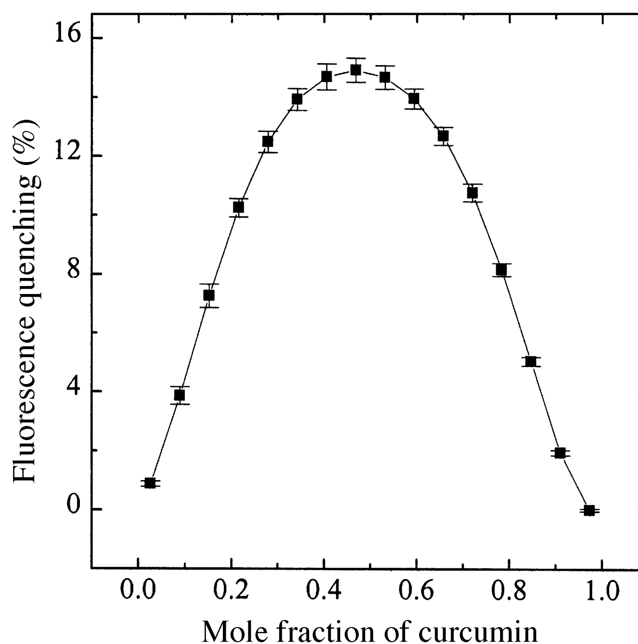

FIG. 2. Job's plot for the interaction of HSA with curcumin ([HSA] + [curcumin] = 10 μM). The error bars refer to the standard error of the mean of three sets of experiments. See Figure 1 for abbreviation.

The stoichiometry of the curcumin–HSA complex was found to be 1:1 ± 0.4. By using a value of $n = 1$ and the extent of maximal interaction obtained from the double reciprocal plot, the mass action plot (Fig. 3) was constructed. From the slope of the mass action plot, the equilibrium constant was found to be $2.0 \pm 0.3 \times 10^5\ M^{-1}$ at 25°C.

Effect of temperature on the curcumin–HSA interaction. The effect of temperature in the range 15 to 45°C on the interaction was followed by determining the equilibrium constant (K_{eq}), which was invariant over the temperature range studied. Thus, van't Hoff's $\Delta H° \approx 0$ kcal/mol and the binding reaction was entropy-driven; $\Delta S° = 25$ cal/mol/K at 27°C with activation free energy of $\Delta G° = -RT\ln K_{eq} = -7.5$ kcal/mol.

CD studies. HSA exhibited broad minima in the near-UV region of 260 to 300 nm. Addition of curcumin (80 µM) did not affect the ellipticity values in this region. Curcumin (80 µM) did not affect significantly the CD bands in the far-UV region (200–260 nm) either. The secondary structure content of HSA was not affected by the addition of curcumin. Curcumin does not exhibit any CD bands in the region of 300–600 nm. However, it becomes optically active upon binding to HSA and exhibits extrinsic CD bands in the region of 300–500 nm. It has a positive band at 400–500 nm and a small negative band at 320–410 nm (Fig. 4). The electronic transitions occurring at 464 nm were used for quantitative measurements. The induced CD amplitude at 464 nm was followed as a function of curcumin concentration. The CD amplitude increased with the concentration of curcumin for the fixed concentration of HSA (Fig. 5A). By assuming that the amplitude at 464 nm for any given molar ratio of curcumin to HSA is directly proportional to the fraction of curcumin that is complexed with HSA, the K_{eq} and Θ_{max} for the complete HSA–curcumin complex were calculated using the equation of Ikeda and Hamaguchi (22)

$$\ln\left[\frac{\Theta}{(\Theta_{max} - \Theta)}\right] = \ln K_{eq} + n \ln C_T \qquad [2]$$

The plot of $\ln[\Theta/\Theta_{max} - \Theta]$ vs. $\ln C_T$ was a straight line (Fig. 5B) with a slope of 1.0 ± 0.1, suggesting that one molecule of curcumin bound to one molecule of HSA. The K_{eq} for the interaction was $2.1 \pm 0.3 \times 10^4\ M^{-1}$. However, the K_{eq} value obtained by fluorescence titration is higher by one order magnitude ($K_{eq} = 2.0 \pm 0.2 \times 10^5\ M^{-1}$), suggesting that HSA has two kinds of affinity sites for curcumin, one with high affinity and the other with lower affinity.

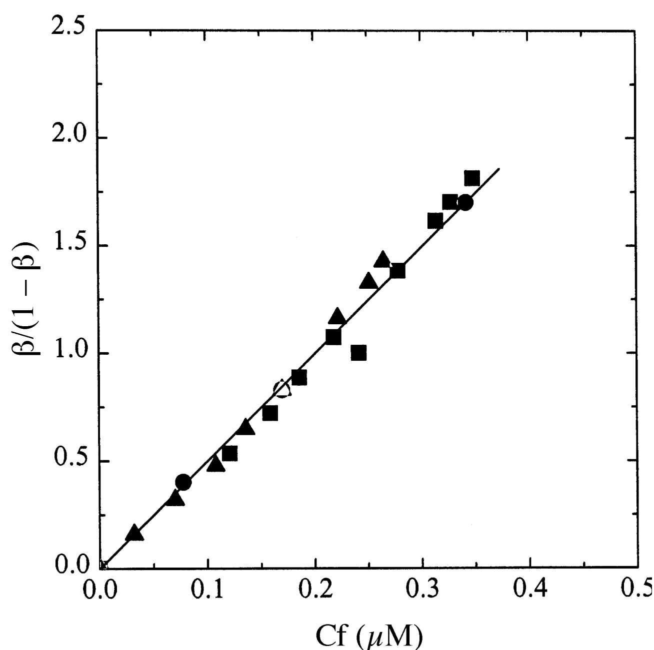

FIG. 3. Mass action plot β/(1 – β) against free curcumin concentration (Cf) at (■) 15°C, (●) 25°C, and (▲) 45°C. The slope of the straight line was used to obtain the equilibrium constant (K_{eq}).

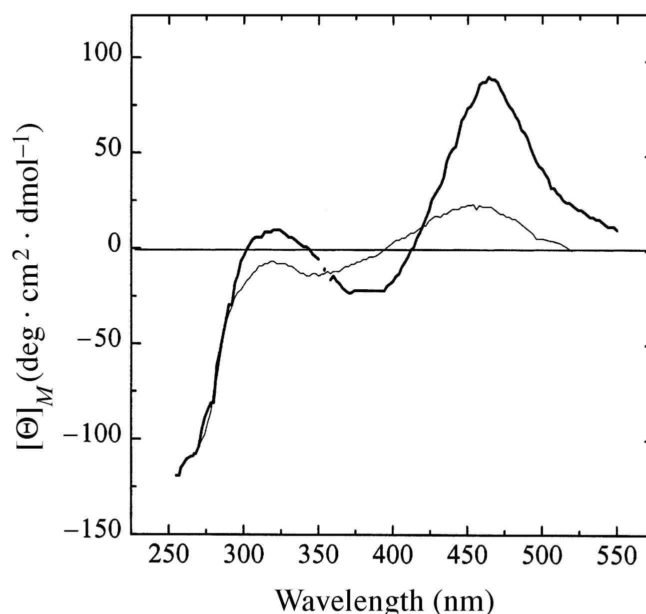

FIG. 4. Circular dichroism (CD) spectra of HSA–curcumin complex. The measurement was made in 1-cm cells with different concentrations of curcumin (solid line is 50 µM and thin line is 10 µM). The HSA concentration was 0.9 mg/mL. See Figure 1 for abbreviation.

DISCUSSION

These results indicate that curcumin binds to HSA with two binding sites characterized by K_{eq} of $2.0 \times 10^5\ M^{-1}$ and $2.1 \times 10^4\ M^{-1}$. The two binding constants obtained were not due to two different physical techniques used. They were due instead to two different types of sites: the high-affinity site (saturated at 7 µM) detected by fluorescence measurements and the low-affinity site (saturated in the concentration range of 0 to 100 µM) detected by CD. Technically, it is not possible to overlap the concentration range of curcumin with the two different techniques used. Curcumin has a phenolic structure with conjugated double bonds; proteins and phenols are thought to reversibly complex with each other *via* hydrogen bonding and hydrophobic interactions (23). The involvement of hydrophobic groups in the formation and stabilization of phenolic tan-

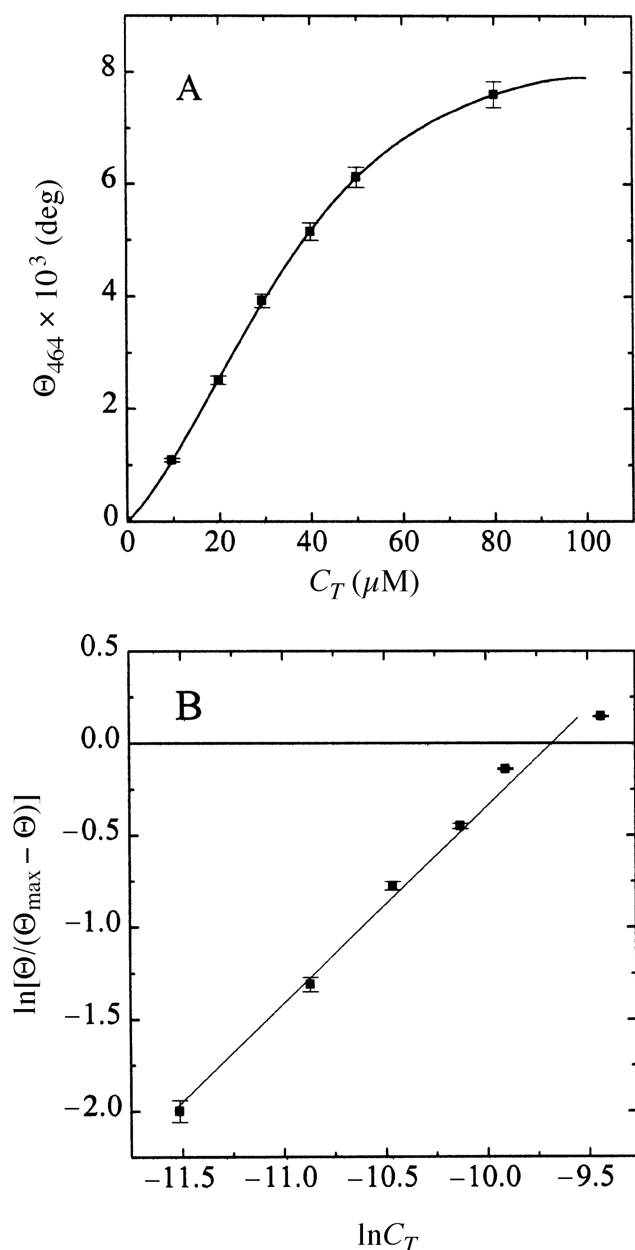

FIG. 5. (A) Variation of the ellipticity values at 464 nm as a function of curcumin concentration. (B) Plot of ln $[\Theta/\Theta_{max} - \Theta]$ vs. ln C_T for HSA–curcumin complex. The error bars indicate the variance of ellipticity values for two sets of experiments.

nins and protein complexes was established in several independent studies (24,25). Oh *et al.* (24) have drawn attention to the fact that hydrophobic interactions may dominate the ligand–protein interaction. The presence of hydrogen donors, in the form of phenolic hydrogen groups in the curcumin molecule and hydrogen acceptors of the peptide linkage of the protein, may lead to the formation of hydrogen bonds. However, our studies on the equilibrium constant as a function of temperature do not favor the concept of hydrogen bonding as a contributing factor for curcumin binding to HSA. The invariance of K_{eq} in the temperature ranges studied supports the role for hydrophobic interactions.

Earlier workers showned (26) that the capacity of ligands to bind to protein is determined by the chemical and physical nature of the phenolic nucleus itself. Both curcumin and protein contain hydrophobic regions. The aromatic nuclei of curcumin, which are essentially hydrophobic, have an affinity for the aliphatic and aromatic side chains of HSA. Binding stoichiometry of curcumin and HSA is indicative of mole-to-mole interaction. Since HSA is known to contain hydrophobic pockets, it is feasible that curcumin possibly binds to the hydrophobic pocket of albumin. The binding constant of curcumin with HSA is comparable with that of caffeic acid ($2.8 \times 10^4\ M^{-1}$), a simple dihydrophenolic compound, where it was shown that the hydroxyl groups play a major role in binding to HSA. Curcumin may also react with the amino acids of proteins through its reactive carbonyl and phenolic hydroxyl groups. Gossypol, a polyphenol present in cottonseed, binds to albumin with an association constant of $2.2 \times 10^6\ M^{-1}$ (27). HSA is the primary carrier of both exogenous and endogenous ligands. These ligands associate with HSA in the plasma by noncovalent linkages and some of them have very high association constants (28). Many of the ligands, like bilirubin, reversibly bind to albumin at neutral pH (29). Bilirubin binds to albumin with 1:1 stoichiometry. However, curcumin binds with 2:1 stoichiometry. Fatty acids in the serum are also carried by albumin. Albumin contains six binding sites for free fatty acids (18,30). Palmitic acid binds to albumin with an association constant of $5.2 \times 10^6\ M^{-1}$. An increase in acyl chain length and degree of acyl chain unsaturation for the same chain length increases the association constant, thus suggesting that fatty acid solubility in aqueous medium may play a significant role in the equilibrium between the fatty acid association with HSA and the aqueous phase. Curcumin with three unsaturated bonds between the two phenyl rings may have a role in the interaction of curcumin–HSA in the aqueous phase, as found in the association between fatty acid and HSA. The curcumin association constant one obtained for the high-affinity site is comparable to that of a free fatty acid binding association constant and gives an indication that curcumin may have binding sites similar to those of fatty acids on albumin. The CD measurements indicated that when curcumin was bound to the protein, it induced a CD band, which was used for quantitative measurement. Curcumin forms a 1:1 complex with HSA with an association constant of $2.0 \pm 0.2 \times 10^5\ M^{-1}$. Recently, we showed (31) that lipoxygenase-1 could be inhibited by phosphatidylcholine (PC)-micelle-bound curcumin. The binding of curcumin with PC micelles involved both hydrophilic and hydrophobic interactions. Similarly, several other studies have shown that curcumin could inhibit membrane-bound enzymes like phospholipase D, protein kinase C, and $pp60^{c\text{-}src}$ tyrosine kinase (32,33). The anticarcinogenic and antioxidant properties of curcumin are potentiated in the hydrophobic environment (13). In this context, the binding of curcumin to HSA was studied. Furthermore, the equilibrium constants obtained for the binding of curcumin with HSA are comparable to many of the K_{eq} reported for both exogenous and endogenous ligands with HSA (21). In conclusion, these measurements sug-

gest a role for hydrophobic interactions in the binding of curcumin to serum albumin. Curcumin may associate with serum albumin by hydrophobic interactions and may thereby be transported to appropriate target cells where it elicits its pharmacological effects.

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Solubilization and Stabilization of Carotenoids Using Micelles: Delivery of Lycopene to Cells in Culture

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ABSTRACT: The use of the organic cosolvents tetrahydrofuran and dimethylsulfoxide was found to be unsuitable for prostate tumor cell cultures because of solvent cytotoxicity and the poor solubility and instability of lycopene. For example, the half-life of lycopene in organic/aqueous solution was found to be less than 2 h. Therefore, a micellar preparation of lycopene was developed for the solubilization and stabilization of lycopene in cell culture media. Neither the micelles themselves nor lycopene solubilized in micelles at concentrations up to 10 μg/mL in the cell culture media produced cytotoxicity or inhibition of cell proliferation in either LNCaP human prostate cells or Hs888Lu human lung cells. Lycopene solubilized in micelles was stable for at least 96 h under standard cell culture conditions so that a constant lycopene supply could be provided to the cells. During the culture process, lycopene was taken up by LNCaP cells and reached a plateau at approximately 12 h. Micelles provide a convenient, inexpensive, and nontoxic vehicle for dissolving and stabilizing carotenes such as lycopene in tissue culture media and then delivering them to cells growing in culture.

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Carotenoids are highly conjugated, intensely colored isoprenoid plant pigments, which may be grouped into the set of hydrocarbons called carotenes or the oxygenated species known as xanthophylls (1). Unstable when exposed to light or oxygen, carotenoids are generally efficient quenchers of singlet oxygen (2). Approximately 50 carotenoids, such as β-carotene, serve as precursors of vitamin A in mammals (3). The carotene lycopene, the abundant red pigment in the tomato, is among the most efficient singlet oxygen quenchers of the carotenoids (4). Although isomeric with β-carotene ($C_{40}H_{56}$), lycopene has no provitamin A activity.

During digestion in humans, the hydrophobic carotenoids are solubilized by bile salts in the small intestine and then absorbed along with other lipids (5). After absorption, carotenoids are transported from the gut to the liver in the chylomicrons, where they are repackaged and transported through the bloodstream in low density lipoproteins (LDL) and high density lipoproteins (HDL) (6). Virtually insoluble in water, carotenoids require solubilization by bile salts, chylomicrons, or lipoproteins in order to be transported in the human body.

Pharmacological actions of carotenoids include enhancement of immune response and possible prevention of initiation, promotion, or progression of cancer (7). In particular, lycopene is under investigation as a possible anticancer or chemopreventive agent (8). In a prospective human study, Gann *et al.* (9) found that low levels of plasma lycopene, but not other abundant dietary carotenoids, were associated with increased incidence of certain cancers. In particular, the chemopreventive evidence for lycopene was strongest for cancer of the prostate, lung, and stomach (10). In this regard, we have been investigating the possible role of lycopene in the prevention of cancer and, in particular, the prevention of prostate cancer. As part of our studies, we needed to examine the uptake and possible effect of lycopene on human prostate cancer cells and other cells growing in tissue culture.

However, existing methods of solubilizing carotenoids were either inadequate or unsuitable for our studies. For example, the use of tetrahydrofuran (THF) as a cosolvent for carotenoid solubilization as reported by Cooney *et al.* (11) can be toxic to some cell lines, is limited to relatively low concentrations of the most hydrophobic carotenoids, such as lycopene, and does not contribute to the stability of lycopene in solution. Ukai *et al.* (12) reported that lycopene may be solubilized and partially stabilized by complexation with cyclodextrin for use as a food additive, but this approach has not been applied to cell culture. El-gorab and Underwood (13) and Martin *et al.* (14) reported the use of micelles to solubilize β-carotene. Because Martin *et al.* (15) found that their micellar preparation did not stabilize carotenoids, they advocated instead the use of human lipoproteins as a vehicle both to solubilize and to stabilize β-carotene. Given the ease of preparation and low cost and availability of reagents for micelles compared to human lipoproteins, we developed a micellar formulation that both stabilized and solubilized lycopene at physiologically relevant concentrations during cell culture.

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Abbreviations: APCI, atmospheric pressure chemical ionization; BHT, butylated hydroxytoluene; HDL, high density lipoprotein; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; LDL, low density lipoprotein; RPMI, Roswell Park Memorial Institute; SRB, sulforhodamine B; THF, tetrahydrofuran; UV/VIS, ultraviolet/visible.

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EXPERIMENTAL PROCEDURES

All chemicals were reagent- or high-performance liquid chromatography (HPLC)-grade and were purchased from either Sigma Chemical Co. (St. Louis, MO) or Fisher Scientific (Fair Lawn, NJ). Purchased from Sigma Chemical Co., all-*trans* lycopene was 95% pure by ultraviolet/visible (UV/VIS) spectrophotometry at 502, 472, and 444 nm. HPLC analysis showed >95% purity (Fig. 1A). THF was high-purity, non-spectrophometric-grade containing butylated hydroxytoluene (BHT; 250 ppm), which was purchased from Burdick & Jackson (Muskegon, MI). Tests for peroxides (peroxide test, EM Science, Cherry Hill, NJ) showed that the THF used in this study contained no detectable peroxides (<1 ppm). Cell culture media were purchased from GibcoBRL (Grand Island, NY). LNCaP human prostate tumor cells and Hs888Lu human fibroblast-like lung cells were obtained from the American Type Culture Collection (Rockville, MD). LNCaP cells were maintained in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 2% L-glutamine, 1% antibiotic/antimycotic (prepared with 10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B in 0.85% saline), 1.5 g/L sodium bicarbonate, 4.5 g/L glucose, 10 mM HEPES, and 1.0 mM sodium pyruvate at 37°C in a humidified atmosphere at 5% CO_2 in air. Hs888Lu cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum, 2% L-glutamine, 1% antibiotic/antimycotic (prepared with 10,000 units/mL penicillin G sodium, 10,000 μg/mL streptomycin sulfate, and 25 μg/mL amphotericin B as Fungizone in 0.85% saline), 1% sodium pyruvate, 1% sodium bicarbonate, and 0.1 mM nonessential amino acids at 37°C in a humidified atmosphere at 5% CO_2 in air.

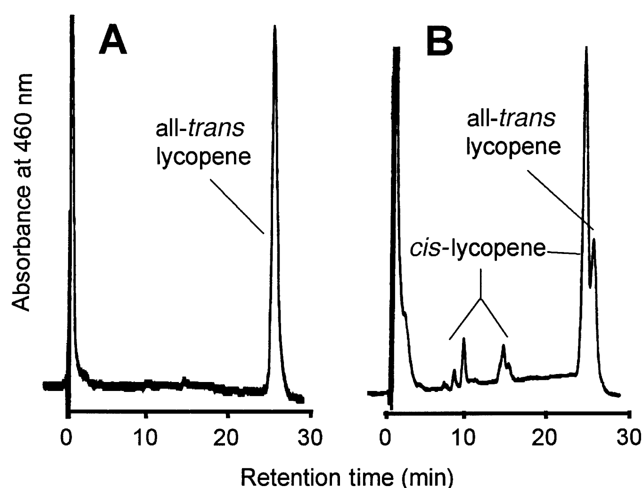

FIG. 1. Chromatograms obtained by high-performance liquid chromatography (HPLC) using a C_{30} carotenoid column showing (A) all-*trans* lycopene standard (5 μg/mL) dissolved in tetrahydrofuran (THF), and (B) lycopene after storage in THF in the dark and in an airtight polystyrene container for 24 h.

Several methods were evaluated for dissolving lycopene in cell culture media at physiological concentrations. Initially, lycopene was dissolved in THF and then diluted into cell culture media according to the method of Cooney *et al.* (11). A variation of this method was investigated in which dimethylsulfoxide was substituted for THF. Subsequently, an alternative approach was developed using a micellar formulation of lycopene as described below.

Preparation of micelles containing lycopene. In a glass container, 13 μL of L-α-phosphatidylcholine, 100 μL monoolein, 100 μL oleic acid, and 0, 5, 10, 20, 50, 100 or 200 μL of lycopene dissolved in THF (500 μg/mL) as the stock solution were combined, and the organic solvent was removed under a stream of nitrogen. Next, 10.76 mg of sodium taurocholate was added to 10 mL of complete cell culture and the mixture was sonicated in a water bath (Branson Ultrasonics Corp. model 2210 R-MTH; Danbury, CT) at room temperature for 15 min to form micelles containing lycopene. The final concentration of each component in the solution was as follows: 100 μM monoolein, 33.3 μM oleic acid, 2 mM sodium taurocholate, and 16.7 μM L-α-phosphatidylcholine. Lycopene concentrations in the cell medium ranged from 0 (for control incubations) to 10 μg/mL (18.63 μM). Each 10-mL batch of cell medium was sterilized by passage through a presterilized 0.22-μm filter.

Lycopene stability in THF or cell culture media. Lycopene dissolved in THF at 9.33 μM or cell culture medium (10 mL) containing 9.32 μM lycopene solubilized in micelles was incubated from 0–96 h using standard cell culture conditions in plastic (Falcon, polystyrene, nonpyrogenic tissue culture dish) or glass containers (Pyrex reusable petri dishes). In some stability experiments, samples were exposed to sunlight. Aliquots of 1 mL were removed at 0, 1, 2, 4, 8, 12, 24, and 48 h and then extracted immediately. First, 1 mL of 0.9% saline and 1 mL ethanol were added, and then the solution was vortexed for 30 s. Then, 3 mL of hexane was added followed by vortexing for another 30 s. The upper hexane layer was removed, and the aqueous solution was extracted twice more with 3-mL portions of hexane. Finally, the hexane fractions were combined in a glass test tube and evaporated to dryness in a vacuum centrifuge.

The amount of residual lycopene was measured using reversed-phase HPLC. All incubations and assays were carried out at least three times and the results were averaged. HPLC analyses were carried out using Waters (Milford, MA) model 501 pumps, 740 data module, and Lamda-Max model 481 UV/VIS absorbance detector. An isocratic solvent system, consisting of methyl-*tert*-butyl ether/methanol/acetic acid (50:49.5:0.5, by vol) at 1.0 mL/min on a YMC (Wilmington, NC) C_{30} 3 μm column (4.6 × 200 mm). The absorbance was measured at 460 nm.

Growth curves and protein assays for LNCaP and Hs888Lu cells. Sterilized medium containing micellar lycopene at 0.05, 0.5, 1.0, 5.0, or 10.0 μg/mL was prepared. LNCaP or Hs888Lu cells were seeded into 96-well plates at a density of 1×10^5 cells/mL (100 μL/well). After an overnight

preincubation, the standard culture medium was decanted, and the lycopene micelle medium solution was added to each well. The plates were incubated for 0–6 d. At each time point of 0, 1, 2, 3, 4, 5, and 6 d, cell viability was assayed as follows: (i) The medium was decanted, cold 20% trichloroacetic acid (100 μL/well) was added, and the cells were incubated at 4°C for 1 h. (ii) The cells were washed with water (1 mL/well) five times and air dried. (iii) The plates were stained using 4% sulforhodamine B (SRB) in 1% acetic acid, 100 μL/well for 30 min at room temperature. (iv) The SRB was decanted, the cells were washed with 1% acetic acid (1mL/time/well) four or five times and air dried again. (v) Then 200 μL 10 mM Tris base (pH 10) was added to dissolve the residue while shaking for 5 min on a gyrating shaker. (vi) The absorbance at 515 nm was measured to indicate protein contained in viable cells. Control incubations were carried out to which micelles containing no lycopene were added, or neither micelles nor lycopene was added.

Cellular uptake of lycopene. LNCaP and Hs888Lu cells were each plated at a density of 1×10^5 cells/mL in 25 cm^2 dishes (10 mL/dish). After preincubation overnight, the standard culture media were decanted and replaced with media containing micellar lycopene at 5 μg/mL. The plates were incubated for 0, 1, 2, 4, 8, 12, or 24 h, and at each time point, cells (alive or dead) were collected and centrifuged in a glass tube. To this tube were added 200 μL of water and 100 μL of internal standard (1 μg/mL retinyl acetate in absolute ethanol). Then 100 μL of absolute ethanol was added and vortexed for 30 s followed by 2 mL hexane (containing BHT, 100 mg/L) and vortexing another 1 min. The tube was centrifuged for 5 min, and the upper hexane layer was removed to another glass tube. The aqueous layer was extracted with another 2 mL hexane. The hexane fractions were combined in the glass tube and evaporated to dryness in a vacuum centrifuge. The residue was redissolved in 50 μL ethyl ether and 150 μL of the HPLC mobile phase to a total volume of 200 μL. Extracts were kept at 0°C until analysis the same day or kept at −30°C overnight.

HPLC was carried out on a Waters Novapak C_{18} column (3.9 × 150 mm, 5 μm particle size) at 1 mL/min with an isocratic elution system containing methanol/acetonitrile/THF (50:45:5, by vol). The HPLC analysis was completed in 15 min. The peaks were detected using a Waters programmable multiwavelength detector (model 490). Two channels were used simultaneously, one for retinyl acetate at 325 nm and the other for lycopene detection at 450 nm.

Measurement of cis/trans *lycopene in cells.* In order to determine the ratio of *cis/trans*-lycopene in cells and in the corresponding culture medium, extracts of LNCaP cell cultures, prepared as described above for the cellular uptake study, were analyzed using liquid chromatography–mass spectrometry (LC–MS). Based on our method for the analysis of β-carotene using LC–MS (16), a new method was developed for the measurement of *cis/trans* isomers of lycopene. Briefly, a Hewlett-Packard (Palo Alto, CA) G1946A LCMSD mass spectrometer equipped with atmospheric pressure chemical ionization (APCI) and an 1100 series HPLC system was used with a YMC C_{30} 3 μm carotenoid column (4.6 × 200 mm). An isocratic mobile phase consisting of 19% methyl-*tert*-butyl ether in methanol at 1.0 mL/min was used without solvent splitting during LC–MS. Under these conditions, the base peak of the positive ion APCI mass spectrum of lycopene was the protonated molecule at *m/z* 537. Therefore, selected ion monitoring of *m/z* 537 with a dwell time of 1 s was used to monitor the elution of lycopene isomers from the C_{30} column. No difference in mass spectrometric response has been observed between lycopene isomers. Other mass spectrometer parameters included a nitrogen nebulizer gas pressure of 60 psi, a nitrogen drying gas temperature of 300°C, a capillary voltage of 4000 V, a fragmenter voltage of 110 V, and a corona current of 4.0 μA.

RESULTS AND DISCUSSION

Because lycopene was found to be much less soluble in dimethylsulfoxide than in THF and the solubility limits of lycopene in dimethylsulfoxide and THF are approximately <0.01 and 4 mg/mL, respectively, only THF was investigated as a cosolvent for the delivery of lycopene to cell culture media. The stability of lycopene (5 μg/mL) in THF was investigated under a variety of conditions, and the results are summarized in Table 1. Lycopene rapidly degraded when exposed to air, was more stable in the dark than in the light (sunlight), and was more stable at 4°C than at room temperature. Unexpectedly, lycopene was much less stable in polystyrene than in glass containers. All-*trans* lycopene in THF solution rapidly isomerized to form at least six *cis* isomers as shown in Figure 1.

When lycopene was dissolved in THF, added to RPMI 1640 culture medium, and then incubated in glass under standard cell culture conditions at 37°C and 5% CO_2 in air, it degraded rapidly with a half-life of approximately 2 h (Fig. 2A). Even if the culture media were changed daily, this instability

TABLE 1
Stability of Lycopene (9.33 μM ; 5 μg/mL) in THF[a]

Incubation conditions	Container	Percent lycopene remaining		
		Incubation time (h)		
		0	24	48
Dark, no air	Glass[b]	100	100	83
4°C	Plastic[c]	100	56	19
Dark, no air	Glass	100	100	94
Room temp.	Plastic	100	20	12
Dark, air	Glass	100	24	6
Room temp.	Plastic	100	20	6
Light[d], no air	Glass	100	100	97
Room temp.	Plastic	100	0	0

[a]Tetrahydrofuran (THF) was high-purity nonspectrophotometric grade containing butylated hydroxytoluene (250 ppm), which was purchased from Burdick & Jackson (Muskegon, MI). Tests for peroxides (Peroxide test, EM Science, Cherry Hill, NJ) showed that the THF used in this study contained no detectable peroxides (<1 ppm), *n* = 3. (SD ± 5%).
[b]Pyrex reusable petri dish.
[c]Falcon polystyrene tissue culture dish.
[d]Sunlight.

raises questions regarding the feasibility of carrying out meaningful cell culture studies using lycopene, since most culture experiments require days to weeks to complete. Therefore, an alternative method was required to solubilize and at the same time stabilize lycopene in cell culture media.

As an alternative approach, lycopene was solubilized using micelles, which enabled lycopene concentrations of a maximum 10 μg/mL (18.6 μM) to be obtained in the cell culture media. This value exceeds the level of lycopene normally observed in human plasma, which is on the order of 0.5 μM (17). Even with dietary supplementation using tomato juice, plasma levels do not usually exceed 1.6 μM (18). Micelles containing lycopene were incubated in the cell culture medium under standard culture conditions for up to 96 h. The concentration of lycopene remaining at each time point was determined using HPLC, and the results show that lycopene is stable in micelles in cell culture media at 37°C and 5% CO_2 in air (see Fig. 2B). Once in micellar form, lycopene in plastic cell culture plates is stable. The next question to be addressed was whether micelles or lycopene produce cytotoxicity.

LNCaP human prostate tumor cells and Hs888Lu human lung cells were incubated for up to 6 d (144 h) with micelles prepared as described in the Experimental Procedures section (see Figs. 3A and 3B). Since the proliferation of Hs888Lu cells in the control incubations containing no micelles was identical to the growth of cells in media containing micelles, no toxicity was observed due to the micelles (Fig. 3B). However, a slight decrease in saturation density of LNCaP cells but not Hs888Lu cells was observed in micelle-treated cells after 4 d (Fig. 3A). In parallel experiments, cells were incubated in media containing five different concentrations of lycopene. Even at the maximal concentration of 10 μg/mL, no toxicity due to lycopene was observed (Fig. 3).

When the cell culture media were replaced daily with fresh media containing additional micelles, inhibition of cell growth was observed. Sodium taurocholate was determined to be the cause of the cytotoxicity, since higher concentrations of this surfactant in the micelles produced greater cytotoxicity. Therefore, the concentration of sodium taurocholate used throughout this investigation was empirically determined to be adequate for micelle formation and lycopene solubilization, and nontoxic when added to the cell culture just once. For comparison, the toxicity of THF was investigated in LNCaP cells. At the concentration of THF used by Cooney *et al.* (11) to solubilize carotenoids, proliferation of LNCaP cells was inhibited (data not shown). Therefore, the combination of poor solubility of lycopene in aqueous solutions of THF, the instability of lycopene in THF solutions, and the toxicity of THF to prostate cells growing in culture makes THF a poor choice for these studies. On the other hand, the solubility, sta-

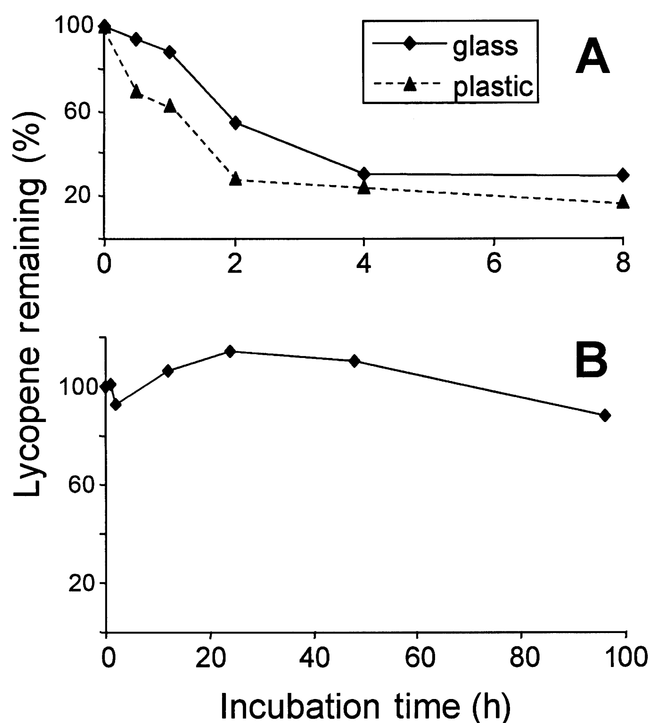

FIG. 2. Stability of lycopene (initial concentration, 263 μg/mL) in Roswell Park Memorial Institute 1640 medium at 37°C under standard cell culture conditions in polystyrene culture dishes measured using HPLC. (A) Lycopene was solubilized using THF, and (B) micelles were used to solubilize lycopene in the cell culture medium (*n* = 3). See Figure 1 for abbreviations.

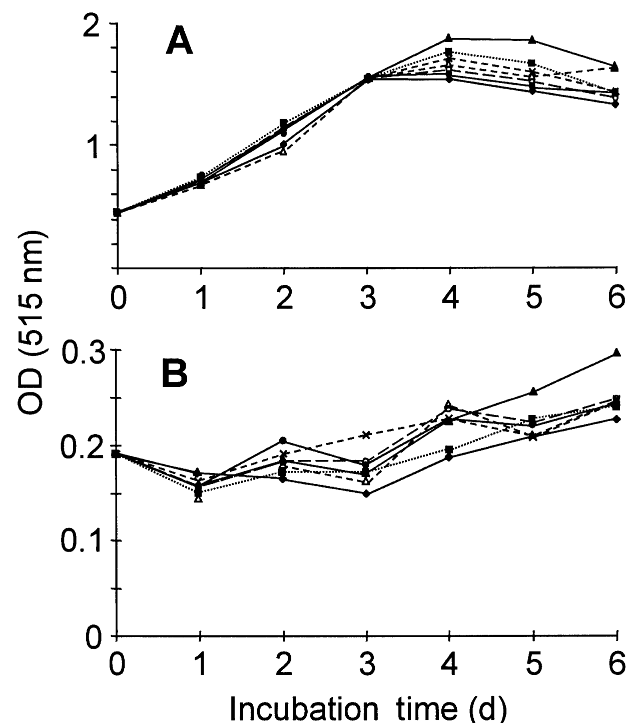

FIG. 3. The effect of media containing no micelles (▲), micelles alone (■), or micelles containing lycopene at final concentrations of (···) 0.05, (x) 0.1, (○) 0.5, (●) 1.0, (△) 5.0, and (◆) 10.0 μg/mL on the growth curves of (A) LNCaP human prostate tumor cells and (B) Hs888Lu human lung cells. Optical density was measured at 515 nm to determine cell protein, which represented cell numbers (*n* = 8).

bility, and minimal toxicity of micellar lycopene are all compatible with prostate cell culture studies.

The next question addressed was whether micellar lycopene could be taken up by prostate cells. LNCaP cells were cultured for up to 24 h in medium containing 5 µg/mL lycopene in micelles. Cells were harvested at different time intervals, rinsed with phosphate buffered saline, and the cellular lycopene was extracted and measured using HPLC. The concentrations of lycopene at each time point were normalized to cellular protein levels, and the results are summarized in Figure 4. Micellar lycopene was taken up by LNCaP cells, and a plateau was reached at approximately 12 h (Fig. 4A). These results show that micellar lycopene in the culture medium can be taken up by LNCaP cells.

The relative proportion of all-*trans* lycopene contained in LNCaP cells during uptake of micellar lycopene over 24 h was measured using LC–MS (16), and the results are shown in Figure 4. During the first 8 h, the concentration of cellular all-*trans* lycopene increased as micellar lycopene was incorporated into the cells (Fig. 4A). Simultaneously, the cellular concentration of *cis*-lycopene increased, too, which was probably the result of all-*trans* lycopene isomerizing as it was released from the stabilizing environment of the micelles during cellular uptake. In contrast, the concentration of extracellular (micellar) all-*trans* lycopene decreased only slightly during this time (Fig. 4B), and the concentration of extracellular *cis*-lycopene remained low. In addition to protecting lycopene from oxidation, the micellar preparation stabilized all-*trans* lycopene and prevented isomerization to its *cis* isomers (see Fig. 5A and 5B). Simultaneously, the proportion of *cis*-lycopene increased substantially within the LNCaP cells (Fig. 5C and 5D).

These results are consistent with those of Clinton *et al.* (19) who reported that the proportion of *cis*-lycopene in human prostate tissue is greater than that in human serum. Specifically, Clinton *et al.* found that lycopene in the prostate of men was 79–88% *cis*, but serum contained 58–73% *cis*-isomers. In our prostate cell culture study, LNCaP cells contained 20–50% *cis*-lycopene after incubation for 24 h. But in the corresponding cell culture medium, the amount of *cis* lycopene was always lower at 0–25%. The explanation for an excess of *cis*-lycopene within prostate cells remains unknown.

Because of the extreme hydrophobicity of carotenes such as lycopene, these compounds are insoluble in tissue culture media. Furthermore, lycopene is highly unstable and degrades rapidly when dissolved in organic solvents or aqueous sys-

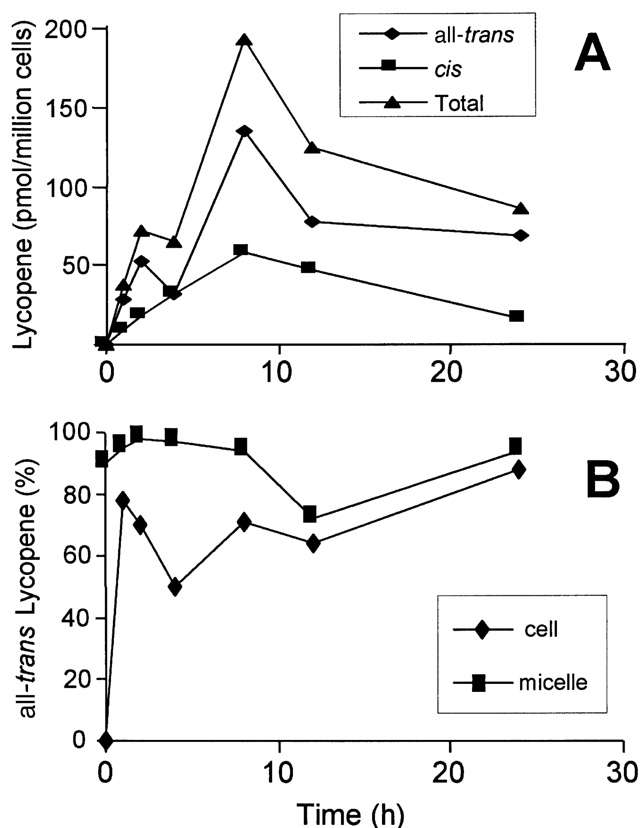

FIG. 4. (A) Cellular lycopene levels in LNCaP human prostate cells (pmol/million cells) measured at different time intervals after addition of 5 µg/mL (9.33 µM) lycopene in micelles. (B) Fraction of all-*trans* lycopene (as % of total lycopene) present in LNCaP cells and remaining in extracellular micelles at each time point measured using liquid chromotography–mass spectrometry (LC–MS) ($n = 2$).

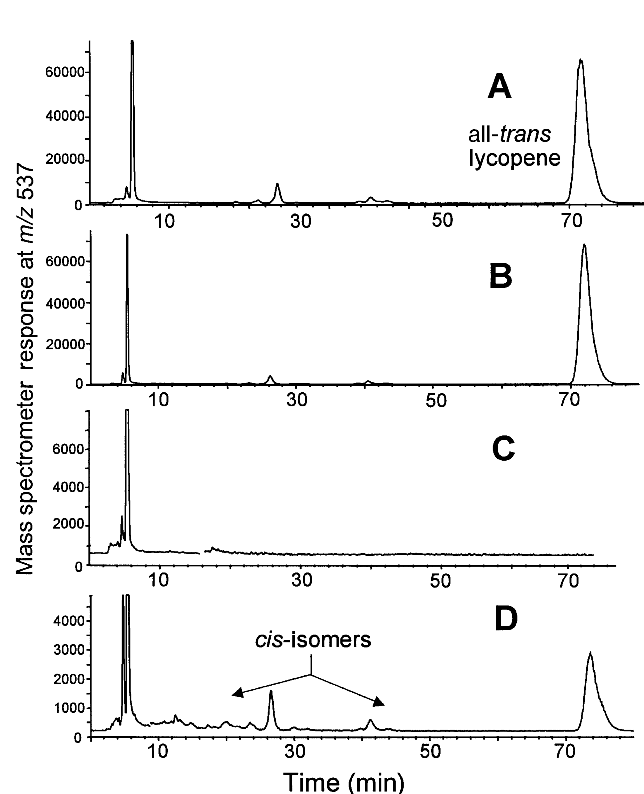

FIG. 5. LC–MS mass chromatograms showing the relative amounts of *cis*- and all-*trans* lycopene (A) in fresh cell culture medium containing micellar lycopene at 5 µg/mL ($t = 0$ h); (B) in the cell culture medium after incubation for 8 h; (C) in LNCaP cells at $t = 0$ h (no lycopene); and (D) in LNCaP cells after incubation with lycopene for 8 h. The proportion of *cis*-lycopene increased only within the cells during incubation. See Figure 4 for abbreviation.

tems containing organic cosolvents and exposed to light and/or air. Micelles provide a convenient, inexpensive, and nontoxic vehicle for dissolving carotenes, such as lycopene, in tissue culture media and then delivering these compounds to cells growing in culture at concentrations spanning the range of physiological levels and even beyond. Our studies show that lycopene contained in micelles may be taken up by cells growing in culture and that the cellular levels generally reflect the concentration of lycopene in the culture medium. Since the half-life of lycopene dissolved in aqueous/organic solutions is only 2 h, meaningful cell culture studies cannot be carried out using lycopene without some means of stabilization. Finally, micelles stabilize lycopene by preventing isomerization and degradation by air so that cell culture studies may be carried out with a constant supply of lycopene in the culture medium.

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Regulation of Mevalonate Synthesis in Low Density Lipoprotein Receptor Knockout Mice Fed n-3 or n-6 Polyunsaturated Fatty Acids[1]

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ABSTRACT: 3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, catalyzes the formation of mevalonate which is also required for cell proliferation. Changes in HMG-CoA reductase may mediate the differential effects of n-3 and n-6 polyunsaturated fatty acids (PUFA) on experimental mammary tumorigenesis, but the mechanisms by which these fatty acids regulate HMG-CoA reductase are unclear. To determine whether the low density lipoprotein receptor (LDL-R) is required for this regulation, groups of female LDL-R knockout (–/–) and wild-type (+/+) mice were fed 7% fat diets rich in either n-3 (menhaden oil) or n-6 (safflower oil) PUFA for 1 wk. Dietary PUFA and deletion of the LDL-R had independent effects on HMG-CoA reductase and serum lipids, and a significant diet–gene interaction was observed. The effects of PUFA on HMG-CoA reductase in the mammary gland, but not the liver, were mediated by the LDL-R. We also observed that differences in HMG-CoA reductase and serum LDL-cholesterol, high density lipoprotein cholesterol, and triglycerides between –/– and +/+ mice were dependent on whether the mice were fed n-3 or n-6 PUFA. Differences between –/– and +/+ mice were much greater when animals were fed n-6 PUFA rather than n-3 PUFA. These results show that the LDL-R mediates the effects of PUFA on HMG-CoA reductase in the mammary gland but not the liver. Furthermore, the composition of dietary PUFA profoundly influences the effects of deleting the LDL-R on HMG-CoA reductase and serum lipids and suggests that diet may influence the phenotype of other knockout or transgenic animals.

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3-Hydroxy-3-methylglutaryl (HMG)-CoA reductase (EC 1.1.1.34) is the rate-limiting enzyme in cholesterol biosynthesis that catalyzes the formation of mevalonate (1). This enzyme is regulated through a multivalent feedback mechanism that is controlled, in part, by intracellular cholesterol levels (2). Cells meet their cholesterol requirements by *de novo* synthesis or uptake of cholesterol-rich lipoproteins through the low density lipoprotein-receptor (LDL-R) or a receptor-independent pathway (2,3). The liver is the major organ regulating serum cholesterol and is a major site of cholesterol biosynthesis (4). When uptake of lipoproteins is enhanced and intracellular cholesterol levels rise, HMG-CoA reductase is down-regulated to prevent excessive accumulation of cholesterol (2).

Mice with a targeted disruption of the *LDL-R* gene were developed as a model of familial hypercholesterolemia and have been used to study atherosclerosis and xanthomatosis (5,6). Deletion of the LDL-R results in a twofold increase in total serum cholesterol and a 14-fold increase in LDL-cholesterol in chow-fed mice (5,7). Steady-state cholesterol concentrations and rates of cholesterol synthesis in various organs, however, are not altered by LDL-R deletion (7).

We recently showed that mammary gland HMG-CoA reductase is lower in rats fed n-3 polyunsaturated fatty acids (PUFA) compared to those fed n-6 PUFA and postulated that this may be a mechanism by which n-3 PUFA inhibit, or n-6 PUFA promote, experimental mammary tumorigenesis. In addition to being a precursor of cholesterol, mevalonate is also required for cell proliferation and has previously been implicated in cancer development (9–12). Competitive inhibitors of HMG-CoA reductase inhibit the development of mammary tumors *in vivo* and the proliferation of human breast cancer cells *in vitro* (13–15). The mevalonate-derived metabolite required for cell-cycle progression has not yet been identified, but is known not to be cholesterol (9).

Spady and colleagues conducted a series of experiments to elucidate the mechanisms by which n-3 and n-6 PUFA regulate cholesterol metabolism (16–18). They observed that dietary n-3 PUFA enhance the internalization of LDL by the liver and various extrahepatic tissues in the rat (16–18), but not the hamster (18). In the rat, enhanced uptake of LDL by the liver appears to be mediated predominantly by the LDL-R and in extrahepatic tissues by a receptor-independent pathway (16). This mechanism may explain how n-3 PUFA lower serum cholesterol and decrease HMG-CoA reductase. The mammary gland, however, is capable of synthesizing choles-

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Abbreviations: ANOVA, analysis of variance; HDL, high density lipoprotein; HMG, 3-hydroxy-3-methylglutaryl; PUFA, polyunsaturated fatty acid(s); LDL, low density lipoprotein; LDL-R, LDL receptor; +/+, wild-type; -/-, knockout; SDS-PAGE, sodium dodecylsulfate-polyacrylamide gel electrophoresis.

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terol for secretion during lactation and may be subject to unique regulatory mechanisms (19).

In the present study, we used wild-type C57Bl/6J mice and the same strain with a targeted disruption of the *LDL-R* gene to investigate whether the LDL-R mediates the effects of PUFA on HMG-CoA reductase and serum lipids. Our results showed that the LDL-R is required for PUFA to regulate HMG-CoA reductase in the mammary gland but not the liver. More importantly, we observed that the composition of PUFA in the diet influences the effects of deleting the LDL-R on serum lipids and HMG-CoA reductase in both the liver and mammary gland.

MATERIALS AND METHODS

Materials. All reagents for electrophoresis and immunodetection were purchased from Bio-Rad Laboratories (Richmond, CA). The anti-HMG-CoA reductase antibody was a generous gift from Dr. D.G. Hardie (University of Dundee, Scotland), and the anti-LDL-R antibody was a generous gift from Dr. A.D. Coooper (Stanford University, Palo Alto, CA). 3-Hydroxy-3-methyl[3-^{14}C]glutaryl-CoA and [5-^{3}H]mevalonolactone were from Du Pont-New England Nuclear (Mississauga, Ontario, Canada), and all other reagents and chemicals were obtained from Sigma (St. Louis, MO).

Animals and diets. Twenty virgin female C57Bl/6J mice that were homozygous for a deletion in the *LDL-R* gene (–/–) and 20 virgin wild-type mice (+/+) were purchased from Jacksons Lab (Bar Harbour, ME) at 7 wk of age. Animals were housed at 23°C and 50% humidity with a 12-h light/dark cycle and acclimatized on the AIN-93G standard reference diet (20) (Dyets, Bethlehem, PA) for 1 wk with free access to food and water. Knockout and wild-type mice (10 per group) were fed one of two diets in which the 7% soybean oil in the AIN-93G diet (devoid of cholesterol) was replaced with 1% soybean oil (to ensure adequate amounts of essential fatty acids) plus either 6% menhaden oil (rich in n-3 PUFA) or 6% safflower oil (rich in n-6 PUFA) for 1 wk. The cholesterol content of the n-3 diet was <0.03%. The fatty acid levels in the diets were determined using the method of Ulberth and Henninger (21). Mice were anesthetized with halothane, blood was collected by cardiac puncture, and the mice were killed during the fourth hour of the light cycle by cervical dislocation. The livers and mammary glands were dissected, immediately frozen in liquid nitrogen, and stored at −80°C until needed for further analysis.

Serum lipids. Blood samples were allowed to coagulate at ambient temperature then centrifuged at 2,500 × *g* for 10 min. Serum was assayed for total cholesterol using a kit obtained from Boehringer Mannheim (Laval, Québec, Canada), and triglycerides and high density lipoprotein (HDL) cholesterol using a kit from Sigma (St. Louis, MO). LDL cholesterol was then calculated as described by Sigma.

Tissue preparation. All tissue preparations were performed over ice or at 4°C. For the preparation of microsomes, tissues were homogenized in buffer A [20 mM Tris-HCl (pH 7.4), 0.25 M sucrose, 70 mM KCl, 5 mM EDTA, 5 mM EGTA, 1 mM dithiothreitol, 50 µM leupeptin] and centrifuged for 10 min at 500 × *g*. Supernatants were centrifuged for 15 min at 9,000 × *g* followed by a final spin for 1 h at 100,000 × *g*. Microsomes were assayed for HMG-CoA reductase activity or were solubilized in Laemmli buffer containing 8 M urea for immunoblotting. Plasma membrane fractions were isolated by homogenizing tissues in buffer B [250 mM Tris-maleate (pH 6.5), 150 mM NaCl, 2 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride, and 2 mM leupeptin]. Homogenates were centrifuged at 9,000 × *g* for 15 min, and the supernatants centrifuged at 40,000 × *g* for 40 min. Membrane pellets were resuspended in buffer B containing 30 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and centrifuged at 40,000 × *g* for 40 min. Solubilized membranes were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under nonreducing conditions. Protein was determined using a protein dye-binding assay (Bio-Rad) according to the manufacturer's protocol with bovine serum albumin as a standard.

Immunoblotting. The 130 kDa mouse LDL-R and 97 kDa HMG-CoA reductase proteins were detected using rabbit polyclonal antibodies as described previously (22,23). Equivalent amounts of protein were applied to SDS-polyacrylamide gels (6% for LDL-R or 7.5% for HMG-CoA reductase), separated by electrophoresis, and transferred to polyvinylidene difluoride membranes using a semidry transfer apparatus. Membranes were blocked overnight at 4°C with 5% nonfat dry milk in Tris-buffered saline (pH 7.4) containing 0.1% Tween-20, probed with anti-HMG-CoA reductase (1:3,000) or anti-LDL-R (1:5,000) for 2 h at room temperature, followed by a 1-h incubation with horseradish peroxidase-conjugated antirabbit antibody (1:5,000) (Santa Cruz Biotechnology, Santa Cruz, CA). Signals were detected using an enhanced chemiluminescence detection system (ECL; Amersham Corp., Arlington Heights, IL), and bands were compared with the migration of prestained molecular weight markers (Bio-Rad). Coomassie blue was used to stain total proteins to ensure equal loading.

HMG-CoA reductase enzyme activity. Total enzyme activity was determined as previously described (24) and expressed as picomoles of mevalonolactone formed per minute and per milligram of microsomal protein. All values are averages of duplicate measurements. Briefly, 50–100 µg of microsomal protein was preincubated at 37°C for 5 min in buffer C [100 mM phosphate (pH 7.4), 70 mM KCl, 10 mM dithiothreitol, 5 mM EDTA, 5 mM EGTA, and 50 µM leupeptin], followed by a further 5 min with an NADPH regenerating system (1 U glucose-6-phosphate dehydrogenase, 20 mM glucose-6-phosphate, 2 mM NADP). The assay was initiated by the addition of ^{14}C-HMG-CoA (20,000 dpm/nmol) at a final concentration of 80 µM and volume of 75 µL, and terminated after 30 min by the addition of 5 µL HCl containing ^{3}H-mevalonolactone as a recovery standard. After a 1-h incubation at 37°C to allow complete lactonization of the product, samples were centrifuged for 1 min at 3,000 × *g* to re-

move the denatured protein, 40 μL of supernatant was applied to a silica gel G plate (Analtech, Newark, DE), and mevalonolactone was separated by thin-layer chromatography using a toluene/acetone (1:1) solvent system. The region corresponding to mevalonolactone (R_f 0.7) was scraped into scintillation fluid and radioactivity measured using a β-counter with a dual-labeling program.

Statistical analyses. All values are shown as means ± SEM. Data were analyzed by one-way analysis of variance (ANOVA) followed by Student's *t*-test with Bonferroni *post hoc* for multiple comparisons. Two-way ANOVA was used to assess diet–gene interactions (GraphPad Prism™ San Diego, CA).

TABLE 2
Serum Lipid Analyses of LDL-R+/+ and LDL-R–/– Mice Fed Either n-3 or n-6 PUFA[a]

	Total cholesterol (mg/dL)	LDL (mg/dL)	HDL (mg/dL)	Triglycerides (mg/dL)
LDL-R+/+				
n-6	113.5 ± 9.5[a]	32.5 ± 5.4[a]	69.3 ± 5.6[a]	88.9 ± 13.3[a]
n-3	64.8 ± 8.9[b]	5.7 ± 2.4[b]	48.9 ± 4.9[b]	58.3 ± 8.9[b]
LDL-R–/–				
n-6	409.9 ± 44.6[c]	306.6 ± 28.0[c]	55.9 ± 4.7[b]	152.1 ± 19.5[c]
n-3	240.8 ± 51.7[d]	157.9 ± 37.0[d]	53.1 ± 9.6[b]	75.9 ± 11.9[a,b]

[a]Values not sharing the same superscript letters within each column differ significantly from each other ($P < 0.05$). LDL, low density lipoprotein cholesterol; HDL, high density lipoprotein cholesterol; (+/+), wild-type mice; (–/–), knockout mice.

RESULTS

Groups of +/+ and LDL-R –/– mice were fed cholesterol-free, 7% fat diets containing either menhaden oil (n-3 diet) or safflower oil (n-6 diet). Lipids were extracted from the diets, and their fatty acid content was analyzed (Table 1). As anticipated, the n-3/n-6 PUFA ratio was 100-fold greater in the n-3 diet than in the n-6 diet. Since chow diets have variable fatty acid and cholesterol contents and the effects of the AIN-93 reference diet on lipid metabolism and mammary carcinogenesis have not been established, we compared the effects of feeding n-3 or n-6 PUFA relative to each other.

The effects of n-3 and n-6 PUFA on cholesterol metabolism were first compared in +/+ mice. Compared to n-6 PUFA, serum triglycerides and total-, LDL- and HDL-cholesterol levels were significantly lower in mice fed n-3 PUFA (Table 2). A radiochemical assay was used to assess the effects on HMG-CoA reductase activity in the livers and mammary glands (Fig. 1). Compared to n-6 PUFA, n-3 PUFA reduced the specific activity of HMG-CoA reductase in the liver from 336 ± 80 to 109 ± 25 pmol/min/mg ($P < 0.005$) (Fig. 1A), and in the mammary glands from 36 ± 10 to 20 ± 9 pmol/min/mg ($P < 0.01$) (Fig. 1B). To determine whether the changes in enzyme activity were due to changes in the levels of HMG-CoA reductase protein, microsomes from livers and mammary glands were subjected to SDS-PAGE and probed using an anti-HMG-CoA reductase antibody. Figure 2 is a representative Western blot showing that the differences we

TABLE 1
Fatty Acid Content of Experimental Diets

	Group (mg fatty acid/g diet)	
Fatty acid	n-3 Diet	n-6 Diet
14:0	6.65	0.35
16:0	10.43	5.97
16:1n-7	7.71	—
18:0	2.27	2.25
18:1n-9	8.01	9.93
18:1n-7	2.25	0.58
18:2n-6	7.57	47.8
18:3n-3	1.32	0.96
18:4n-3	1.04	—
20:5n-3	8.67	—
22:5n-3	1.29	—
22:6n-3	2.7	—
Other	10.1	2.16
Total	70.0	70.0
n-3/n-6 Ratio	2.0	0.02

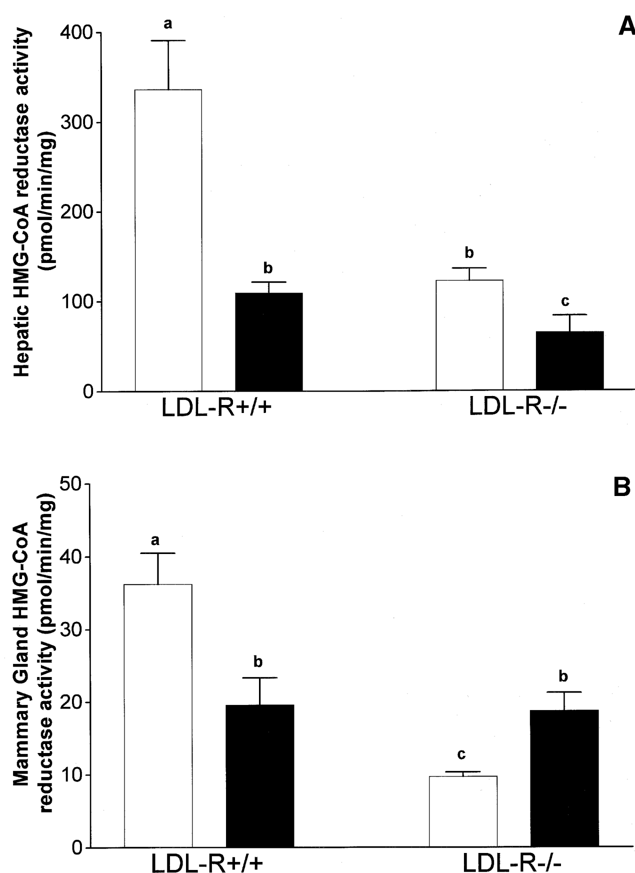

FIG. 1. Effects of n-3 (■) or n-6 (□) polyunsaturated fatty acids (PUFA) on 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase activity in low density lipoprotein receptor (LDL-R) knockout (–/–) and wild-type (+/+) mice. Liver (A) and mammary gland (B) HMG-CoA reductase enzyme activities were assayed from tissue microsomes prepared from LDL-R–/– and LDL-R+/+ mice fed either n-3 (menhaden oil) or n-6 (safflower oil) PUFA for 1 wk. Values are mean ± SEM ($n = 10$). Groups not sharing the same letter differ significantly ($P < 0.05$).

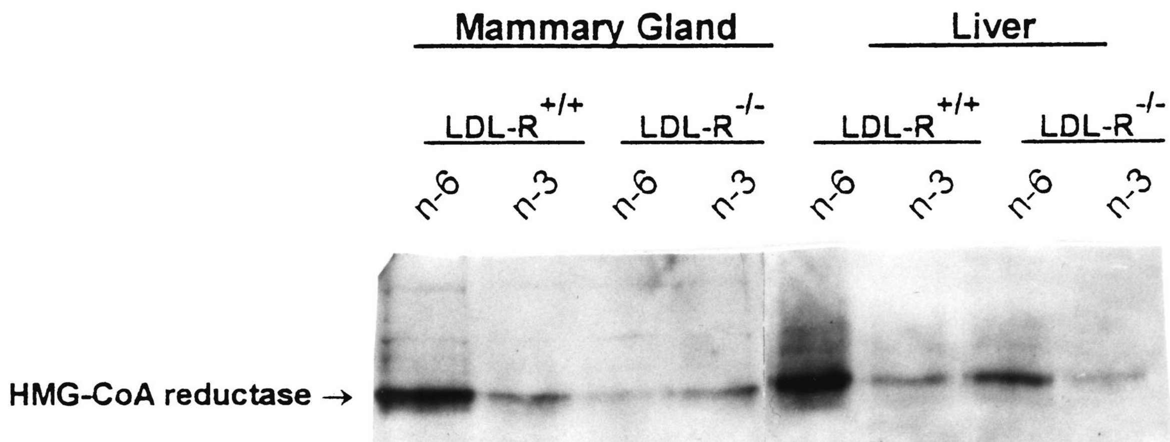

FIG. 2. Representative Western blot of HMG-CoA reductase. Microsomes from mammary glands and livers of LDL-R–/– and LDL-R+/+ mice fed either n-3 (menhaden oil) or n-6 (safflower oil) PUFA for 1 wk were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and probed with a rabbit polyclonal anti-HMG-CoA reductase antibody. The blot shows a representative sample from each group. For abbreviations see Figure 1.

observed in enzyme activity are due, at least in part, to differences in the amount of HMG-CoA reductase protein. LDL-R protein levels were not different in the liver or mammary glands of +/+ mice fed either of the two diets (Fig. 3).

Next, we compared the effects of n-3 to n-6 PUFA in –/– mice to determine the role of the LDL-R in mediating the changes we observed in +/+ mice. In –/– mice fed n-3 PUFA, serum triglycerides and total- and LDL-cholesterol levels were also lower than in those fed n-6 PUFA (Table 2). HDL-cholesterol levels, however, did not differ between the two diets (Table 2). n-3 PUFA reduced hepatic HMG-CoA reductase activity from 123 ± 27 to 65 ± 38 pmol/min/mg ($P < 0.05$) (Fig. 1A), but unexpectedly increased activity in the mammary gland from 10 ± 2 to 19 ± 6 pmol/min/mg ($P < 0.05$) (Fig. 1B). These differences in enzyme activity corresponded with differences in the levels of HMG-CoA reductase protein (Fig. 2).

Comparisons between +/+ and –/– mice by two-way ANOVA revealed that the *LDL-R* gene had an effect, independent of PUFA, on HMG-CoA reductase and serum lipids, but a diet–gene interaction ($P < 0.05$) determined the magnitude of the differences. Serum LDL-cholesterol was 27-fold greater in –/– than in +/+ mice fed n-3 PUFA and only 10-fold greater in mice fed n-6 PUFA. HDL-cholesterol levels were lower and serum triglycerides were higher in –/– than in +/+ mice fed n-6 PUFA, but no differences were observed among those fed n-3 PUFA. Total serum cholesterol was approximately fourfold greater in –/– than in +/+ mice, regardless of the diet. Dietary PUFA also modulated the effects of LDL-R deletion on HMG-CoA reductase. Compared to +/+

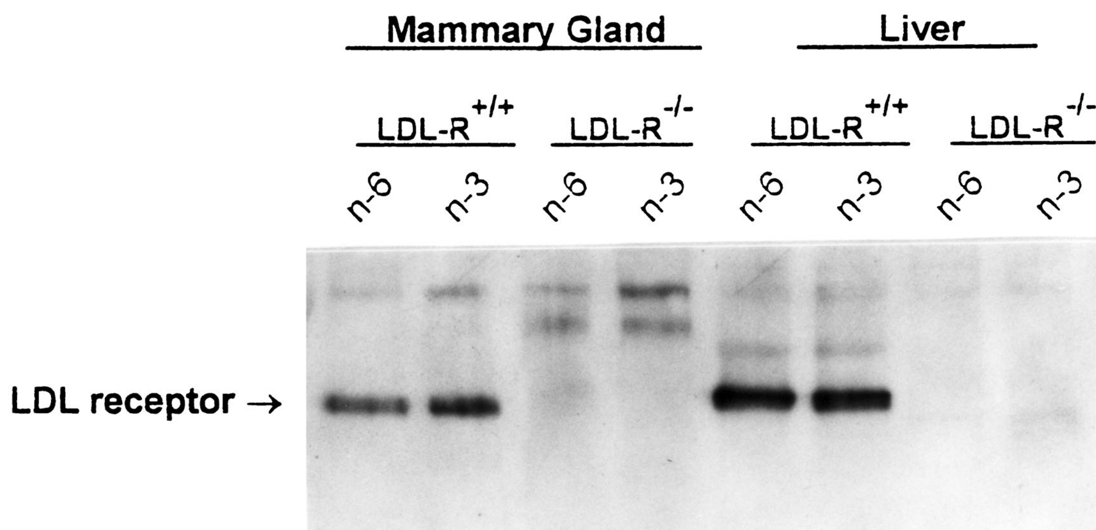

FIG. 3. Representative Western blot of LDL-R. Plasma membrane fractions were isolated from mammary glands and livers of LDL-R–/– and LDL-R+/+ mice fed either n-3 (menhaden oil) or n-6 (safflower oil) PUFA for 1 wk. Equal amounts of protein were resolved on 6% polyacrylamide gels and blotted proteins were probed using a rabbit polyclonal anti-LDL-R antibody. For abbreviations see Figure 1.

mice, hepatic HMG-CoA reductase was 2.7-fold lower in –/– mice when the groups were fed n-6 PUFA, but was only 1.7-fold lower when they were fed n-3 PUFA (Fig. 1A). Deletion of the LDL-R did not alter mammary gland HMG-CoA reductase in mice fed n-3 PUFA, but resulted in a significant decrease in enzyme activity (3.6-fold) and protein levels in those fed n-6 PUFA (Figs. 1B and 2). These analyses show that the composition of PUFA in the diet can profoundly influence the effects of deleting the LDL-R on serum lipids and HMG-CoA reductase.

DISCUSSION

We previously showed that n-3 and n-6 PUFA differentially regulate mammary gland HMG-CoA reductase and hypothesized that this may be a mechanism by which these fatty acids modulate experimental mammary tumorigenesis (8). To investigate the functional significance of the mevalonate pathway, we sought to identify an animal model in which the regulation of HMG-CoA reductase by PUFA may be perturbed. The present studies were designed to determine whether LDL-R –/– mice qualify as such a model. By comparing the effects of n-3 and n-6 PUFA in both +/+ and LDL-R –/– mice, we established that the LDL-R is indeed required for PUFA to regulate mammary gland HMG-CoA reductase. A two-way ANOVA also revealed that the LDL-R has an independent effect on HMG-CoA reductase as well as serum lipids. When we compared +/+ to –/– mice, it became clear that the differences in serum lipids and HMG-CoA reductase between these two groups were markedly dependent on the type of PUFA in the diet. These results provide an example of what has been described as diet–gene interactions (25,26).

LDL-R deficiency has previously been shown to have no effect on hepatic cholesterol synthesis in mice (7), but it leads to a 40% decrease in rabbits (27). Although this discrepancy may be due to genetic differences between these species, a role for the different diets consumed by these animals cannot be excluded. Chow diets used commonly in genetic studies vary considerably in their composition and may produce conflicting observations. Moreover, the effects of pharmacological or dietary factors in genetically altered animals may either be masked or augmented depending on the composition of the basal diet. This could ultimately lead to an incomplete understanding of the normal function of a gene.

Our previous studies (8) and those of others (16–18) on the regulation of HMG-CoA reductase or cholesterol biosynthesis by PUFA used rats. Since cholesterol metabolism is known to differ between species (4,28) and since the effects of PUFA in mice were not known, we needed to establish in the present experiment whether the mouse responds like the rat to the effects of PUFA on serum lipids and HMG-CoA reductase. Compared to mice fed n-6 PUFA, those fed n-3 PUFA had lower levels of serum triglycerides and total-, LDL-, and HDL-cholesterol and decreased HMG-CoA reductase in the liver and mammary glands. These results demonstrate that mice respond like rats to the effects of dietary PUFA. Although the fish oil we used contained low levels of cholesterol, the decrease in the HMG-CoA reductase is likely to be due to the long-chain n-3 PUFA; purified n-3 PUFA have effects on cholesterol metabolism similar to the fish oils from which they are derived (16,17).

To determine whether any of the effects we observed are mediated by the LDL-R, we also fed the experimental diets to mice lacking a functional LDL-R. Compared to n-6 PUFA, n-3 PUFA reduced total- and LDL-cholesterol levels while reducing hepatic HMG-CoA reductase. Although the rate of LDL uptake was not measured directly in these experiments, the effects observed are consistent with enhanced uptake of LDL by the liver in mice fed n-3 PUFA (28). It is also possible that n-3 PUFA decrease cholesterol secretion from the liver as has previously been shown in the rat (17). In the mammary glands of –/– mice, HMG-CoA reductase was greater in those fed n-3 compared with those fed n-6 PUFA. This unexpected difference may have been a response to the lower serum LDL-cholesterol levels in the n-3 PUFA group that were caused by enhanced receptor-independent uptake of LDL in the liver. Importantly, this observation demonstrates that the LDL-R is required for n-3 PUFA to decrease mammary gland HMG-CoA reductase but is not required for n-3 PUFA to decrease hepatic HMG-CoA reductase or serum lipoproteins.

The first report on LDL-R –/– mice showed a twofold increase in total cholesterol, a seven- to ninefold increase in intermediate density lipoproteins and LDL, and no significant changes in HDL or triglyceride levels compared to +/+ littermates (5). Osono *et al.* (7) were the first to examine the effects of LDL-R deletion on rates of cholesterol synthesis and cholesterol balance across the plasma space. They observed that deletion of the LDL-R results in a 14-fold increase in serum LDL and a significant increase in very low density lipoprotein and HDL (7). There were no changes in the rates of cholesterol synthesis in the liver or various extrahepatic tissues. The mice in those two studies (5,7), however, were fed chow diets that contain low levels of cholesterol and undefined fatty acids. In the present study, we observed that the type of PUFA in a semisynthetic diet profoundly influenced the effect on serum lipoproteins of deleting the LDL-R. For example, LDL-cholesterol was 27-fold greater in –/–than in +/+ mice when both groups were fed n-3 PUFA, but was only 10-fold greater when they were fed n-6 PUFA. Interestingly, HDL-cholesterol levels were slightly higher in –/– than in +/+ mice fed n-3 PUFA but were significantly lower when they were fed n-6 PUFA (Table 2).

The types of dietary PUFA were also important determinants of the effects of deleting the LDL-R on HMG-CoA reductase. In the liver, deletion of the LDL-R decreased HMG-CoA reductase to a greater extent in mice fed n-6 PUFA than in mice fed n-3 PUFA. These decreases in HMG-CoA reductase are likely due to receptor-independent uptake of LDL-cholesterol, which is stimulated by the extremely high circulating levels in –/– animals. A more striking effect was observed in the mammary gland where deletion of the LDL-R

had no effect on HMG-CoA reductase activity or protein levels when n-3 PUFA were fed but resulted in a marked (3.6-fold) decrease when n-6 PUFA were the major source of dietary fat. This decrease caused by LDL-R deletion in mice fed n-6 PUFA may, in part, be a consequence of the elevated serum cholesterol that enters the cells through a receptor-independent pathway, thereby down-regulating HMG-CoA reductase. However, the lack of an effect on HMG-CoA reductase by LDL-R deletion among mice fed n-3 PUFA suggests other mechanisms may also be involved. An alternative interpretation is that n-6 PUFA stimulate mammary gland HMG-CoA reductase through the LDL-R, whereas n-3 PUFA have no effect. This mechanism is unlikely since n-3 PUFA lower serum cholesterol and decrease cholesterol synthesis whereas n-6 PUFA do not have this effect compared to a diet rich in saturated fatty acids (16).

In addition to dietary differences, the background strain of LDL-R knockout mice used in previous studies also differs from the one used in the present study. Earlier studies used the B129 strain (5–7), which is a cross between the 129Sv and C57Bl/6J strains. The LDL-R-deficient mice we used in the present study are in a C57Bl/6J background and were developed by Jackson Labs by backcrossing the B129 with the C57Bl/6J strain. Mouse strains differ in their metabolism of cholesterol and expression levels of HMG-CoA reductase. Comparisons between some studies may therefore be further confounded by these genetic differences.

In summary, we have shown that the LDL-R mediates the effects of PUFA on HMG-CoA reductase in the mammary gland but is not required for the decrease in serum lipids or hepatic HMG-CoA reductase caused by n-3 PUFA. These results may lead to a better understanding of the mechanisms by which dietary PUFA modulate biochemical pathways that may be involved in mammary carcinogenesis. Furthermore, our observations show that the composition of dietary PUFA is a major determinant of the effects of LDL-R deletion on cholesterol metabolism and may alter the interpretation of studies using LDL-R knockout mice. This paradigm may also be applicable to other dietary factors and to other knockout or transgenic animals.

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Synthesis and Estimation of Calorific Value of a Structured Lipid–Potential Reduced Calorie Fat

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ABSTRACT: The majority of reduced calorie fats and fat substitutes available today, though similar in texture and flavor to natural fats, contain fatty acids that are not usually present in edible oils and fats and thus do not fully match the chemistry and functions of natural fats. For example, such products do not provide nutritionally important essential fatty acids (EFA). In this investigation, we prepared and evaluated a reduced calorie fat, prepared entirely from natural fats, taking advantage of the fact that long-chain saturated fatty acids (LCSFA), such as behenic acid (22:0), are poorly absorbed. Mustard oil (MO) and sunflower oil (SO) were used as substrates to yield a structured lipid (SL). The product, being derived from a natural vegetable oil, would thus provide EFA, as would a native fat, a feature not provided by the low-calorie fats available in the market. Erucic acid (22:1) was isolated from MO by a lipase (EC 3.1.1.3)-catalyzed reaction. It was then hydrogenated to behenic acid, the ethyl ester of which was subsequently enzymatically transesterified with SO to yield a plastic fat containing about 30–35% behenic acid. Absorption of this fat was studied in Wistar rats. In a preliminary single oral dose experiment, rats were fed equal amounts (2 mL) of SO and the SL. Plasma triacylglycerol (TAG) levels were estimated after 1, 2, and 3 h of feeding. The significantly lower concentration of plasma TAG in the 2-h sample, observed in the SL-fed group compared to the SO-fed group ($P < 0.001$), indicated poor absorption of the SL. In order to estimate the calorific value of the SL, we conducted a restricted diet growth experiment over 21 d on weanling Wistar male rats with SO as caloric control. Diets for the test groups were modified by adding 5, 10, and 15% SO for the control groups, and 5 and 10% SL for the experimental groups. Food consumption of the test groups was restricted to 50% of the feed containing 5% SO that had been consumed by the *ad libitum* group the previous day. Body weights were recorded during the experiment. Calorific value of the SL was estimated by comparing the 21st-d mean body weight gain of the control group with that of the experimental group. Estimated calorific value of the SL was 5.36 kcal/g. Most of the behenic acid fed was excreted, as indicated by the analysis of the fatty acids of plasma and fecal total lipid. A second growth experiment on *ad libitum* diet was conducted over 21 d on weanling Wistar male rats to compare the absorption behavior of the SL with that of natural oil. SO (10%) was added to the diet of the control group, and SL (10%) was added to the diet of the experimental group. Feed consumption, as well as body weights, was recorded during the experiment. The growth pattern of the experimental group was identical to that of the control group during the period of study. The mean feed intake (9.8 g/d/rat for the control group vs. 9.9 g/d/rat for the experimental group) indicated good palatability of the product. In conclusion, the enzymatically synthesized SL containing EFA and natural antioxidants has nutritional properties almost identical to those of natural fats, and can be used as a reduced calorie fat.

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The most energy-rich component of an animal's diet is fat, providing 9 kcal/g compared to the 4 kcal/g of carbohydrate and protein. Dietary fat also serves as a source of essential fatty acids (EFA) and is the carrier for fat-soluble vitamins and antioxidants (1,2). Moreover, the texture and flavor of fat are its two most pleasing organoleptic characteristics (3). However, high fat intake is linked to degenerative diseases such as heart disease, diabetes, high blood pressure, and cancer (4). With increasing consumer awareness of the risks associated with high fat intake, a market for reduced calorie fats and fat replacers has opened up. In addition to reduced calorie fats, fat replacers based on carbohydrates and proteins have entered the market. However, the latter two types are useful only in low-temperature applications, such as frozen desserts. They cannot be used where elevated temperatures are involved, as in cooking or deep frying (5). Only lipid-based fat substitutes can possibly mimic all the attributes of a natural fat.

Generally, structured lipids (SL) used in food products are triacylglycerols (TAG) composed of mixtures of long-chain with medium- or short-chain fatty acids that are randomly arranged on the glycerol molecule (6). They are beneficial to human nutrition, as they can be tailor-made to target specific diseases and metabolic conditions. Manipulations of their fatty acid compositions allow them to be used as reduced calorie fats. Reduced calorie SL are designed by taking advantage of either the limited absorption of long-chain satu-

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Abbreviations: BHT, butylated hydroxy toluene; EFA, essential fatty acids; FFA, free fatty acids; GC, gas-liquid chromatography; LCSFA, long-chain saturated fatty acid; MAG, monoacylglycerol; MO, mustard oil; NMR, nuclear magnetic resonance; prep-TLC, preparative thin-layer chromatography; RP-HPLC, reversed-phase high-performance liquid chromatography; SL, structured lipid; SO, sunflower oil; TAG, triacylglycerol.

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rated fatty acids (LCSFA) or the lower caloric density of short-chain fatty acids (6,7). Caprenin, one of the first SL in the United States, was offered as a potential cocoa butter substitute. Developed as a reduced calorie fat with 5 kcal/g, Caprenin contains behenic acid, obtained from hydrogenated rapeseed oil, and two medium-chain fatty acids—caprylic and capric acids—from coconut and palm kernel oils (8–10). SALATRIM, another commercially availabile SL, contains long-chain (stearic acid) and short-chain (acetic, propionic, or butyric acids) aliphatic acids (11,12).

In this investigation, we wanted to synthesize a SL from natural vegetable oils for use as a reduced calorie fat. Such a product would contain EFA and natural antioxidants and would be close to native oils in its properties and metabolic functions. Behenic acid was incorporated into the *sn*-1 and *sn*-3 positions of sunflower oil (SO) TAG by a lipase-catalyzed transesterification reaction. A 1,3-specific lipase (Lipozyme from *Mucor miehei*) was used for this reaction. The product thus retained unaltered EFA in the *sn*-2 position, a desirable feature because fatty acids at the *sn*-2 position are better absorbed in the animal body than at *sn*-1 or *sn*-3 (13).

Literature suggests that LCSFA, like stearic acid and behenic acid, are poorly absorbed (14,15). This property of behenic acid was employed here for the synthesis of SL. Mustard oil (MO) was the source of behenic acid. Enzymatic hydrolysis of the oil under modified conditions (16) using *Candida cylindracea* lipase yielded 1,3-dierucin, which was hydrogenated to yield 1,3-dibehenin. Chemical transesterification of 1,3-dibehenin yielded ethyl behenate, which was used in the preparation of the SL.

The SL was studied for its absorption behavior and its calorific value. A preliminary single oral dose experiment was conducted on overnight-fast Wistar rats, with SO as control. This was followed by restricted diet growth studies and by growth studies on *ad libitum* diet, again using SO as control. The protocol of Finley *et al.* (17) was used for the estimation of calorific value of the SL. The basal diet for test groups in the restricted diet study was based on 50% of the diet consumed by a pilot *ad libitum* group the previous day. All absorption studies were conducted on inbred weanling Wistar rats.

EXPERIMENTAL PROCEDURES

Materials. Refined SO and MO of well-known brands were purchased from the local market. Lipases from *C. cylindracea* and from porcine pancreas (Type II) were obtained from Sigma Chemical Co. (St. Louis, MO), and Lipozyme IM20 from *Mucor miehei* in the immobilized form on a macroporous anion exchange resin was a generous gift of Novo Nordisk (Bangalore, India). All solvents needed for the preparation as well as analysis including the high-performance liquid chromatography (HPLC)-grade solvents, were purchased from Qualigen India Ltd. (Bombay, India). Chemicals needed for the estimation of plasma TAG, such as 2,4-pentanedione (acetyl acetone), potassium hydroxide, acetic acid, aluminum oxide (neutral), and sodium meta periodate, were obtained from Sisco Research Laboratory Pvt. Ltd. (Mumbai, India). Triolein, heptadecanoic acid, a standard mixture of fatty acid methyl esters (product no. O-7756), a standard mixture of TAG (product no. 178-12), and butylated hydroxytoluene (BHT) were purchased from Sigma Chemical Co. A standard mixture of fatty acid methyl esters (GLC-68) was purchased from Nu-Chek-Prep, Inc. (Elysian, MN). All ingredients needed for the preparation of AIN-93G diet, formulated for growth, were purchased from the local market. All feeding experiments were conducted on inbred Wistar/NIN male rats, bred at National Institute of Nutrition, Hyderabad, India.

Preparation of ethyl behenate. This was prepared from 1,3-dierucin isolated from MO by a lipase-catalyzed process developed in our laboratory (16). The crystallized 1,3-dierucin (203 g, melting point 45°C) was hydrogenated with 10% palladium on carbon (10 g) at 40 psi hydrogen pressure in chloroform (600 mL) for 4 h. The reaction mixture was filtered hot to separate the catalyst, and 1,3-dibehenin was crystallized from acetone (yield 180 g, melting point 87°C). The 1,3-dibehenin was transesterified with ethanol (200 mL) containing 2% H_2SO_4 to yield ethyl behenate, which was recrystallized from hexane (yield 140 g, melting point 52°C). The product had the composition: 16:0, 0.2%; 18:0, 0.6%; 20:0, 4.3%; 22:0, 91.9%; and 24:0, 3%.

Preparation of SL. SO (358 g) and ethyl behenate (165.5 g) were taken in a round-bottomed flask and stirred for 30 min at 60°C under vacuum. Lipozyme (2% w/w of total substrate) was added, and the reaction mixture was stirred at 60°C for 3 h. The product was filtered to remove the enzyme, and the enzyme was washed with hexane. The solvent was removed from the combined filtrate containing transesterified TAG and ethyl esters of fatty acids. The mixture was purified by silicic acid (60–120 mesh) column chromatography with a solvent system of 1% ethyl acetate in hexane to elute the ethyl esters of fatty acids followed by 5% ethyl acetate for the elution of the SL (yield 300 g).

Gas–liquid chromatography (GC). Analysis of the fatty acid methyl esters was carried out on a Hewlett-Packard (Palo Alto, CA) 5890 Series II gas chromatograph equipped with a flame-ionization detector. A fused-silica capillary column SP 2330 (30 m × 0.25 mm i.d. × 0.20 µm film thickness) was used with N_2 as carrier gas. The column was operated at 100°C for 5 min, raised to 160°C at a rate of 30°C/min, and held at this temperature for 1 min. Finally, the column temperature was raised again at a rate of 5°C/min to the final temperature of 235°C and held at this temperature until completion of the analysis. The injection port and detector temperatures were maintained at 235 and 260°C, respectively. Peak identification was accomplished by injecting a standard fatty acid methyl ester mixture. The relative percentage of individual fatty acids was identified and quantified using Chem Station software (Hewlett-Packard) based on the relative response of an internal standard of pure heptadecanoic acid of known concentration.

Analysis of TAG. (i) Measurement of slip melting points and solid fat content. The slip melting point of the SL was de-

termined by open capillary tube method (18). The solid fat content was measured by pulse nuclear magnetic resonance (NMR) (19) by using a Bruker MINISPEC NMS 120 Series NMR analyzer (Bruker Analytische Messtechnik Gmbh, Rheinstetten, Germany).

(ii) *Reversed-phase high-performance liquid chromatography (RP-HPLC)*. TAG molecular species of SO and the SL were analyzed by RP-HPLC on a Shimadzu LC system (model LC 10A) equipped with an evaporative light-scattering detector (Varex ELSD II A; Rockville, MD). The separations of the molecular species were effected on a 3.5 × 150 mm Nova-pak C18 (Waters) steel cartridge column. The ELSD was set at 105°C at a nebulizer gas (N_2) pressure of 30 psi, and the injection volume was 20 µL. The mobile phase consisted of 35% acetonitrile and 65% acetone for the initial 10 min at a flow rate of 1 mL/min. After 10 min, the composition of the mobile phase was changed over a period of 5 min to 25% acetonitrile and 75% acetone at a flow rate of 1 mL/min. The composition was further changed over a period of 10 min to 15% acetonitrile and 85% acetone at a flow rate of 1.2 mL/min. Finally, the mobile phase composition was brought back to 35% acetonitrile and 65% acetone over a period of 5 min at a flow rate of 1 mL/min. The total run time was 30 min. Peak identification was based on equivalent carbon number, and incorporated use of a standard TAG mixture whenever possible. Quantification of molecular species was made by a calibration method using the standard TAG mixture (20).

Porcine pancreatic lipase hydrolysis of the SL. A method modified from Luddy *et al*. (21) was followed. The SL (50 mg) was dissolved in diethyl ether (100 µL) in a stoppered vial. Tris buffer (1 M, pH 8.0, 1 mL), calcium chloride (2.2%; 0.25 mL), and bile salts (0.05%, 0.25 mL) were added to the SL solution. The entire solution was allowed to equilibrate at 40°C in a waterbath for 2–3 min with occasional vortexing. Porcine pancreatic lipase (50 mg) was added, and the reaction mixture was vortexed for 3–4 min. The reaction was quenched immediately by the addition of 1 mL of ethanol and 1 mL of 6 N HCl, and the reaction solution was extracted with 3 × 2 mL of diethyl ether. The ether layer was washed with water and dried over anhydrous sodium sulfate. Finally, the mixtures of products were separated by preparative thin-layer chromatography (prep-TLC) with hexane/ether/acetic acid (80:20:2; by vol) as solvent system. The monoacylglycerol (MAG) band was scraped off, converted to methyl ester, and analyzed by GC.

Diets and animal study designs. The experimental diets were prepared according to the recommendation of the American Institute of Nutrition (22). AIN-93G diet, formulated for growth, was taken as basal diet for the two growth experiments and contained (g/kg diet) corn starch 400, casein 200, α-corn starch 132, sucrose 100, cellulose 50, L-cystine 3, choline chloride 2.5, mineral mixture (AIN-93G-MX) 40, and vitamin mixture (AIN-93G-VX) 10. The dietary levels of calcium and magnesium per gram were 0.357 and 0.024 mg, respectively. Diets used in the growth studies were identical in all respects except for the sources of added fat and their percentages.

Three experiments were conducted for the absorption study of the SL. These were (i) single oral dose experiment, (ii) growth study on restricted diet, and (iii) growth study on *ad libitum* diet. In all these experiments SO was used as control. Results from the growth study on restricted diet were used to estimate the calorific value of SL. The protocol described below was reviewed and pre-approved by the Committee of Ethics on Animal Experiments of National Institute of Nutrition (Indian Council of Medical Research) and was carried out under the guidelines for animal studies of the Institute.

(i) Single oral dose experiment. An initial single oral dose experiment was conducted on male Wistar rats maintained at a temperature of 22 ± 2°C and a relative humidity of 55 ± 5%. Two sets of studies were conducted on rats having different body weights. The first group had a body weight of 120–130 g, and the second, 165–200 g. In each study, rats were divided into two groups of three rats, and all of them were fasted overnight. One group of rats was administered orally by gastric intubation 2 mL of SO (control group) and the other group was given 2 mL of the SL (experimental group). Blood (1.0 mL) samples were drawn from the orbital sinus of rats from both groups, before feeding (zero hour) and 1, 2, and 3 h after fat loading. The level of TAG in the plasma was estimated according to the method described by Foster and Dunn (23). During the period of experiment, no feed was provided to the test rats. After the 3-h blood withdrawal, all the rats were transferred to metabolic cages (Nalgene) and provided with standard animal feed containing 5% fat. Fecal samples were collected for the next 2 d.

(ii) Growth study on restricted diet. This experiment is meant for estimation of calorific value of the SL and is in line with the experiment of Finley *et al.* (17). In this experiment, 42 weanling Wistar/NIN male 21-d-old rats with initial body weights of 39–40 g were fed AIN-93G diet containing 5% SO for 1 wk prior to the start of the experiment. During this period, typical body weights attained were 65–70 g. Rats were then distributed randomly into six groups of seven rats each. Because animals bred within the facility were used and because the initial body weight range was narrow, the variability was minimal at the beginning of the experiment. Rats were housed separately in metal cages with numbered tags for identification. The room temperature was maintained at 22 ± 2°C with a 12-h light/dark cycle. Fresh, sterile deionized water was provided *ad libitum* by a glass bottle with stainless steel nozzle. Food was provided daily at the same time in a straight-walled glass container to minimize spillage. One group of rats was maintained on *ad libitum* diet containing 5% SO (pilot group). All other rats in the five test groups received 50% of the mean diet consumed by the pilot group the previous day. These diets were modified by adding 5, 10, and 15% of SO, respectively, for the three control groups and 5 and 10% of the SL, respectively, for the two experimental groups. Feed consumption was monitored daily. Rats on restricted diet consumed all feed with no spillage. Body weights were recorded on days 0, 3, 6, 9, 14, 17, and 21. After 21 d of the experiment, all rats were transferred to metabolic cages (Nalgene) and were fasted overnight. Blood sam-

ples and 24-h fecal samples of all the groups were collected for fatty acid composition analysis.

(iii) Growth study on ad libitum *diet*. In this experiment, 14 weanling Wistar/NIN male rats weighing 39–40 g were divided randomly into two groups. One group of rats was fed *ad libitum* AIN-93G diet supplemented with 10% of SO (control group), and the other group was fed *ad libitum* AIN-93G diet containing 10% of the SL (experimental group). Rats were housed separately in metal cages with number tags for identification. Fresh, sterile deionized water was provided *ad libitum* by a glass bottle with stainless steel nozzle. Diet consumption was monitored for all the rats on every day. Body weights were recorded on days 0, 3, 5, 7, 10, 13, 17, 19 and 21. The animals were maintained at 22 ± 2°C with 12-h light/dark cycle. After 21 d of the experiment, all rats were transferred to metabolic cages (Nalgene) and were fasted overnight. Blood samples and 24-h fecal samples of all the groups were collected for fatty acid composition analysis.

Extraction of plasma and fecal total lipid. Blood samples (1.5 mL) were centrifuged at 3000 rpm at 5°C for 15 min; the plasma layers were transferred to plastic tubes and stored under nitrogen at −78°C until the time of analysis. Plasma total lipid was extracted according to the method of Folch *et al.* (24). Plasma (100 µL) was mixed with 5 mL of chloroform/methanol (2:1, vol/vol) solution containing BHT (25 mg/L) and 1 mL of water. Before lipid extraction, 50 µL of heptadecanoic acid (1 mg/mL in chloroform) was added to the sample. Transmethylation of total lipid was carried out with 10 mL of 2% H_2SO_4 in methanol at 55°C for 5–6 h. After ether extraction, the methyl esters of fatty acids were purified by prep-TLC with a solvent system of hexane/ethyl acetate (95:5, vol/vol). The methyl ester band was scraped off and eluted with 3 × 5 mL of ether. The combined ether layer was evaporated to dryness by carefully flushing with N_2. Chloroform (1 mL) was added to the tube, washing the side of the test tube so that the entire extract was collected at the bottom of the tube, and 0.4 µL was injected into the gas chromatograph for fatty acid composition analysis. For extraction of total fecal lipid, the entire fecal sample collected over a period of 24 h (3.5–4 g) was crushed into a homogeneous mass and neutral lipids were extraccted with hexane for 16 h in a Soxhlet apparatus. The hexane was removed from the sample in a rotary evaporator, and 300 µL of heptadecanoic acid (1 g/100 mL in chloroform) was added. The neutral lipids were converted to methyl esters by refluxing with 50 mL of 2% H_2SO_4 in methanol for 4 h. After evaporating the methanol, the methyl esters were extracted with ether and washed with water until neutral. The ether layer was dried over anhydrous sodium sulfate and concentrated in a round-bottomed flask to a minimal volume. The methyl esters were finally purified by prep-TLC with a solvent system of hexane/ethyl acetate (95:5, vol/vol) as described above and analyzed by GC for fatty acid composition. For extraction of salts of fatty acids of fecal total lipid, the hexane-extracted fecal material was taken in a screw-cap test tube, soaked in 5 mL of 6 N HCl for 1 h, crushed with a spatula, and vortexed. The mixture was left at 50°C for 8 h with occasional vortexing. The mixture was cooled to room temperature, and 1 mL of heptadecanoic acid (1 g/100 mL in chloroform) was added to the tube followed by 6 mL of ether. The tube was vortexed for 3–4 min and then centrifuged, and the supernatant ether layer was placed in a second screw-cap test tube. This operation was repeated three times and the entire ether layer was washed free of mineral acid and dried over anhydrous sodium sulfate. The fatty acids were converted to methyl esters by treatment with diazomethane and purified by prep-TLC with a solvent system of hexane/ethyl acetate (95:5, vol/vol) as described before and analyzed by GC for fatty acid composition.

Statistical analysis. The data, presented as mean ± SE, were analyzed by a paired Student's *t*-test to evaluate the level of statistical significance. Differences were assessed by one-way analysis of variance. A *P*-value of less than 0.05 was considered significant.

RESULTS

By using the 1,3-specific lipase, Lipozyme, behenic acid was incorporated predominantly into *sn*-1 and/or *sn*-3 position of SO. Pancreatic lipase hydrolysis of the product showed the following composition for the MAG isolated from the hydrolysis products: 16:0, 4.4; 18:0, 6.3; 18:1, 20.6; 18:2, 63.2; 22:0, 3.7; and 24:0, 1.8%. The fatty acid compositions of SO and the SL are given in Table 1. An incorporation of 34.7% of behenic acid into SO gave a plastic fat having a slip melting point of 37°C. The solid fat content of the SL as determined by NMR showed that SL contained 11.5% of solid fat

TABLE 1
Fatty Acid Composition of SO, SL, and Different Test Diets[a]

	Fatty acid (wt%)						
Fatty acid	SO	SL	5% SO diet	10% SO diet	15% SO diet	5% SL diet	10% SL diet
16:0	4.8	2.8	17.4	12	11.9	14.5	7.5
18:0	4.9	4.2	6.9	6.2	6	5.8	3.7
18:1	24.6	17.1	26	24.5	26.6	24	24.7
18:2	64.8	38.9	49.4	56.6	55.5	25.2	33.2
20:0	0.9	1.7	0.3	0.7		1.9	1.8
22:0		34.7				28.4	29.1
24:0		0.6				0.2	ND

[a]SO, sunflower oil; SL, structured lipid; ND, not detected.

TABLE 2
Triacylglycerol Species of SO and SL as Measured by RP-HPLC[a]

	SO		SL	
ECN	TAG	Wt%	Probable TAG	Wt%
C 42	LLL	37.1	LLL	12.1
C 44	LLO	28.9	LLO	10.8
C 44	LLP	10.1	LLP	2.8
C 46	SLL; POL	9.4	SLL; POL	3
C 46	LOO	8.2	LOO	2.4
C 48	SOL; OOO	6.3	SOL; OOO	2.6
C 50			BLL	32.5
C 52			BLO	12.2
C 52			BLP	2.4
C 54			BOO	1.9
C 54			BOP	2.1
C 56			BOS	1.7
C 58			BLB	11.4
C 60			BOB	2.1

[a]ECN, equivalent carbon number; RP-HPLC, reversed-phase high-performance liquid chromatography; TAG, triacylglycerol; L, linoleic acid; O, oleic acid; P, palmitic acid; S, stearic acid; B, behenic acid. For other abbreviations see Table 1.

at 15°C, 9.25% at 25°C, 3.64% at 35°C, and 0% at 40°C. RP-HPLC analysis of the molecular species of the SL was carried out and was compared with that of SO. As shown in Table 2, 66.3% of the total molecular species of the product contained at least one molecule of behenic acid.

Animal studies. The purpose of the initial single oral dose experiment was to elucidate the absorption characteristics of SL in Wistar rats, taking SO as control. The plasma TAG levels in two different groups of rats having different body weights 1, 2, and 3 h after fat or oil loading are indicated in Table 3. The concentration of TAG increased after 1 h of feeding, reached a peak value at 2 h, and thereafter decreased to initial levels in all the groups. TAG concentration always was higher in the control group than the experimental group. Plasma TAG concentration in the control group was significantly higher ($P < 0.001$) than the experimental group at the peak level, i.e., at 2 h in both studies. To check for the presence of 22:0 in the lipids, plasma and fecal total lipid fatty acids were analyzed. No behenic acid was measurable in the plasma lipids of either group. The absence of 22:0 in the plasma of the experimental group indicated that behenic acid did not enter the normal metabolic pathway and was excreted. This observation was confirmed by the analysis of fecal total lipid, where a large amount of 22:0 was observed. Excretion of 22:0 was greater in the form of salts than as neutral lipid. By calculation, the experimental group excreted 85% of the 22:0 fed within 48 h after fat loading.

All diets prepared for the two growth experiments conducted on weanling rats, with varying levels of SO for the control groups and of SL for the experimental groups, were analyzed for fatty acid composition. The results (Table 1) are means of two readings, before starting the experiment and after 21 d of the experiment.

Two basic assumptions are made in the assay of Finley *et al.* (17): (i) The weight gain of the rats is linearly related to kilocalories added to the diet, and, (ii) the weight gain is linear over 14 d of the experiment. The mean weight gains per gram of diet of all the test groups on restricted diet and also the pilot *ad libitum* group were recorded for each of the time intervals when body weights were recorded over the 21 d of the experiment and are given in Table 4, as are the slopes, intercepts and regression coefficients (r^2). Unlike the assay of Finley *et al.* (17), we prolonged our experiment for 21 d. When mean body weight gain was regressed against 21 d of assay for all the groups, regression coefficients were greater than 0.987. This fulfills the first assumption of this assay. Similarly, mean body weight gain of the control groups fed varying levels of SO was also regressed against kilocalories added to the diet on each day that body weights were recorded over 21 d of the experiment. Linearity was observed from ninth day of the study, satisfying the other assumption of this assay. When the 21st-d mean body weight gains of all the control groups were regressed against kilocalories added to the diet, a r^2 of 0.993, intercept of 21.13, and slope of 0.2111 were obtained. The slope and intercept calculated from the standard regression curve were used in the following formula to estimate the calorific value of the SL (17):

$$\text{kcal}_x = \frac{\text{BWG}_x - \text{INT}}{\text{SLP} \times K_x} \quad [1]$$

where kcal_x is the estimated kilocalories per gram of the unknown oil, i.e., the SL in this assay, BWG_x is the 21st-d mean body weight gain for rats fed diets containing 5% of the SL, INT is the intercept from the standard curve, SLP is the slope of the standard curve and K_x is the amount of SL added to the diet (g/100 g of diet). By substituting these values into Equation 1, the estimated calorific value of the SL was 5.36 kcal/g.

Six rats were chosen randomly from each group for the fatty acid composition analysis of plasma total lipid. The

TABLE 3
Mean Level of Plasma TAG Following Oral Administration of SO and SL in Single Oral Dose Experiment

Body weights (g)	Plasma TAG levels (mg%), $n = 3$[a]							
	SO group				SL group			
	0 h	1 h	2 h	3 h	0 h	1 h	2 h	3 h
165–200	83 ± 16.7	162 ± 13.2	247 ± 17	93 ± 8.7	100 ± 8.7	119 ± 18.3	128[b] ± 18.9	97 ± 7.8
120–130	37 ± 8.4	50 ± 7.4	82 ± 3.8	58 ± 4.4	36 ± 3.7	46 ± 6.1	57[b] ± 1.5	34[b] ± 2.3

[a]Mean ± SE; n, number of rats.
[b]Significantly different from control group: $P < 0.001$. For abbreviations see Tables 1 and 2.

TABLE 4
Mean Body Weight Gains/g of Diet for Various Days and Caloric Addition in the Restricted Diet Growth Experiment

	Days of study regression analysis (±SE)								
Groups	0–3	0–6	0–9	0–14	0–17	0–21	Intercept[a]	Slope[a]	$r^{2\,a}$
Ad libitum	12.9 ± 0.383	27 ± 0.694	40.87 ± 1.094	54.69 ± 2.647	71.56 ± 2.34	87.5 ± 2.576	1.884	4.046	0.997
5% SL	−2.629 ± 0.824	−0.375 ± 0.632	3.81 ± 0.875	6.75 ± 0.963	11.94 ± 0.997	15.47 ± 1.801	−6.28	1.068	0.997
10% SL	−1.625 ± 0.363	1.062 ± 0.485	6.937 ± 0.643	10.94 ± 0.873	18.31 ± 0.958	22.87 ± 0.919	−6.82	1.462	0.996
5% SO (353 kcal)	−1.4 ± 0.085	−0.78 ± 0.46	5.87 ± 0.67	9.75 ± 1.75	15 ± 1.75	21.5 ± 1.07	−7.07	1.35	0.987
10% SO (378 kcal)	−1.187 ± 0.37	2.12 ± 0.62	6.06 ± 0.79	11.87 ± 0.95	19.87 ± 1.18	25.68 ± 1.346	−7.29	1.59	0.994
15% SO (403 kcal)	0.5 ± 0.834	4.68 ± 1.01	10.75 ± 1.157	16.62 ± 1.291	25.62 ± 1.451	32.05 ± 1.1498	−6.02	1.859	0.997
Intercept[b]	—	—	5.12	9.312	14.8	21.13			
Slope[b]	—	—	0.098	0.1374	0.2124	0.2111			
$r^{2\,b}$	—	—	0.780	0.976	0.998	0.993			

[a]Regression analyses of mean body weight gain at various times for each group.
[b]Regression analysis of mean body weight gains against kilocalories added to the diet in the SO groups (control). For abbreviations see Table 1.

mean individual fatty acid concentration (mg/100 mL plasma) and composition (in mol%) after 21 d of the 5 and 10% experiments are given in Table 5. No behenic acid was detected in the experimental groups (5 and 10% SL). The data indicate significantly higher amounts of 18:2 ($P < 0.001$) and 20:4 ($P < 0.05$) in the 5% SO group compared to the 5% SL group. No such differences were observed between 10% SO and 10% SL when individual fatty acid concentrations were compared statistically. This indicates that at 10% added fat level, the requirement of all major fatty acids was met in the experimental group.

Absence of 22:0 in the plasma of the experimental groups indicates that 22:0 did not enter the normal metabolic pathway and was excreted. This was confirmed by the analysis of fatty acid composition of fecal total lipid, collected under conditions of overnight fasting for a period of 24 h after 21 d of experiment. Three rats were chosen randomly from all the groups of the 5 and 10% experiments. Both neutral lipids and fatty acid salts were isolated and analyzed as detailed in the Experimental Procedures section. Mean fatty acid compositions (in mol%) of the excreted lipids are presented for comparison in Table 6. No excretion of behenic acid was found in the control groups, which is a clear evidence of excreted behenic acid being a direct consequence of dietary intake. Data indicate a significantly higher amount of excretion of total fecal lipid in the experimental group than the control group ($P < 0.001$).

TABLE 5
Plasma Total Lipid Fatty Acid Concentrations and Compositions After 21 d of Restricted Diet Growth Experiment[a]

	5% SO		5% SL		10% SO		10% SL	
Fatty acid	Conc.	Compn.	Conc.	Compn.	Conc.	Compn.	Conc.	Compn.
14:0	3.7 ± 0.57	2.4 ± 0.29	2.5 ± 0.3	2.3 ± 0.14	1.8 ± 0.2	1.3 ± 0.07	1.6 ± 0.09	1.3 ± 0.04
14:1	1.2 ± 0.28	0.7 ± 0.2	0.4 ± 0.06	0.3 ± 0.08	0.2 ± 0.04	0.3 ± 0.04	0.3 ± 0.04	0.2 ± 0.06
14:2	2.8 ± 0.49	1.8 ± 0.29	1.5 ± 0.19	1.4 ± 0.13	1.2 ± 0.08	0.9 ± 0.05	1.0 ± 0.08	0.8 ± 0.06
16:0	46.0 ± 2.2	26.5 ± 0.5	37.8 ± 3.5	30.6 ± 0.5[b]	35.5 ± 1.1	22.9 ± 0.1	33.6 ± 1.8	24.3 ± 0.5
16:1	10.9 ± 1.8	6.2 ± 0.9	5.8 ± 0.82	4.7 ± 0.48	3.3 ± 0.33	2.2 ± 0.13	3.3 ± 0.5	2.4 ± 0.24
18:0	20.5 ± 0.8	9.6 ± 0.28	16.5 ± 1.6	12.0 ± 0.4[b]	20.7 ± 0.8	12.0 ± 0.1	19.0 ± 0.6	12.6 ± 0.6
18:1	23.2 ± 1.9	13.0 ± 0.4	21.4 ± 2.3	15.7 ± 0.8[c]	18.7 ± 2.2	10.6 ± 0.8	15.4 ± 2.3	9.8 ± 0.85
18:2	37.3 ± 3.0	18.6 ± 0.5	14.2 ± 1.4[b]	10.5 ± 0.3[b]	41.6 ± 4.8	23.6 ± 2.0	31.2 ± 3.4	20.4 ± 1.0
20:0	1.0 ± 0.08	0.5 ± 0.03	0.5 ± 0.03	0.4 ± 0.05	0.7 ± 0.04	0.4 ± 0.01	0.8 ± 0.06	0.5 ± 0.02
18:3	0.7 ± 0.08	0.4 ± 0.02	0.3 ± 0.01	0.2 ± 0.09	0.4 ± 0.04	0.3 ± 0.05	0.3 ± 0.04	0.2 ± 0.03
20:3	0.7 ± 0.4	0.4 ± 0.26	2.6 ± 0.7	1.6 ± 0.4[d]	0.6 ± 0.22	0.4 ± 0.08	1.2 ± 0.2	0.5 ± 0.19
20:4	39.5 ± 3.9	16.9 ± 1.1	24.9 ± 3.8[d]	17.2 ± 2.1	39.8 ± 4.8	22.5 ± 2.4	38.7 ± 2.1	23.9 ± 1.3
24:0	2.2 ± 0.32	0.9 ± 0.13	1.8 ± 0.2	1.2 ± 0.14	1.7 ± 0.14	0.7 ± 0.04	2.0 ± 0.16	1.0 ± 0.09
22:4	0.9 ± 0.05	0.5 ± 0.01	0.3 ± 0.05	0.3 ± 0.02	1.0 ± 0.01	0.5 ± 0.05	0.9 ± 0.02	0.5 ± 0.06
24:1	1.7 ± 0.2	0.8 ± 0.06	1.0 ± 0.16	0.7 ± 0.09	1.8 ± 0.24	0.8 ± 0.08	1.5 ± 0.16	0.7 ± 0.02
22:5	ND	ND	ND	ND	ND	ND	ND	ND
22.6	1.1 ± 0.22	0.8 ± 0.08	1.1 ± 0.02	1.0 ± 0.1	1.1 ± 0.01	0.6 ± 0.1	1.7 ± 0.55	0.9 ± 0.1

[a]Concentrations, mg/100 mL plasma; compn., compositions, mol%; mean ± SE; $n = 6$.
[b]$P < 0.001$.
[c]$P < 0.01$.
[d]$P < 0.05$. ND, not detected. For other abbreviations see Table 1.

TABLE 6
Fatty Acid Composition of the Fecal Total Lipid After 21 d of the Restricted Diet Growth Experiment[a,b]

	Neutral lipid (n = 3)				Soap (n = 3)			
Fatty acids	5% SO	5% SL	10% SO	10% SL	5% SO	5% SL	10% SO	10% SL
14:0	3.8 ± 0.21[b]	2.7 ± 0.15[b]	1.7 ± 0.18	1.4 ± 0.05	3.7 ± 0.29	1.8 ± 0.21[d]	2 ± 0.03	0.8 ± 0.05[c]
16:0	34.2 ± 1.33	19.4 ± 0.15[c]	24.7 ± 0.06	15.7 ± 0.06	35.4 ± 1.72	16.3 ± 1.11[c]	28.6 ± 1.39	11.6 ± 0.75[c]
16:1	3.9 ± 0.41	0.8 ± 0.25[d]	1.6 ± 0.17	0.4 ± 0.12[d]	3.97 ± 1.92	0.2 ± 0.08	3.2 ± 1.32	0.2 ± 0.05
18:0	20.3 ± 1.51	10 ± 0.08[d]	23.8 ± 0.72	10.9 ± 0.41[c]	26.3 ± 0.63	10 ± 0.29[c]	27 ± 1.55	9.8 ± 0.57[c]
18:1	16.1 ± 1.04	10.2 ± 0.15[d]	19.3 ± 0.78	13 ± 0.34[d]	12 ± 1.05	2 ± 1.05[c]	17.6 ± 0.8	3.8 ± 0.26[c]
18:2	6.3 ± 0.46	4.2 ± 0.06[b]	11 ± 1.38	7.3 ± 0.23	4.7 ± 0.37	0.7 ± 0.28[c]	7.5 ± 0.8	2.1 ± 0.09[d]
20:0	2.3 ± 0.08	2.6 ± 0.45	2.3 ± 0.17	3.3 ± 0.03[d]	2.3 ± 0.27	3.5 ± 0.03[b]	2.1 ± 0.12	2.8 ± 1.21
18:3	2.4 ± 0.76	1 ± 0.53	3.1 ± 0.4	2.5 ± 0.58	2.8 ± 0.12	0.3 ± 0.14[c]	1.9 ± 0.51	0.3 ± 0.23[e]
20:3	6.1 ± 0.36	ND	7.1 ± 0.54	ND	5.2 ± 0.92	ND	6 ± 0.7	ND
22:0	ND	46.3 ± 0.27	ND	43.1 ± 1.27	ND	61.3 ± 2.36	ND	65.2 ± 1.84
24:0	4.3 ± 1.13	1.8 ± 0.05	4.8 ± 0.75	2 ± 0.07[b]	3.5 ± 0.69	2.1 ± 0.11	3 ± 0.29	2.1 ± 0.07[e]
Amount of excreted lipid (mg/d)	0.866 ± 0.2	17.83 ± 1.4[c]	1.93 ± 0.19	7.38 ± 0.4[c]	3.62 ± 0.4	76.82 ± 7.6[c]	9.05 ± 0.8	122.01 ± 7.9[c]

[a]Data reported as mean ± SE: percentage calculated as (mol fatty acid/mol total fatty acid) × 100. n, number of rats.
[b]Values with different superscript roman letters are significantly different from the group fed SO-containing diet according to this designation: [b]$P < 0.02$, [c]$P < 0.001$, [d]$P < 0.01$, [e]$P < 0.05$. For abbreviations see Table 1.

In the second growth experiment on *ad libitum* diet, no difference in growth pattern was observed between the two groups over 21 d of the experiment (Table 7), indicating no abnormality in the absorption behavior of the SL. Similarly, no difference was observed between the two groups in their mean food intake. Mean food intake of the control group was 9.8 g/d/rat and that of the experimental group was 9.9 g/d/rat. This means that the flavor and palatability of the SL are very similar to natural oils, and the absorbable portion of SL meets the requirements of fat in the animal system and provides usable energy to sustain growth and development.

Blood samples from all rats of both groups were analyzed for the fatty acid composition of plasma total lipid. The mean individual fatty acid concentrations (mg/100 mL plasma) and compositions (in mol%) of plasma total lipid in the control and experimental group are given in Table 8. There was no difference in the individual fatty acid concentrations in the plasma lipids between the two groups. Three rats were chosen randomly from both the groups for fecal lipid fatty acid composition analysis (Table 9). The major fatty acids were 16:0 ($P < 0.01$), 18:0 ($P < 0.01$), 18:1 ($P < 0.001$), and 18:2 ($P < 0.01$) in the control group and 22:0 in the experimental group, both as neutral lipids and as salts. Consistent with earlier results, excretion of large amount of behenic acid was observed, mostly as salts. Compared to control, a significantly higher amount of total fecal lipid was excreted by rats in the experimental group. It was estimated that 78.8% of the fed behenic acid was recovered in the feces.

DISCUSSION

An SL was synthesized by incorporation of behenic acid into the TAG of SO to produce a plastic fat melting at ~37°C and containing EFA and natural antioxidants, as present in native oils. The presence of these latter components makes the SL clearly superior to other reduced-calorie fats currently available. The synthetic process is simple, and the 1,3-specific lipase, Lipozyme, used for transesterification reaction, incorporates behenic acid mainly at *sn*-1 and/or *sn*-3 position of the glycerol backbone, leaving the EFA at the *sn*-2 position mostly untouched. While carrying out enzymatic transesterification reactions, different reaction parameters like enzyme concentration, temperature, reaction time and substrate ratios were optimized to generate a product having a slip melting point of ~37°C. The solid fat index did not show a sharp decline, which may be due to the presence of different kinds of molecular species and lower amounts of saturated fatty acids in the SL. RP-HPLC analysis of the SL showed that the majority of molecular species produced by behenic acid incorporation contained one molecule of the acid. Species containing two behenic acid molecules constituted only 13.5% of the total. Overall, 66.3% of molecular species of the SL contained at least one behenic acid molecule (Table 2).

In the digestion of dietary TAG, major hydrolytic activity can be attributed to pancreatic lipase. Over 95% of dietary lipids are digested and absorbed in the upper intestine (25). Pancreatic lipase hydrolyzes TAG in association with co-li-

TABLE 7
Mean Body Weight Gain/g of Diet of the Control and Experimental Group in the Growth Study on *ad libitum* Diet

	Days of study							
Groups	0–3	0–5	0–7	0–10	0–13	0–17	0–19	0–21
Control	50.5 ± 0.66	59 ± 0.87	69.6 ± 0.72	75.1 ± 1.9	89.2 ± 1.6	103 ± 0.48	109.6 ± 2.1	112 ± 2.7
Experimental	50.2 ± 0.63	59.8 ± 0.79	67.8 ± 0.78	78.9 ± 2.3	90 ± 1.5	102.8 ± 1.5	109 ± 1.89	111.3 ± 2.5

TABLE 8
Plasma Total Lipid Fatty Acid Concentration and Compositions After 21 d of Growth Experiment on *ad libitum* Diet[a]

	10% SO (control)		10% SL	
Fatty acid	Concentration	Composition	Concentration	Composition
14:0	2.4 ± 0.26	1.4 ± 0.17	2.4 ± 0.21	1.4 ± 0.06
16:0	51.0 ± 2.8	24.6 ± 0.42	44.6 ± 1.0	23.8 ± 0.53
16:1	3.8 ± 0.51	3.1 ± 0.36	4.5 ± 1.0	3.1 ± 0.36
18:0	28.0 ± 1.1	12.3 ± 0.53	25.1 ± 0.74	12.2 ± 0.6
18:1	31.5 ± 2.1	13.8 ± 0.3	26.7 ± 1.3	12.8 ± 0.35
18:2	43.9 ± 4.1	19.2 ± 0.93	36.4 ± 1.6	19.4 ± 0.26
20:0	0.7 ± 0.16	0.3 ± 0.08	1.0 ± 0.2	0.4 ± 0.06
18:3	0.5 ± 0.16	0.2 ± 0.06	0.6 ± 0.1	0.2 ± 0.06
20:3	3.3 ± 0.6	1.5 ± 0.42	3.5 ± 0.6	1.7 ± 0.21
20:4	52.2 ± 4.1	20.8 ± 0.85	51.2 ± 2.1	22.8 ± 0.37
24:0	2.4 ± 0.16	1.0 ± 0.16	1.6 ± 0.2	0.6 ± 0.06
22:4	0.6 ± 0.01	0.3 ± 0.05	0.6 ± 0.05	0.3 ± 0.01
24:1	1.7 ± 0.29	0.8 ± 0.08	1.2 ± 0.2	0.8 ± 0.05
22:5	ND	ND	ND	ND
22:6	1.1 ± 0.1	0.4 ± 0.05	0.8 ± 0.02	0.5 ± 0.05

[a]Concentration, mg/100 mL plasma; composition, mol%; mean ± SE; $n = 7$. For abbreviations see Tables 1 and 5.

pase and bile salts to produce 2-MAG and free fatty acids (FFA). The physicochemical state of lipid in the intestinal lumen during the digestion of fat is that of a micellar phase, containing mainly the products of lipolysis solubilized in bile salt solutions, which are in continuous equilibrium with an emulsified oil phase (26). Because the digestion of fats takes place in a micellar phase, and because this condition is best achieved as FFA (i.e., the hydrolysis product of fats), fatty acid is the principal form in which fat is absorbed. Hence, the equilibrium between the oil and the aqueous phase, and in particular the rate and extent of formation by the FFA of mixed micelles in the system, is important in controlling the absorption of fatty acids. This means that when fatty acids are released into oil–bile salt dispersions, the rate at which the fatty acids establish equilibrium between the two phases is important for their absorption. This rate depends on chain length degree of unsaturation of fatty acids, i.e., the solubility of the fatty acid in aqueous solution. Poor diffusion of fatty acid from oil phase to the aqueous phase, because of very low solubility in water, may inhibit the enzyme action by block-

TABLE 9
Fatty Acid Composition of Fecal Total Lipid after 21 d of the Control and the Experimental Group in the Growth Experiment on *ad libitum* Diet[a,b]

	Neutral lipid (n = 3)		Soap (n = 3)	
Fatty acid	10% SO	10% SL	10% SO	10% SL
14:0	1.6 ± 0.35	0.5 ± 0.03[b]	1.5 ± 0.23	0.2 ± 0.06[c]
14:1	1.8 ± 0.31	0.6 ± 0.09[b]	ND	ND
14:2	1.8 ± 0.41	0.4 ± 0.05[b]	ND	ND
16:0	22.6 ± 0.41	14.7 ± 1.57	29.8 ± 1.9	9.3 ± 0.95[d]
16:1	2.1 ± 0.56	ND	ND	ND
18:0	19.4 ± 1.02	12.5 ± 0.18[c]	31.1 ± 2.3	10.6 ± 0.73[c]
18:1	21.4 ± 0.48	9.4 ± 0.96[d]	16.1 ± 1.8	1.5 ± 0.43[c]
18:2	12.9 ± 0.89	3.9 ± 0.97[c]	7.5 ± 1.8	0.8 ± 0.03[d]
20:0	2.2 ± 0.15	1.9 ± 1.03	1.5 ± 0.34	4.4 ± 0.08[c]
18:3	2.4 ± 0.32	ND	0.8 ± 0.41	ND
20:3	6.8 ± 0.81	ND	8 ± 1.32	ND
22:0	ND	52.4 ± 4.52	ND	70.8 ± 1.2
24:0	4.4 ± 0.17	2.9 ± 0.56	3.2 ± 1.2	2.2 ± 0.13
Amount of excreted lipid (mg/d)	2.9 ± 0.75	22.97 ± 4.36[c]	5.8 ± 2.1	134.7 ± 9.7[d]

[a]Data reported as mean ± SE: percentage calculated as (mol fatty acid/mol total fatty acid) × 100; n, number of rats.
[b]Values with different superscript roman letters are significantly different from the group fed 10% SO-containing diet according to this designation: [b]$P < 0.05$, [c]$P < 0.01$, [d]$P < 0.001$. For abbreviations see Table 1.

ing the interface. The solubility and the distribution coefficient of the fatty acid also depends on the pH of the medium and the concentration of divalent cations, like calcium and magnesium ions. The interaction of divalent cations and pH with the distribution coefficients of fatty acids has important physiological implications. In the presence of Ca^{2+} above a critical concentration of 1 meq/L and at pH 6.4 (which is the pH of the jejunal-ileal region of the intestine), the distribution in the micellar phase of very LCSFA having carbon numbers 18 and above becomes restricted. Under such circumstances, very LCSFA may react with divalent cations to form insoluble soap (27). Soap may constitute 5–50% of total fecal fat depending on the kind of fatty acid intake and the level of calcium and magnesium ions in the diet. Bliss *et al.* (28) found a linear relationship between the amount of fecal fat and the amount of calcium and magnesium that is excreted as insoluble soap. Mattson *et al*. (14) found poor absorption of stearic acid (18:0) from the *sn*-1,3 position of glycerol moiety. They also observed that the absorption of 18:0 depends on the kind of other fatty acid esterified along with it and the level of divalent cations in the diet. There is absolute absorption of 18:0 if it is esterified at the *sn*-2 position. In studying SALATRIM, Finley *et al*. (29) observed an identical absorption behavior of 18:0. The other very LCSFA whose non- or poorly absorptive nature was advantageously employed by researchers in designing a reduced calorie structured lipid is behenic acid. Yoshida *et al*. (30) in their absorption studies with saturated TAG having one LCSFA (18:0 or 22:0) and two medium-chain fatty acids (8:0 and 10:0) found higher absorption of 18:0 compared to 22:0 when it was esterified at the *sn*-1,3 position of glycerol moiety. But, unlike 18:0, they found poor absorption of 22:0 even when it was esterified at the *sn*-2 position. Behenic acid esterified at *sn*-2 position will be excreted in the feces as 2-MAG. In their work with Caprenin, a reduced calorie fat substitute, Peters *et al*. (10) found poor absorption of 22:0 in rats and hamsters. Caprenin provided usable energy to sustain growth, although the calorific availability of Caprenin was 5 kcal/g. Up to 75–82% of 22:0 remained unabsorbed, and 3–15% of that which was absorbed was recovered from total carcass fat at the end of the 28-d study. Fecal excretion of major portions of ingested 22:0 did not significantly affect calcium and iron balance in rats. Caprenin was nontoxic to rats when consumed for 28 consecutive days at level of up to 15% in the diet.

In the present work the absorption behavior of a structured TAG containing 22:0 in combination with SO fatty acids was studied. The enzymatically prepared SL had 34% of 22:0 esterified mainly at *sn*-1 and/or *sn*-3 position of SO. The presence of oleic and linoleic acids mostly at the *sn*-2 position of the SL makes it nutritionally improved compared to Caprenin and SALATRIM. In metabolic studies with Caprenin (9), corn oil was added as a source of EFA, whereas in our studies with the SL, no such supplementation was necessary as the product is already enriched with EFA.

The literature states that there will be an increase in the level of plasma TAG shortly after a fatty meal, reaching a peak in a period of time typical of the species and then decreasing to the pre- (or post-) absorptive level (31). An estimation of these elevated plasma TAG levels may help us understand not only the rate of metabolism but also the absorption of the particular fat product. Our initial single oral dose experiment was designed as a preliminary experiment to find the absorption pattern of SL in comparison with SO. This particular type of fat-loading experiment requires less time, fewer animals, and hence is less expensive. A series of experiments were conducted to find the time after dosing at which the peak value of plasma TAG is attained. This peak value was found to be 2 h after fat-loading. A significant reduction in the level of plasma TAG was obtained in the experimental group ($P < 0.001$) when peak values, obtained after 2 h, were compared with those from the control group. The near-total excretion of 22:0 observed in this short-term fat-loading experiment is an early indication of lower calorific availability from SL, which was confirmed in the growth studies.

The growth experiment on restricted diet was designed to estimate the calorific availability of the SL, taking SO as the caloric standard. The method is almost in line with the study of Finley *et al.* (17), which is a modified version of the method adopted by Rice *et al*. (32). The authors have tested the method by estimating the calorific availability of three fats (cocoa butter, tallow, and lard) and two simple esters (ethyl stearate and ethyl oleate). Good agreement was found between the calculated value and the reported value (17). The method provides good reproducibility within and between experiments. The modified method requires fewer animals, is simple to conduct, does not require highly specialized metabolic chambers or radiolabeled material, and provides an accurate estimate of calorific availability. The only difference was that we carried out our experiment over 21 d, instead of 14 d. The two basic assumptions of this method were found to apply even when the experiment was extended to over 21 d. A linear response was observed in all the groups when mean body weight gains were regressed against days of the study. Mean body weight gains of the control groups were also regressed against kilocalories added to the diet. An initial reduction in mean body weight gain of the 5% SO group, continuing to 6 d after initiation of the study, restricted our regression study. The reduction in mean body weight observed on the very first day on restricted diet became positive only after the sixth day of the experiment. A negative mean weight gain was also observed in the 10% SO group until the third day of the study. In the 15% SO group, no such negative mean weight gain was observed. When the ninth day mean weight gain of the control groups was regressed against kilocalories added to the diet, an r^2 of 0.780 was obtained. The slight deviation from linearity was due to poor weight gain in the 5% SO group. The r^2 value improved with time. The calorific value of the SL was estimated to be 5.36 kcal/g, which is 59.5% of the calories provided by natural oils.

The metabolic behavior of the SL can be determined from the fatty acid composition of the plasma as well as fecal lipid. For this purpose, the rats were fasted overnight, and the blood

and fecal samples were collected on the next day. No 22:0 was found in the plasma lipids of animals from the experimental groups. Significant differences were found in the level of 18:2 and 20:4 between the 5% SO group and the 5% SL group. When the oil content of the diet was increased from 5 to 10%, no such difference was observed. In the fecal lipid, a significantly higher amount of excretion of total fecal lipid was observed in animals of the experimental group ($P < 0.001$) than the control group, and 22:0 was the major fatty acid excreted in the experimental group. All these findings indicate that excretion of behenic acid is proportionally large. Behenic acid, in turn, may disturb the equilibrium between the oil and bile salt micelle, resulting in poor absorption of other fatty acids.

The growth experiment on *ad libitum* diet was designed to find how closely the SL resembles a natural oil in its metabolic behavior. An identical growth pattern of the control and experimental groups was observed (Table 7). The study was carried out for only 21 d, and a prolonged study would have probably shown differences in weight gain. In a similar study on SALATRIM (33), differences in weight gain were noticed only after 21 d. No difference was observed in mean food intake between the two groups, indicating that the taste and palatability of the SL were very similar to native fats. No difference was observed between the control and experimental groups in the levels of major fatty acids of the plasma total lipids. Fecal lipid analysis gave identical results to those observed in the previous growth experiment on restricted diet.

To conclude, an SL was synthesized from easily available renewable resources like MO and SO. The synthesized product delivered a reduced calorie value of 5.36 kcal/g, which is 59.5% of the calories provided by a natural oil. Because the substrate is a natural oil, the EFA and natural antioxidants present in the oil are carried over into the final product. The product thus is a definite improvement over the existing reduced calorie fats. The plastic nature of the fats widens the scope of utility of the product. The product may find applications in regular cooking and deep-frying as well as a bakery shortening or as a base for zero-*trans* margarine. The melting point of the product can be conveniently modified by altering the level of incorporation of behenic acid. Thus, we believe that the product has sufficient versatility to find varied applications.

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Effects of γ-Linolenic Acid and Docosahexaenoic Acid in Formulae on Brain Fatty Acid Composition in Artificially Reared Rats

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ABSTRACT: This study evaluated the effects of dietary supplementation with γ-linolenic acid (GLA, 18:3n-6) and docosahexaenoic acid (DHA, 22:6n-3) on the fatty acid composition of the neonatal brain in gastrostomized rat pups reared artificially from days 5–18. These pups were fed rat milk substitutes containing fats that provided 10% linoleic acid and 1% α-linolenic acid (% fatty acids) and, using a 2 × 3 factorial design, one of two levels of DHA (0.5 and 2.5%), and one of three levels of GLA (0.5, 1.0, and 3.0%). A seventh artificially reared group served as a reference group and was fed 0.5% DHA and 0.5% arachidonic acid (AA, 20:4n-6); these levels are within the range of those found in rat milk. The eighth group, the suckled control group, was reared by nursing dams fed a standard American Institute of Nutrition 93M chow. The fatty acid composition of the phosphatidylethanolamine, phosphatidylcholine, and phosphatidylserine/phosphatidylinositol membrane fractions of the forebrain on day 18 reflected the dietary composition in that high levels of dietary DHA resulted in increases in DHA but decreases in 22:4n-6 and 22:5n-6 in brain. High levels of GLA increased 22:4n-6 but, in contrast to previous findings with high levels of AA, did not decrease levels of DHA. These results suggest that dietary GLA, during development, differs from high dietary levels of AA in that it does not lead to reductions in brain DHA.

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The period of brain development in mammals is a time of rapid accrual of lipids, including the long-chain polyunsaturated fatty acids (LCP) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6) (1). Unlike cholesterol and the saturated fatty acids, which are preferentially formed *de novo* in the developing brain (2,3), DHA and AA must be obtained from dietary sources or derived from the 18-carbon precursor essential fatty acids, α-linolenic acid (LN, 18:3n-3) and linoleic acid (LA, 18:2n-6), respectively, through a series of metabolic steps involving the processes of chain elongation, desaturation, and β-oxidation (4). The extent to which these steps occur in the liver (5) and/or brain (6) is not known. In the adult, the LCP composition of the brain is relatively resistant to short-term alterations in dietary levels of n-3 and n-6 fatty acids (7). On the other hand, because of the rapid deposition of LCP into brain membranes during early development, alterations in dietary levels of n-3 and n-6 fatty acids have been shown to affect the composition of brain cell membranes (1).

Research into the relationship between the type and amount of n-6 and n-3 fatty acid in the diet, and subsequent LCP composition of the developing brain, has been hindered by our inability to directly manipulate the fatty acid composition of the nutrient supply during a large portion of the period of brain development. While manipulations of the fatty acid composition of the maternal diet have been informative (8), multigeneration feeding studies appear necessary to produce substantial alterations in brain fatty acid composition. A limitation of this maternal feeding model is the potential for effects on maternal milk composition secondary to changes in dietary n-6 or n-3 fatty acids. We have attempted to resolve at least part of this problem by using the artificial rearing (AR) model in infant rats. In this model, rat pups are removed from their mothers and fed, *via* gastrostomy tube, a rat milk substitute which closely resembles the composition of rat milk, and whereby the fatty acid composition can be altered experimentally. The brain of the rat is relatively immature at birth, and a greater proportion of its growth occurs during the first three postnatal weeks (9,10). This artificial feeding model thus allows us to manipulate precisely the dietary fatty acid composition throughout most of the brain growth spurt.

Rats reared by the AR method and fed formulae deficient in all n-3 fatty acids exhibited substantially lower levels of DHA in the brain (11). A subsequent study using this model established that LN-fed rats did not achieve brain DHA levels equivalent to the DHA-fed rats (12). We recently completed a 3 × 3 factorial study in which AR rats were fed diets containing fats with 1% (by weight) LN and 10% LA and either 0, 0.5, or 2.5% DHA and AA. In this study, we found significant correlations between the formula levels of these fatty acids and

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Abbreviations: AA, arachidonic acid; AR, artificial rearing, DHA, docosahexaenoic acid; GLA, γ-linolenic acid; LA, linoleic acid; LCP, long-chain polyunsaturated fatty acid; LN, linolenic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS/PI, phosphatidylserine/phosphatidylinositol.

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levels in brain membrane phospholipids (13). Specifically, supplementation of DHA, without concurrent AA, increased DHA levels in the brain but also reduced AA levels, while supplementation of AA, without concurrent DHA, increased AA levels in the brain but decreased DHA levels. These effects generally were most noticeable after supplementation of relatively high (2.5%) levels. Whether such modifications in AA and DHA levels in the brain have functional consequences remains uncertain. Using the AR model with formula containing 1% LN, 10% LA, and similarly extreme levels of AA or DHA (2.5%), which allowed us to obtain a wide range of brain AA and DHA, we found no effects on spatial working memory in the Morris water maze (14). A previous study found that mice born to mothers who were fed high n-3 LCP diets with a low n-6/n-3 ratio throughout pregnancy and lactation performed more poorly in the Morris water maze, but that these differences could be accounted for by decreased swimming speed rather than by deficits in learning *per se* (15).

Dietary γ-linolenic acid (GLA, 18:3n-6), a metabolite of LA and precursor of AA, may offset the reduction in AA levels in the brain that results from diets containing high levels of DHA (16). In pregnant and lactating mice fed a fish-oil supplement, both AA and GLA offset the effects of long-chain n-3 fatty acids on brain AA composition (17). In the present study, infant rats were fed milk substitutes with fat containing 10% LA, 1% LN, and, using a 2 × 3 design, DHA (0.5 or 2.5%) and GLA (0.5, 1.0, or 3.0%). Our previous study had shown decreases in brain levels of AA as dietary DHA levels increased (13). In the present study, we were interested in the effects of dietary GLA in the presence of long-chain n-3 LCP, and thus all groups received at least some DHA in formula. Furthermore, these groups were compared to two different control groups. One group was artificially reared on a formula in which the essential fatty acid composition consisted of 10% LA, 1% LN, and 0.5% each of AA and DHA. The LCP composition was within the range noted for rodent breast milk (12,17,18). The other control group was nursed by dams. We hypothesized that levels of AA in the brain would be lower when dietary DHA levels were increased to 2.5% and that the addition of GLA to formula would prevent these reductions in brain AA levels resulting from high dietary DHA levels.

MATERIALS AND METHODS

Animals. Offspring of timed-pregnant, Long-Evans rats, acquired at 10–12 d gestation from a commercial supplier (Harlan Sprague Dawley, Indianapolis, IN), were used. Upon arrival in our lab, the pregnant rats were housed individually under a reversed dark/light cycle at 22 ± 1°C with free access to American Institute of Nutrition 93M laboratory chow (19) (Dyets Inc., Bethlehem, PA) and tap water. Litters were culled to 12 pups within 24 h of birth.

Diets. The procedure for making the rat milk substitutes used in this study has been described elsewhere (13), and the composition is shown in Table 1. The dietary oils were a mixture of medium-chain triglyceride oil (MCT oil), coconut, soy, high-oleic safflower, and borage oils (Ross Laboratories, Columbus, OH), to which DHA was added in the form of the single-cell microbial oil, DHASCO, a trademarked product of Martek Biosciences Corp. (Columbia, MD). All dietary oils were formulated to provide 10% LA and 1% LN. The compositions of these oils were determined by gas chromatographic analysis and are shown in Table 2. It should be noted that the protein and whey components of the diets were determined, again by gas chromatographic analysis, to contain 0.25 and 2.54% fat, by weight, respectively. The calculated amounts (in g/100 g formula) of the various essential fatty acids in the formula which were contributed by the combined protein powder were therefore very low. Specifically, the individual levels were as follows: LA, 0.423 g; GLA, 0.007 g; AA, 0.045 g; LN, 0.151 g; 20:5n-3, 0.027 g; 22:5n-3, 0.072 g; DHA, 0.0047 g. The fatty acid composition of the rat breast milk has been published previously (13).

Experimental design. This study used a 2 × 3 factorial design with two levels of DHA (0.5 or 2.5% of fatty acids) and three levels of GLA (0.5, 1, or 3% of fatty acids) (7–10 pups

TABLE 1
Composition of Rat Milk Substitutes

Ingredient	Diet (g/kg)
Protein	
Casein extract (SO79841)[a]	46.5
Whey extract (SO84826)[a]	46.5
Amino acid mixture[b]	0.85
Carbohydrate	
Lactose	28.6
Dietary oils	
Fat mixture	123.2
Vitamins	
Teklad vitamin mixture #40060[c]	3.52
Supplemental vitamin mixture[d]	0.48
Minerals	
Calcium mixture[e]	7.92
Noncalcium mineral mixture[f]	5.30
$CuSO_4$ solution (0.03 kg/L)	0.85
$ZnSO_4$ solution (0.38 kg/L)	0.26
Other ingredients	
Carnitine (0.1 kg/L)	0.35
Creatine solution (0.01 kg/L)	6.16
Ethanolamine	0.03

[a]Ross Laboratories, Columbus, OH.
[b]Arginine (512 g/kg), glycine (310 g/kg), taurine (156 g/kg), picolinic acid (22 g/kg).
[c]*p*-Aminobenzoic acid (11.01 g/kg), ascorbic acid (97.5%) (101.66 g/kg), biotin (0.044 g/kg), vitamin B_{12} (0.1%) (2.97 g/kg), calcium pantothenate (6.6 g/kg), choline dihydrogen citrate (349.69 g/kg), folic acid (0.20 g/kg), inositol (11.01 g/kg), menadione (4.96 g/kg), niacin (9.91 g/kg), pyridoxine HCl (2.20 g/kg), riboflavin 2.20 g/kg), thiamine HCl (2.20 g/kg), vitamin A palmitate (500,000 U/g) (3.96 g/kg), vitamin D (500,000 U/g) (0.44 g/kg), vitamin E acetate (500 U/g) (24.23 g/kg), corn starch (466.69 g/kg).
[d]Riboflavin (16.7 g/kg), niacin (26 g/kg), pyridoxal (13.9 g/kg), inositol (929.4 g/kg).
[e]Calcium phosphate (720 g/kg), calcium chloride (205 g/kg), calcium hydroxide (75 g/kg).
[f]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), $Al_2(SO_4)_3$ (0.156 g/kg), $MnSO_4$ (0.042 g/kg).

TABLE 2
Selected Fatty Acid Composition of the Dietary Oils[a]

Fatty acid	Low DHA Low GLA	Low DHA Medium GLA	Low DHA High GLA	High DHA Low GLA	High DHA Medium GLA	High DHA High GLA	Reference control
8:0	14.04	14.82	14.79	13.99	14.67	13.73	13.99
10:0	8.94	9.46	8.91	8.69	8.78	8.28	8.86
12:0	23.45	23.41	21.87	20.68	21.49	20.24	22.05
14:0	9.61	9.61	8.98	9.05	9.35	8.84	9.09
16:0	7.26	7.31	7.08	7.32	7.40	7.24	7.17
18:0	2.44	2.44	2.33	2.35	2.36	2.27	2.53
16:1	0.06	0.06	0.07	0.13	0.13	0.14	0.06
18:1n-9	19.63	16.93	17.52	20.58	17.82	18.97	21.32
18:1n-7	0.38	0.38	0.50	0.40	0.38	0.51	0.37
18:2n-6	10.81	11.28	11.20	11.25	11.31	11.15	11.20
18:3n-6	0.58	1.23	2.90	0.63	1.19	2.82	0.03
20:4n-6	ND	ND	ND	ND	ND	ND	0.59
18:3n-3	0.84	0.87	1.05	0.90	0.88	1.07	0.83
18:4n-3	ND	ND	0.03	ND	0.02	0.03	ND
22:6n-3	0.52	0.59	0.53	2.52	2.52	2.49	0.54

[a]DHA, docosahexaenoic acid; GLA, γ-linoleic acid; ND, not detected; values are expressed as total fatty acids (g/100 g).

per group). A seventh group of eight pups was fed a diet in which the fat contained 0.5% each of both AA and DHA. This group constituted the reference control group. An eighth group of 10 pups was fostered to nursing dams and constituted a suckled control group.

AR procedure. The procedures used in this study were in compliance with the Animals for Research Act of Ontario (Revised Statutes of Ontario) and the Guide for the Care and Use of Experimental Animals from the Canadian Council on Animal Care, and were reviewed and approved by the Animal Care Committee at the University of Waterloo. On day 27 post-conception (approximately day 5 after birth), pups were separated from the nursing dam and reared artificially according to the procedure described in detail elsewhere (13). Briefly, gastrostomy tubes were implanted into anesthetized pups, who were then housed in an incubator and fed, *via* gastrostomy tube, their respective rat milk substitute for 10 min every h by an automated infusion pump system. The amount fed over each 24-h period was equivalent to 29% of their body weight, increasing to 36% of body weight by approximately 10 d of age. Pups were reweighed every day and their infusion volume was adjusted accordingly. The survival rate for gastrostomized Long-Evans rats in this study ranged from approximately 70 to 80%, with most mortality occurring during the first 48 h. There was no effect of dietary treatment on mortality in this study. Suckled control pups were raised and fed by nursing dams and, except for daily weighings, were not disturbed throughout the duration of the AR period. On day 40 postconception (day 18 after birth), the pups were killed under Halothane anesthesia, and their forebrains removed and stored at −80°C.

Biochemical analyses. Forebrain lipids were extracted by the method of Folch *et al.* (20) and separated into phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine/phosphatidylinositol (PI/PS) fractions by thin-layer chromatography using chloroform/methanol/water/triethylamine (4:5:1:4, by vol) as the developing solvent system. Fatty acid methyl esters were produced from these fractions by the method of Morrison and Smith (21) and analyzed by capillary gas chromatography (Hewlett-Packard 5890 II Plus; Hewlett-Packard, Palo Alto, CA) using a 30-m capillary column (i.d. = 0.32 mm, 0.25 µm film thickness; Omegawax, Supelco, Bellefonte, PA). Oven temperature was programmed to increase from 120 to 205°C at a rate of 4°C/min and to hold for 15 min. Heptadecanoic acid was used as an internal standard, and each fatty acid was identified by authentic standard mixtures (Nu-Chek-Prep, Elysian, MN).

Statistical analyses. Data were analyzed using SAS version 6.0.9 (SAS Institute, Cary, NC). The general linear models approach for analysis of variance was used to assess main and interactive effects of both DHA and GLA, and significant effects were further analyzed by planned comparisons (22). Effects of each dietary treatment were analyzed by comparing levels of one treatment collapsed across levels of the other treatment, and interactions were interpreted by comparing the simple main effects of one treatment among the levels of the other. The physiological and the suckled control groups were each compared with all other groups using Tukey's *t*-test to control for multiple comparisons. The significance level was set at 0.05.

RESULTS

The effects of GLA and DHA dietary supplementation were generally consistent across the PE, PC, and PS/PI phospholipid fractions (Tables 3, 4, and 5; Fig. 1). High dietary levels of GLA increased forebrain 22:4n-6, but had no significant effect on 20:4n-6, 22:5n-6, or 22:6n-3, nor on the n-6/n-3 ratio. High dietary levels of DHA had no significant effect on 20:4n-6, but resulted in lower levels of 22:4n-6 and 22:5n-6 and higher levels of 22:6n-3 and a lower n-6/n-3 ratio. High dietary levels (2.5%) of DHA also increased 18:2n-6 and

TABLE 3
Selected Fatty Acid Composition of PE Fraction from Forebrain of Rat Pups Fed Experimental Formulae Containing Either Low (0.5%), Medium (1%), or High (3%) Levels of GLA and Low (0.5%) or High (2.5%) Levels of DHA on Postnatal Days 5–18[a,b]

		Reference group	0.5% DHA			2.5% DHA			Two-way ANOVA		
Fatty acid	Suckled control	0.5% DHA 0.5% AA	0.5% GLA	1% GLA	3% GLA	0.5% GLA	1% GLA	3% GLA	DHA[c]	GLA[c]	Int[c]
16:0	9.25 ± 0.25	8.10 ± 0.33	8.78 ± 0.40	8.65 ± 0.39	8.05 ± 0.34	8.15 ± 0.34	8.29 ± 0.34	8.66 ± 0.29	NS	NS	NS
18:0	16.08 ± 1.19	16.97 ± 1.32	19.25 ± 1.11	16.37 ± 1.15	18.84 ± 1.17	18.43 ± 1.16	17.80 ± 1.23	17.32 ± 1.46	NS	NS	NS
18:1	8.49^{y} ± 0.13	$9.20^{x,y}$ ± 0.15	9.63^{x} ± 0.23	$8.92^{x,y}$ ± 0.22	9.19x,y ± 0.18	9.55^{x} ± 0.17	9.33^{x} ± 0.09	9.39^{x} ± 0.10	NS	$P < 0.05$	NS
18:2n-6	0.48^{z} ± 0.02	$0.54^{y,z}$ ± 0.03	$0.54^{y,z}$ ± 0.04	$0.62^{x,y,z}$ ± 0.02	$0.56^{y,z}$ ± 0.04	0.73^{x} ± 0.05	0.74^{x} ± 0.04	$0.64^{x,y}$ ± 0.04	$P < 0.001$	NS	NS
20:3n-6	0.47^{v} ± 0.02	$0.60^{v,w}$ ± 0.03	$0.66^{z,v,w}$ ± 0.05	$0.74^{z,v}$ ± 0.04	$0.82^{y,z}$ ± 0.04	1.09^{x} ± 0.07	1.07^{x} ± 0.07	$1.00^{x,y}$ ± 0.06	$P < 0.001$	NS	NS
20:4n-6	21.41 ± 0.80	22.01 ± 0.77	20.02 ± 0.93	21.04 ± 0.83	20.83 ± 0.84	18.77 ± 0.97	19.65 ± 0.87	20.12 ± 1.02	NS	NS	NS
22:4n-6	6.17^{x} ± 0.15	6.13^{x} ± 0.19	$5.88^{x,y}$ ± 0.11	$5.69^{x,y}$ ± 0.16	6.28^{x} ± 0.16	4.80^{z} ± 0.13	4.99^{z} ± 0.11	$5.28^{y,z}$ ± 0.09	$P < 0.001$	$P < 0.01$	NS
22:5n-6	$2.39^{x,y}$ ± 0.12	$2.42^{x,y}$ ± 0.12	2.61^{x} ± 0.14	$2.24^{x,y}$ ± 0.09	2.56^{x} ± 0.13	1.50^{z} ± 0.19	1.67^{z} ± 0.09	$1.96^{y,z}$ ± 0.15	$P < 0.001$	NS	NS
22:6n-3	24.07^{y} ± 0.91	$25.46^{x,y}$ ± 0.94	23.46^{y} ± 1.10	$24.80^{x,y}$ ± 0.88	23.92^{y} ± 0.98	28.94^{x} ± 1.44	$28.24^{x,y}$ ± 1.14	$27.54^{x,y}$ ± 1.37	$P < 0.001$	NS	NS
n-6/n-3 ratio	$1.44^{x,y}$ ± 0.05	$1.45^{x,y}$ ± 0.05	1.51^{x} ± 0.06	$1.46^{x,y}$ ± 0.06	1.57^{x} ± 0.06	1.15^{z} ± 0.05	1.20^{z} ± 0.04	$1.22^{y,z}$ ± 0.04	$P < 0.001$	NS	NS

[a]Values shown as means ± SEM in weight percent of fatty acids, $n = 7–10$.
[b]Groups that share the same superscript are not significantly different (*post-hoc* Tukey's test, $P > 0.05$).
[c]AA, arachidonic acid; ANOVA, analysis of variance; DHA, main effect of DHA; GLA, main effect of GLA; Int, interaction effect; NS, $P > 0.05$; PE, phosphatidylethanolamine; for other abbreviations, see Table 2.

TABLE 4
Selected Fatty Acid Composition of PC Fraction from Forebrain of Rat Pups Fed Experimental Formulae Containing Either Low (0.5%), Medium (1%), or High (3%) Levels of GLA and Low (0.5%) or High (2.5%) Levels of DHA on Postnatal Days 5–18[a,b]

		Reference group	0.5% DHA			2.5% DHA			Two-way ANOVA		
Fatty acid	Suckled control	0.5% DHA 0.5% AA	0.5% GLA	1% GLA	3% GLA	0.5% GLA	1% GLA	3% GLA	DHA[c]	GLA[c]	Int[c]
16:0	49.89 ± 0.23	49.89 ± 0.33	49.81 ± 0.51	50.01 ± 0.36	49.76 ± 0.32	50.05 ± 0.57	50.34 ± 0.40	50.01 ± 0.64	NS	NS	NS
18:0	7.60 ± 0.56	8.00 ± 0.82	8.83 ± 0.64	7.61 ± 0.69	8.58 ± 0.61	8.33 ± 0.77	7.89 ± 0.69	7.71 ± 0.85	NS	NS	NS
18:1	21.10 ± 0.31	21.98 ± 0.41	22.74 ± 0.40	21.24 ± 0.38	22.09 ± 0.28	22.25 ± 0.39	21.90 ± 0.31	21.79 ± 0.51	NS	NS	NS
18:2n-6	1.29^{z} ± 0.05	$1.50^{y,z}$ ± 0.08	$1.33^{y,z}$ ± 0.11	$1.63^{x,y,z}$ ± 0.07	$1.43^{y,z}$ ± 0.06	2.00^{x} ± 0.12	1.95^{x} ± 0.10	$1.69^{x,y}$ ± 0.11	$P < 0.001$	NS	NS
20:3n-6	0.31^{v} ± 0.02	$0.38^{v,w}$ ± 0.01	$0.38^{v,w}$ ± 0.07	$0.51^{z,v}$ ± 0.03	$0.59^{y,z}$ ± 0.03	0.83^{x} ± 0.05	0.80^{x} ± 0.05	$0.73^{x,y}$ ± 0.06	$P < 0.001$	NS	$P < 0.05$
20:4n-6	9.90^{x} ± 0.36	$9.49^{x,y}$ ± 0.37	$8.59^{x,y}$ ± 0.36	$9.52^{x,y}$ ± 0.41	$9.11^{x,y}$ ± 0.32	7.94^{y} ± 0.42	$8.46^{x,y}$ ± 0.36	$9.14^{x,y}$ ± 1.51	NS	NS	NS
22:4n-6	0.81^{x} ± 0.03	$0.70^{x,y}$ ± 0.07	$0.57^{x,y}$ ± 0.10	$0.65^{x,y}$ ± 0.07	0.79^{x} ± 0.02	0.37^{y} ± 0.11	0.41^{y} ± 0.09	0.34^{y} ± 0.12	$P < 0.001$	NS	NS
22:5n-6	0.32^{x} ± 0.07	$0.13^{x,y}$ ± 0.05	$0.12^{x,y}$ ± 0.06	$0.12^{x,y}$ ± 0.05	$0.20^{x,y}$ ± 0.05	0.03^{y} ± 0.03	0.04^{y} ± 0.03	0.03^{y} ± 0.03	$P < 0.01$	NS	NS
22:6n-3	$3.97^{x,y}$ ± 0.17	$3.89^{x,y}$ ± 0.18	3.55^{y} ± 0.17	$3.96^{x,y}$ ± 0.16	$3.71^{x,y}$ ± 0.16	4.41^{x} ± 0.22	$4.32^{x,y}$ ± 0.17	4.42^{x} ± 0.21	$P < 0.001$	NS	NS
n-6/n-3 ratio	$2.87^{x,y}$ ± 0.16	$3.07^{x,y}$ ± 0.15	$3.19^{x,y}$ ± 0.13	$2.88^{x,y}$ ± 0.16	3.32^{x} ± 0.12	2.65^{y} ± 0.05	2.74^{y} ± 0.09	$2.72^{x,y}$ ± 0.10	$P < 0.001$	NS	NS

[a]Values shown as means ± SEM in weight percent of fatty acids, $n = 7–10$.
[b]Groups that share the same superscript are not significantly different (*post-hoc* Tukey's test, $P > 0.05$).
[c]DHA, main effect of DHA; GLA, main effect of GLA; Int, interaction effect; NS, $P > 0.05$; PC, phosphatidylcholine; for other abbreviations, see Table 2.

TABLE 5
Selected Fatty Acid Composition of PS/PI Fraction from Forebrain of Rat Pups Fed Experimental Formulae Containing Either Low (0.5%), Medium (1%), or High (3%) Levels of GLA and Low (0.5%) or High (2.5%) Levels of DHA on Postnatal Days 5–18[a,b]

Fatty acid	Suckled control	Reference group 0.5% DHA 0.5% AA	0.5% DHA 0.5% GLA	0.5% DHA 1% GLA	0.5% DHA 3% GLA	2.5% DHA 0.5% GLA	2.5% DHA 1% GLA	2.5% DHA 3% GLA	Two-way ANOVA DHA[c]	Two-way ANOVA GLA[c]	Two-way ANOVA Int[c]
16:0	4.98 ± 0.23	4.66 ± 0.28	4.74 ± 0.19	4.76 ± 0.18	4.73 ± 0.16	4.50 ± 0.12	4.53 ± 0.32	4.79 ± 0.24	NS	NS	NS
18:0	31.64 ± 1.77	32.73 ± 2.31	35.10 ± 1.94	32.21 ± 1.81	35.33 ± 1.81	34.99 ± 1.87	33.69 ± 2.12	32.68 ± 2.35	NS	NS	NS
18:1	9.58 ± 0.25	9.66 ± 0.21	9.93 ± 0.28	9.36 ± 0.20	9.48 ± 0.27	9.78 ± 0.24	9.29 ± 0.26	9.79 ± 0.18	NS	NS	NS
18:2n-6	$0.35^{x,y}$ ± 0.05	0.19^{y} ± 0.06	$0.25^{x,y}$ ± 0.07	$0.32^{x,y}$ ± 0.05	$0.22^{x,y}$ ± 0.06	0.48^{x} ± 0.04	$0.39^{x,y}$ ± 0.08	$0.22^{x,y}$ ± 0.08	NS	$P < 0.05$	NS
20:3n-6	0.45^{w} ± 0.02	$0.56^{v,w}$ ± 0.08	$0.57^{u,v,w}$ ± 0.05	$0.72^{u,v,z}$ ± 0.04	$0.79^{y,y,z}$ ± 0.04	1.10^{x} ± 0.06	$0.99^{x,y}$ ± 0.09	$0.89^{x,y,z}$ ± 0.09	$P < 0.001$	NS	$P < 0.01$
20:4n-6	18.15 ± 0.45	18.15 ± 0.76	17.11 ± 0.48	17.74 ± 0.37	17.41 ± 0.50	16.20 ± 0.54	17.03 ± 0.52	17.33 ± 0.70	NS	NS	NS
22:4n-6	4.66^{x} ± 0.08	$3.74^{y,z}$ ± 0.48	$4.18^{x,y,z}$ ± 0.09	$4.41^{x,y}$ ± 0.06	$4.46^{x,y}$ ± 0.07	3.49^{z} ± 0.09	3.49^{z} ± 0.07	$3.68^{y,z}$ ± 0.15	$P < 0.001$	$P < 0.05$	NS
22:5n-6	$2.87^{x,y}$ ± 0.09	$2.73^{x,y}$ ± 0.29	2.96^{x} ± 0.15	3.01^{x} ± 0.08	3.00^{x} ± 0.09	2.02^{z} ± 0.08	2.07^{z} ± 0.08	$2.30^{y,z}$ ± 0.09	$P < 0.001$	NS	NS
22:6n-3	24.04 ± 1.03	25.37 ± 1.25	22.83 ± 1.24	24.97 ± 0.92	23.07 ± 0.90	25.95 ± 1.31	26.52 ± 1.21	26.36 ± 1.43	$P < 0.01$	NS	NS
n-6/n-3 ratio	$1.06^{x,y}$ ± 0.04	$1.00^{x,y}$ ± 0.05	1.12^{x} ± 0.04	$1.02^{x,y}$ ± 0.03	1.13^{x} ± 0.03	0.91^{y} ± 0.02	0.91^{y} ± 0.03	0.94^{y} ± 0.04	$P < 0.001$	NS	NS

[a]Values shown as means ± SEM in weight percent of fatty acids, $n = 7–10$.
[b]Groups that share the same superscript are not significantly different (*post-hoc* Tukey's test, $P > 0.05$).
[c]DHA, main effect of DHA; GLA, main effect of GLA; Int, interaction effect; NS, $P > 0.05$; PS/PI, phosphatidylserine/phosphatidylinositol; for other abbreviations, see Table 2.

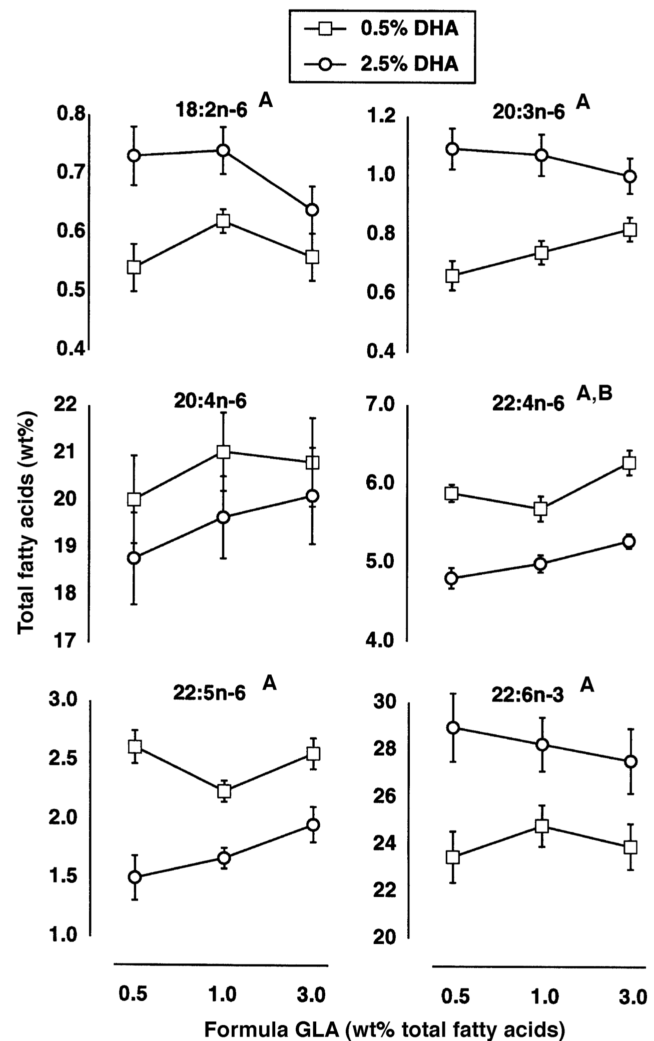

FIG. 1. Mean concentrations of selected forebrain polyunsaturated fatty acids in phosphatidylethanolamine membrane fraction from 18-d-old rats fed experimental formula from day 5. (A) Denotes main effect of docosahexaenoic acid (DHA), (B) denotes main effect of γ-linoleic acid (GLA).

20:3n-6, and individual group comparisons showed that these effects were present only when dietary GLA was low. There were no noteworthy effects of GLA or DHA supplementation on the saturated and monounsaturated fats. Similarly, the two control groups did not differ from each other in any meaningful way. In a comparison of the control groups with the experimental groups, groups fed high levels of DHA most often differed from both control groups, generally having higher levels of 18:2n-6 and 20:3n-6, and lower levels of 22:4n-6 and 22:5n-6, than the control groups. The levels of DHA in PC and PS/PI were generally similar for experimental and control groups while in the PE fraction, the group fed 2.5% DHA with 0.5% GLA had significantly higher levels of DHA than those of the suckled control group.

DISCUSSION

As with our previous study using this AR procedure (13), feed-

ing of formulae with different levels of DHA resulted in changes in the fatty acid composition of the rat brain at 18 d of age. In the present study, as expected, high dietary levels of DHA (2.5%) resulted in higher levels of 22:6n-3 and lower levels of 22:4n-6 and 22:5n-6 in the brain than did 0.5% DHA. In comparison, high dietary levels of GLA (3%) increased 22:4n-6 relative to medium and low levels, but had no significant effect on 22:6n-3. Neither DHA nor GLA at the levels used in this study had significant effects on levels of 20:4n-6. Nonetheless, the data did show trends in the directions expected, i.e., 20:4n-6 was decreased by DHA and increased by GLA, suggesting that the lack of statistical significance may be related to the large variability apparent in these data relative to the other fatty acids. High levels of DHA increased 20:3n-6 in the brain, but the effect was present only at the lower levels of GLA, and high levels of DHA also increased 18:2n-6.

It should be noted that the highest levels of DHA in the formulae in this study (2.5%) are beyond any reported physiological level for rat milk. For instance, the amount of DHA in milk of rats (11,18) and mice (17) fed diets containing LCP during pregnancy and lactation has been reported to range from 0.4–1.5% of total fatty acids. Therefore, findings based upon the use of high levels of DHA, while important for understanding the relationships among dietary LCP during brain development, should be interpreted with caution when studying variation in levels of LCP closer to the reported physiological range.

In order to compare these findings with our previous work (13), it is necessary first to consider the differences between the designs of the two studies. The 3 × 3 design of our previous study included groups that were not supplemented with either DHA or AA (0%), as well as those supplemented with low (0.4%) and high (2.4%) levels of DHA and AA. The inclusion of the unsupplemented groups in the first study allowed us to determine the amount of DHA and AA that was necessary to achieve a brain LCP composition similar to that of the suckled control group. The results indicated that this could be achieved by supplementation with low levels (0.5%) of both AA and DHA, and this group was thus considered the appropriate reference control group for the AR groups in the present study. In contrast to the first study, all AR groups (other than the reference control group) in the present study were fed diets that were supplemented with both DHA and GLA, at either low (0.5%) or high (2.5%) levels of DHA, and at either low (0.5%), medium (1%), or high (3%) levels of GLA. Generally the differences between the effects of AA in the first study and those of GLA in the second study were differences in magnitude. For example, in the first study, high levels (compared with low levels) of AA significantly increased both 20:4n-6 and 22:4n-6, and at the same time they significantly decreased DHA in the brain. In the present study, although high levels of GLA did signficantly increase 22:4n-6, the effects on 20:4n-6 were not significant, and there was not a significant overall decrease in 22:6n-3. Such differences in effectiveness between GLA and AA on tissue n-6 LCP levels might be expected, based upon their relative positions in the metabolic pathway. GLA is a precursor for 20:3n-6, which is then subsequently desaturated to form 20:4n-6 and then elongated to form 22:4n-6. When compared to the reference group (0.5% AA, 0.5% DHA), the groups fed formula containing 0.5% DHA and either 0.5 or 1% GLA had similar brain LCP compositions. What these results suggest therefore is that dietary GLA in the range of 0.5 to 3% total fatty acids prevents the reduction in levels of some 20- and 22-carbon n-6 fatty acids in the brain, which can occur in the presence of high dietary DHA levels. Furthermore, they suggest that even high levels of dietary GLA (3%) differ from high dietary levels of AA in that they do not lead to reductions in brain DHA.

In contrast to the effects on other n-6 LCP, high dietary levels of DHA led to increases in 20:3n-6 in the brain, which is consistent with previous findings in our lab (15,17,23,24) and with the findings of a study on the effects of DHA supplementation on lung LCP in piglets (25). Furthermore, a previous study in which AR rats were fed n-3 deficient diets found decreased levels of 20:3n-6 in the brain in n-3 deficient rats (11). This provides further support for the suggestion that high levels of dietary DHA may inhibit Δ5 and Δ6 desaturase activity and thereby decrease the formation of AA from 18:2n-6 (17). The finding that DHA also increased levels of 18:2n-6 would be consistent with this explanation. It is interesting to note that the addition of 3% GLA to formula acted to counter the effect of DHA.

While levels of most LCP were much lower in PC than in other phospholipid fractions, the effects of dietary DHA and GLA in the present study were generally consistent across all fractions. This is in contrast to the previous study, where effects in PC were less consistent with those in the PE fraction (13). Different effects on PC relative to PE and PI/PS, accompanied by low levels of long-chain LCP in PC relative to those in PE, also have been reported by others (26,27).

With the exception of 22:4n-6 in PS/PI, which was greater in the suckled control pups, there were no significant differences between the two control groups in this study. This is consistent with the findings of our previous study which found that pups fed a formula containing small amounts (0.5%) of both DHA and AA had a brain LCP composition similar to that of a suckled control group (13). As stated in the previous report, this is interesting in light of the fact that the milk of the dams did not contain detectable levels of DHA, although it did contain small amounts (0.7%) of 20:5n-3. This likely reflects the fact that the American Institute of Nutrition 93M chow fed to the dams comprised a mixture of corn and soybean oil and therefore contained LN and LA, but no LCP. While we did not assess the fatty acid composition of the rat milk in this study, it is likely to be similar to that found previously since the dams in the present study were fed the same diet, for the same period of time, as were those in the previous study.

In summary, formula-feeding of GLA offsets the effects of high levels of DHA in formula on n-6 LCP levels in the brain while leaving DHA levels unchanged. Additionally, these results confirm that the fatty acid composition of the develop-

ing brain can be affected by the type and amounts of n-3 and n-6 fatty acids provided in the diet.

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Infant Cerebellar Gray and White Matter Fatty Acids in Relation to Age and Diet

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ABSTRACT: There is little evidence as to the fatty acid composition of the cerebellum in infancy and it remains uncertain whether milk diet can influence its composition. We therefore examined cerebellar gray and white matter of infants less than 6 mon old who had died unexpectedly. The fatty acid content of 33 gray and 21 white matter specimens from infants born at term and 6 gray and 5 white matter specimens from preterm infants was assessed by gas chromatographic/mass spectrometric analysis. Infants were grouped according to whether they had received human or manufactured formula milk. Whereas cerebellar cortex docosahexaenoic acid (DHA, 22:6n-3) concentrations were significantly lower ($P < 0.01$) in the formula-fed than breast-fed infants, no differences existed between the term (n = 10) and preterm (n = 5) Scientific Milk Adaptation (SMA) formula-fed infants. Cerebellar white matter DHA concentrations were similarly lower ($P < 0.01$) in the SMA formula-fed infants (n = 8) than in an age-matched breast-fed group. Low concentrations of cerebellar white matter lignoceric (24:0) and nervonic acid (24:1n-9) in two 7-wk-old preterm infants appeared to correlate with postgestational rather than chronological age. Dietary long-chain polyunsaturated fatty acids, particularly DHA, are probably essential for normal development of the infant cerebellum.

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The cerebellum is composed of two hemispheres displaying numerous deep fissures with folia between and connected by the vermis in the midline. Like the cerebrum, it is also divided into lobes, the most primitive being the flocculonodular, followed by the anterior and posterior. The cerebellum acts to modulate and balance the sensory stimuli reaching the cerebral cortex with that of the generated neurological response, and its principal function is in the coordination of movement and balance. However, it may also undertake some of the higher sensory functions, which have been, until recently, considered the preserve of the cerebral cortex (1,2). In evolutionary terms, after the brain stem, the cerebellum was next to develop from the anterior dorsal part of the primitive midbrain. In humans it was believed that the increased cerebellar complexity, particularly the lateral expansion of the posterior lobe, accounted for our manual dexterity. However, positron emission tomography shows maximal activity in the cerebellar posterior lobe in response to cognitive and linguistic tasks simultaneously performed, with activity generally centered in the prefrontal cortex (3).

In comparison to the infant cerebrum, the cerebellum has a faster but shorter growth spurt, starting later but achieving adult proportions earlier (4). This growth necessitates incorporation of polyunsaturated fatty acids (PUFA) (5) and as it includes a period of rapid myelination, a supply of very long chain fatty acids (VLCFA) such as nervonic and lignoceric is essential. Our previous research identified dietary-related deficits in infant cerebral gray and white matter long-chain PUFA and VLCFA (6–8), and therefore one must consider that these may also affect cerebellar development during a critical time in infant nutrition (9). The cerebellums of animals fed a restricted diet have disproportionately fewer cells, especially granular neurons and glial cells, and the brain lipids, especially those characteristic of myelin, are permanently reduced (10). Martinez (11) found no difference in the concentration of cerebellar myelin-associated galactolipids when comparing a group of undernourished infants with those fed a normal diet.

This study reports cerebellar grey and white matter fatty acid compositions in early infancy with specific reference to diet. Cerebellar white matter glycosphingolipid (galactocerebroside and sulfatide) contents are also reported.

SUBJECTS AND METHODS

Tissue samples from both gray and white matter were obtained from precise loci in the folia of the anterior cerebellar lobe, distant from the nuclei. Specimens were obtained from 34 babies who had died in the first 6 mon of life from sudden infant death syndrome, after a pathologist's examination had excluded any other cause of death. Tissues were stored at −60°C pending analysis. Full medical and dietary histories were obtained soon after death and infants were grouped according to which milk diet they had received.

Formula and human milk compositions from a local population of mothers have been reported previously (6–8). In this

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Abbreviations: CGOST, Cow Gate and Farley's Ostermilk formulas; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; PUFA, polyunsaturated fatty acids; SMA, Scientific Milk Adaptation; VLCFA, very long chain fatty acids.

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TABLE 1
Details Regarding Infant and Cerebellar Cortex Total Lipid Fatty Acid Concentrations in Relation to Infants' Diet[a]

	Dietary group[b]			
	Breast milk	SMA	CGOST	SMA (prem)
Infant details				
Birth weight (g)	3356 (517)	2996 (314)	3000 (618)	1968 (427)
Gestational age (wk)	39.9 (1.2)	38.6 (1.2)	39.3 (1.6)	33.4 (2.1)
Age (wk)	9.2	10.1	13.2	15.4
Age range (wk)	1–19	0.5–19	6.5–21	7–25
Male/female	3/7	8/2	5/2	4/1
Fatty acid (wt%)				
14:0	1.85 (0.28)	1.90 (0.29)	1.76 (0.35)	1.85 (0.46)
16:0	28.50 (1.06)	29.77 (1.33)	28.93 (1.04)	29.44 (0.68)
16:1n-7	3.21 (0.73)	3.13 (0.70)	3.08 (0.76)	3.32 (0.51)
18:0	18.19 (1.17)	18.17 (0.98)	18.86 (1.87)	18.05 (0.78)
18:1n-7 + n-9	20.04 (1.04)	19.52 (1.17)	20.77 (0.61)	20.41 (1.36)
18:2n-6	0.87 (0.44)	1.07 (0.28)	0.86 (0.14)	1.35 (0.25)
20:3n-6	2.02 (0.66)	2.43 (0.66)	2.62 (0.42)	2.70 (0.49)
20:4n-6	11.15 (0.60)	11.10 (0.69)	11.23 (0.89)	11.35 (0.78)
22:4n-6	4.74 (0.54)	5.12 (0.60)	5.35 (0.83)	5.04 (0.36)
22:5n-6	0.69 (0.27)	1.08 (0.25)[a]	1.76 (0.37)[b]	1.38 (0.42)[a]
22:6n-3	8.44 (0.79)	6.54 (1.13)[b]	4.98 (0.98)[b]	6.16 (1.28)[a]

[a]Details regarding infants and fatty acid concentrations are given as means, with standard deviations in parentheses.
[b]Significant differences in fatty acid concentrations between the breast milk-fed group and the term and preterm formula-fed groups, calculated by Student's *t*-test (two-tailed), are shown. [a]$P < 0.01$; [b]$P < 0.001$.

study period (April 1986–June 1996), all formula milks were devoid of both long-chain PUFA and very long-chain monounsaturated fatty acids. Term-born infants were subsequently classified as either exclusively breast-fed ($n = 10$), Scientific Milk Adaptation formula (SMA)-fed ($n = 11$), or fed a regimen designated CGOST ($n = 7$), which included infants fed either Farley's Ostermilk (Heinz, United Kingdom) or Cow & Gate Premium (Cow & Gate, Trowbridge, United Kingdom). In addition, tissue from seven preterm infants was obtained, five of which were SMA-fed (delivered at 30, 32, 34, 35, and 36 wk gestation, respectively), one (34 wk gestation) CGOST fed (grey matter only), and the other (32 wk gestation) fed both human and SMA formula and designated mixed-fed (white matter only). α-Linolenic acid (18:3n-3) was present at 1.5% of the total fatty acids in SMA (linoleic/α-linolenic ratio *ca.* 10), whereas the CGOST formulas contained <0.4% as α-linolenic acid with a linoleic/α-linolenic ratio of about 40.

Age comparability between feeding groups was considered sufficient for the assessment of dietary-related differences in cerebellar grey matter fatty acid compositions. However because of the rapid postnatal changes in brain white matter fatty acid compositions (8,12) it was deemed essential to age-match (±1 wk) infants in the cerebellar white matter dietary groups. This proved possible with the breast-fed and SMA-fed groups but led to the exclusion of a 9-wk-old breast-fed and 17-wk-old SMA-fed infant. In addition, no cerebellar white matter was retained from a 7-wk-old breast-fed or 9- and 16-wk-old SMA-fed infants, although the last was substituted by a further specimen from an infant of the

TABLE 2
Details Regarding Infants and Cerebellar White Matter and Myelin Total Lipid Fatty Acid Concentrations in Relation to Infants' Diet[a]

	Dietary group	
	Breast milk	SMA
Infant details[b]		
Birth weight (g)	3208 (506)	3178 (438)
Gestational age (wk)	39.6 (1.4)	38.5 (1.3)
Age (wk)	9.5	9.3
Age range (wk)	1–19	0.5–19
Male/female	3/5	6/2
Fatty acid (wt%)[c]		
14:0	1.30 (1.13–2.22)	1.25 (0.72–1.44)
16:0	20.00 (16.74–22.93)	20.77 (16.68–24.12)
16:1n-7	2.58 (2.03–3.69)	2.68 (1.78–3.43)
18:0	21.69 (19.38–22.24)	22.37 (21.13–23.26)
18:1n-7 + n-9	26.98 (22.74–31.41)	27.16 (21.36–30.69)
20:1n-9	1.58 (0.99–2.55)	1.43 (0.60–2.31)
20:2n-6	0.72 (0.49–0.97)	0.74 (0.42–0.95)
20:3n-6	1.45 (0.98–1.96)	1.30 (0.97–1.80)
20:4n-6	7.37 (5.81–9.55)	7.13 (5.94–9.89)
22:4n-6	5.70 (5.00–6.58)	6.42 (5.23–7.20)[a]
22:6n-3	6.26 (4.32–10.24)	5.30 (3.10–8.16)[b]
24:0	1.87 (1.29–2.48)	2.19 (1.60–2.47)[a]
24:1n-9	2.30 (1.12–3.12)	2.29 (0.91–4.09)

[a]Significant differences in fatty acid concentrations between feeding groups were calculated by the Wilcoxon signed rank test for paired data ($n = 8$). [a]$P < 0.02$; [b]$P < 0.01$.
[b]Details regarding infants are given as means with standard deviations in parentheses.
[c]Fatty acid concentrations are expressed as weight percentage of total fatty acids and presented as medians and ranges.

same age. An insufficient degree of age-matching also excluded a cerebellar white matter CGOST group from the analysis. Details regarding infants and feeding group compositions are presented in Tables 1 and 2.

Tissues were extracted as previously described (5), and gray and white matter fatty acids were derivatized with boron trifluoride in methanol (BDH, Poole, United Kingdom) (14% wt/vol) and anhydrous methanolic 3 N HCl (Sigma-Aldrich, Poole, United Kingdom), respectively. The resulting fatty acid methyl esters were analyzed by gas chromatography/mass spectrometry (Hewlett-Packard 5972, Stockport, United Kingdom) using a 30-m Supelco Omegawax capillary column (Sigma-Aldrich). The column temperature was initially held at 150°C for 2 min, then programmed to 220°C at 5°C/min. Detector and injector temperatures were 280 and 250°C, respectively, and helium was used as carrier gas. Cerebellar grey and white matter fatty acid concentrations with respect to dietary group are shown in Tables 1 and 2.

To relate fatty acid compositions in the cerebellar white matter (particularly the VLCFA) with the lipid species present, we analyzed tissue extracts (nonderivatized) by high-performance thin-layer chromatography on silica gel plates (LHPK Silica Gel 60A, Whatman International Ltd., Maidstone, United Kingdom). One microliter (*ca.* 20 µg lipid) was spotted as a narrow band beside a mixed standard (10 µg) containing equal amounts of sulfatides, galactocerebrosides, and the major phospholipids all derived from bovine brain and obtained from Sigma-Aldrich. Plates were run at room temperature in 40 mL methyl acetate/propan-2-ol/chloroform/methanol/0.25% aqueous KCl (25:25:25:10:9, by vol) for 40 min. After drying, they were dipped in the developing reagent (3% copper acetate in 8% orthophosphoric acid) and charred at 160°C for 20 min. The resultant bands were analyzed by scanning densitometry (Shimadzu, Milton Keynes, United Kingdom) on reflectance mode at 340 nm wavelength. After correction for relative absorption, lipid classes were quantified after reference to the standard values.

Between-group differences in cerebellar cortex fatty acids were assessed by Student's *t*-test (two-tailed). After correction for multigroup analysis a significance level of $P < 0.01$ was adopted. Cerebellar white matter fatty acid data were assessed using the Wilcoxon signed rank test solely for the age-matched breast-fed and SMA-fed infants. The white matter results from the SMA-fed preterm infants are provided for information only, without statistical analysis. Distributions of age-related results, corrected for gestation, are provided for cerebellar cortex docosahexaenoic acid (DHA) (Fig. 1) and docosapentaenoic acid (DPA, 22:5n-6) (Fig. 2) and cerebellar white matter DHA (Fig. 3), nervonic acid (Fig. 4) and lipid subfractions (Fig. 5).

RESULTS

Cerebellar gray matter. None of the saturated or mono-unsaturated fatty acid concentrations differed between groups (Table 1). Cortical DHA concentrations in the human milk-fed group were generally above 8% of total fatty acids during the first 6 mon (Fig. 1) and were significantly greater than in the SMA-fed ($P < 0.001$), CGOST-fed ($P < 0.001$) and SMA-fed preterm infant ($P < 0.01$) groups where DHA levels declined

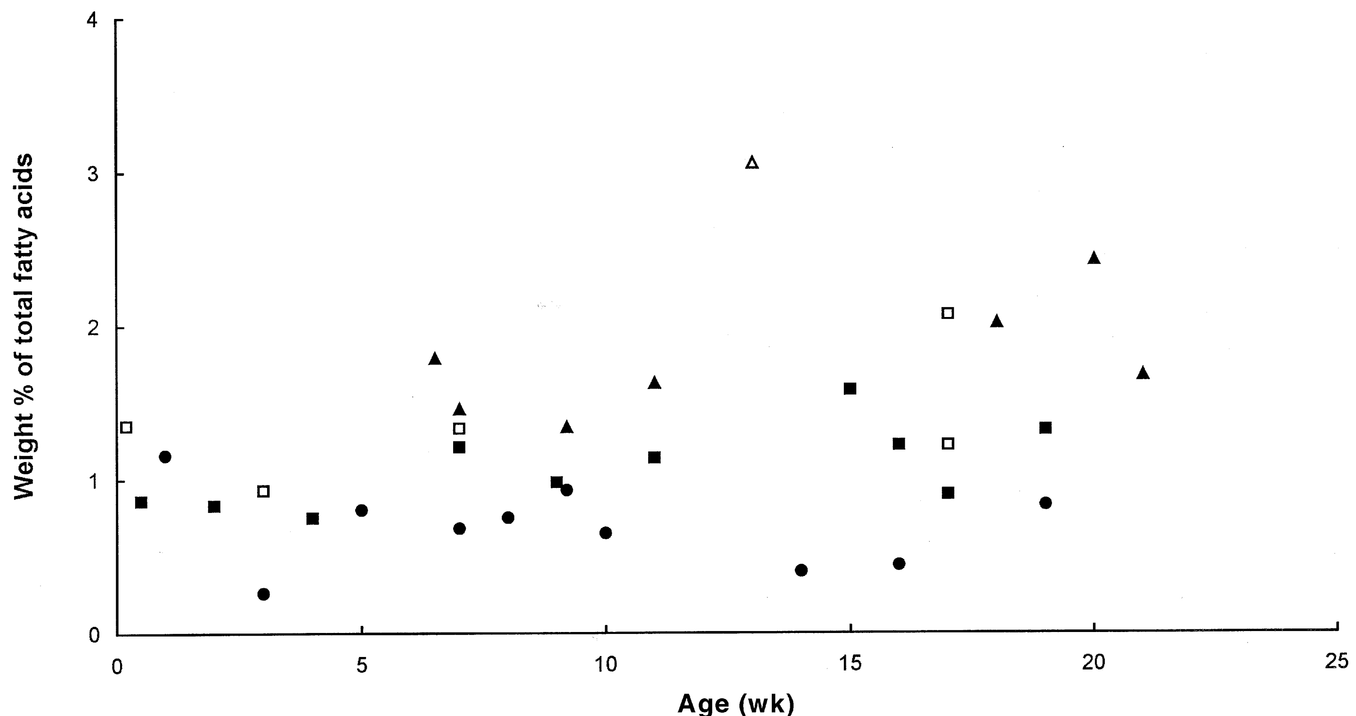

FIG. 1. Cerebellar cortex phospholipid docosapentaenoic acid (22:5n-6) in relation to infants' diet and age. (●, ■, ▲), term infants; (○, □, △), preterm infants. (●,○), breast-fed infants; (■,□), SMA-fed infants; (▲,△), CGOST-fed infants.

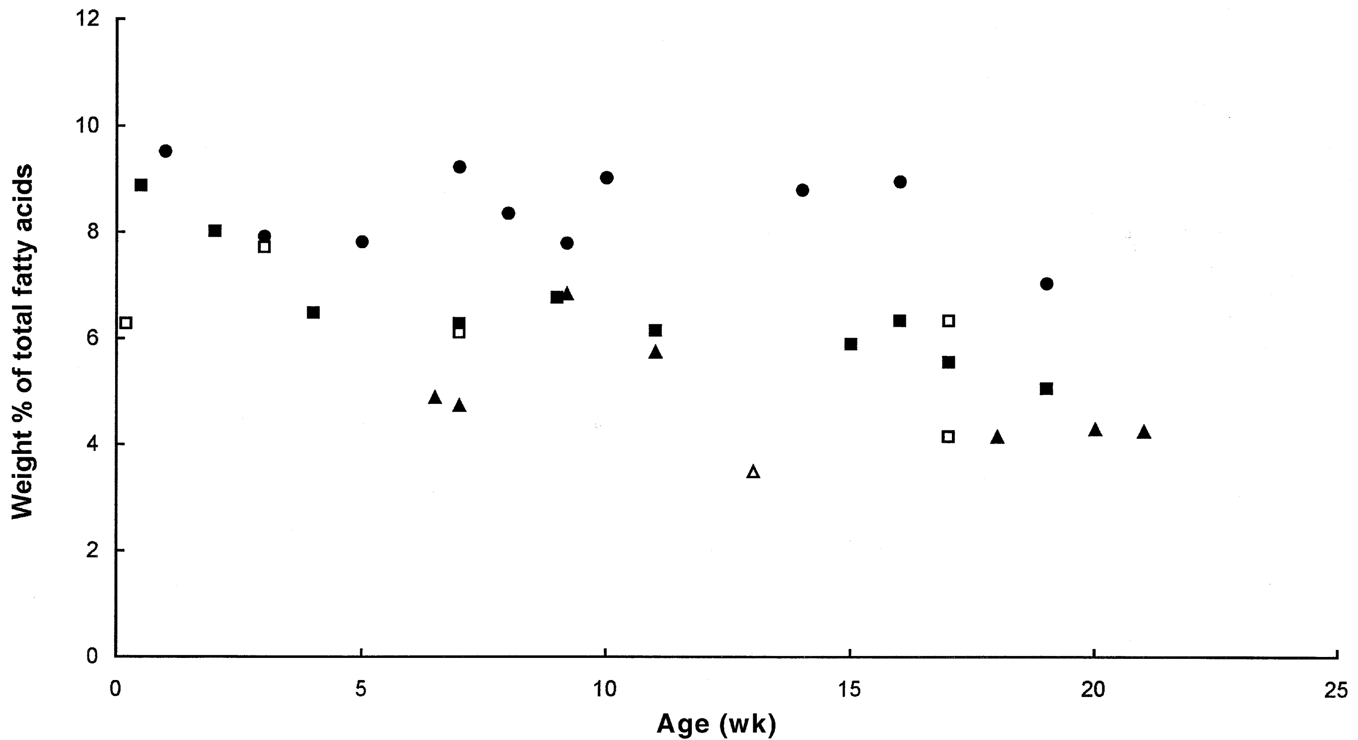

FIG. 2. Cerebellar cortex phospholipid docosahexaenoic acid (22:6n-3) in relation to infants' diet and age. For symbols see Figure 1.

over the same period to about 4–5%. Partial substitution is suggested by a generalized increase in n-6 series PUFA, which, however, only reached statistical significance in DPA (Table 1). A similar pattern with greater DHA ($P < 0.01$) and lower DPA ($P < 0.001$) concentrations in the SMA-fed than CGOST-fed infants was observed. No significant differences in fatty acid compositions were found between the term and preterm SMA-fed groups. Overall, the highest DPA (3.06%) and lowest DHA (3.49%) concentrations were present in the 19-wk-old CGOST-fed preterm (34-wk gestation) infant (Figs. 1,2).

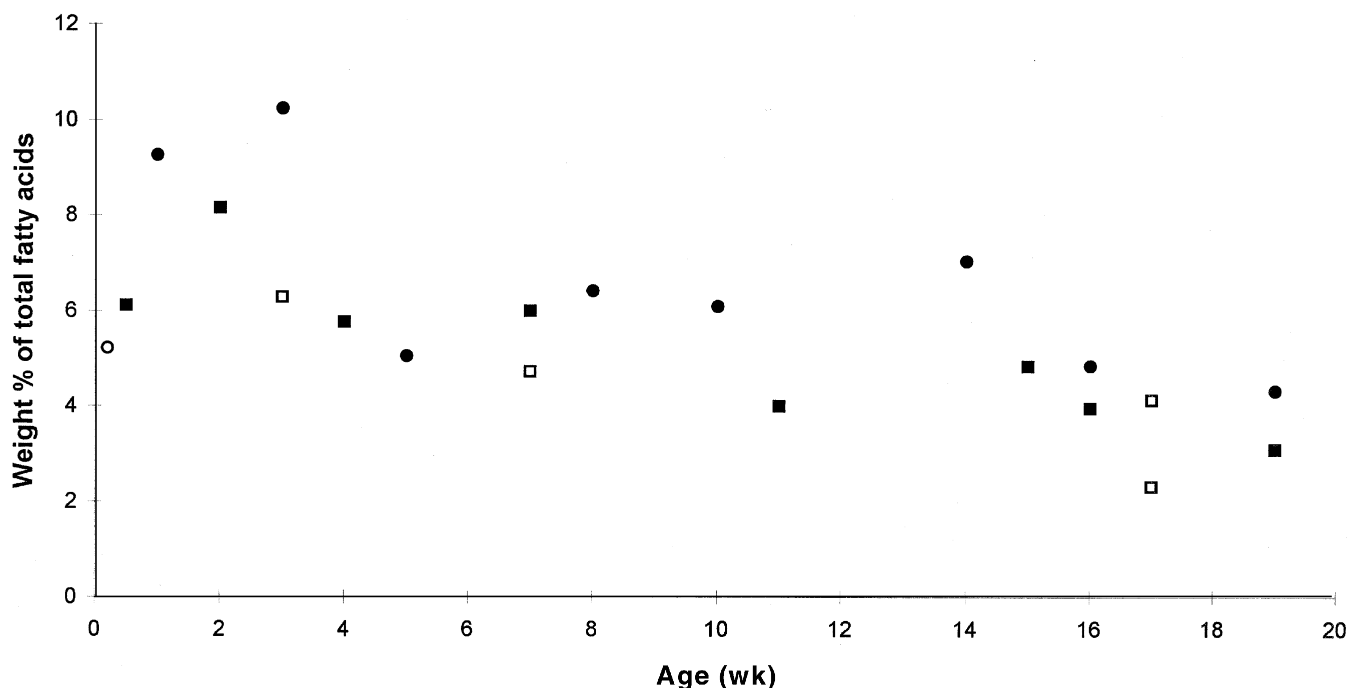

FIG. 3. Cerebellar white matter total lipid docosahexaenoic acid (22:6n-3) in relation to infants' diet and age. For symbols see Figure 1.

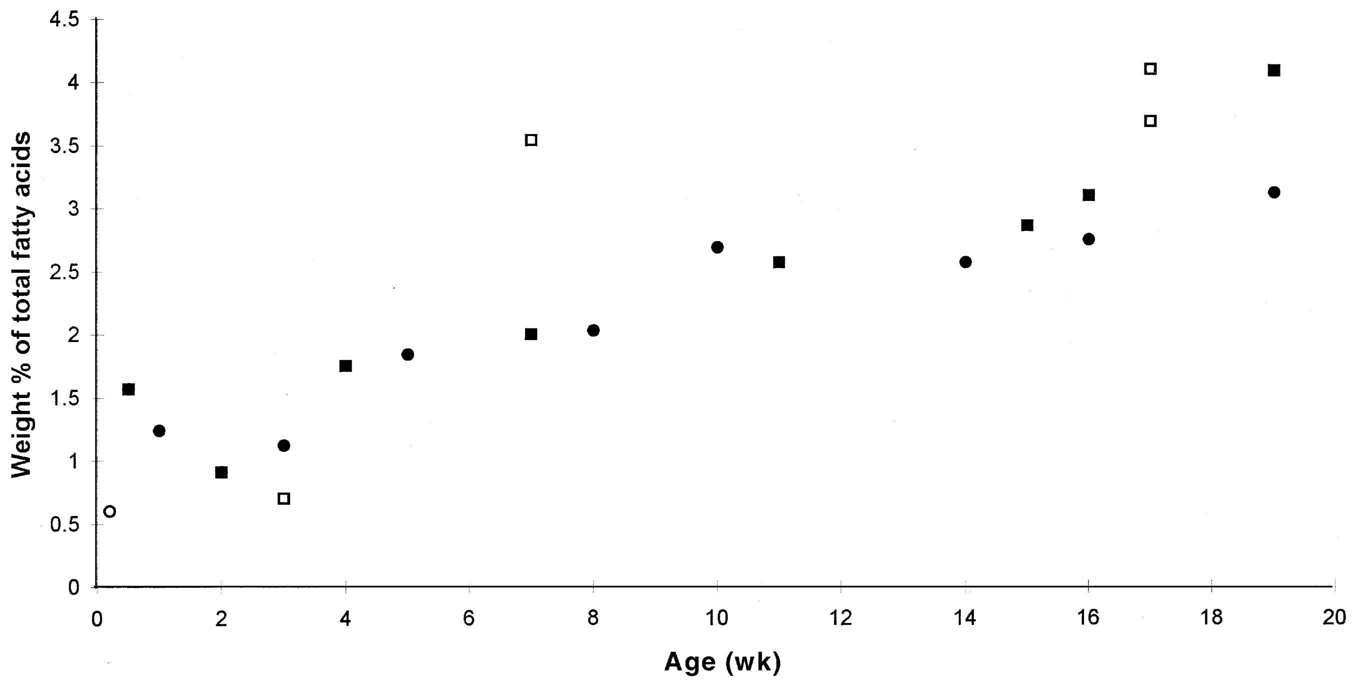

FIG. 4. Cerebellar white matter total lipid nervonic acid (24:1n-9) in relation to infants' diet and age. For symbols see Figure 1.

Cerebellar white matter. Again, no significant differences were found in mono-unsaturated fatty acids in respect of diet. Cerebellar white matter DHA incorporation was greater in the breast-fed group ($P < 0.01$) than the age-matched SMA group (Table 2) although a steady decline in concentrations with age to less than 5% of total fatty acids was a feature common to all feeding groups. In contrast to the cerebellar cortex, DPA was not prominent in cerebellar white matter, however, both docosatetraenoic (22:4n-6) ($P < 0.02$) and lignoceric acid concentrations ($P < 0.02$) were greater in the SMA- than the

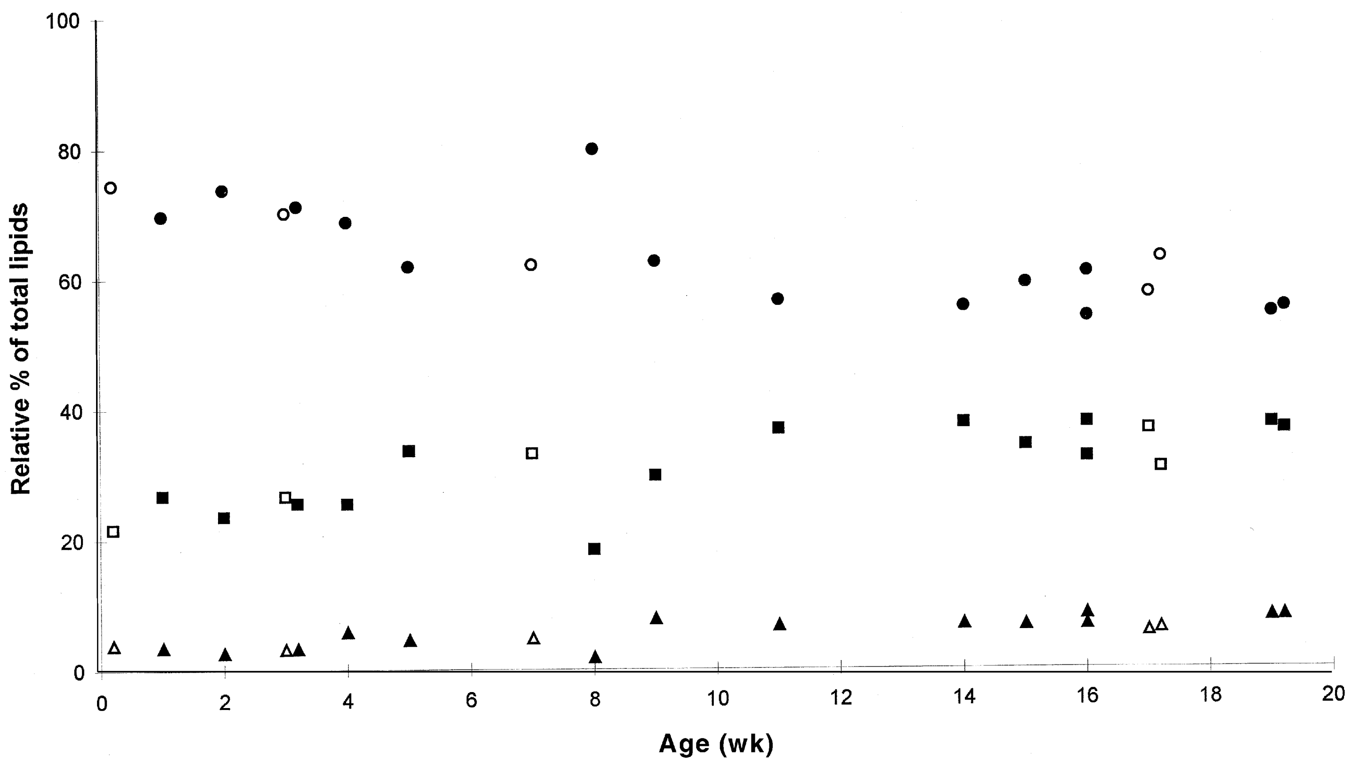

FIG. 5. Infant cerebellar white matter lipid compositions with respect to age. (●, ■, ▲), term infants; (○, □, △), preterm infants. (●,○), phospholipids; (■,□), galactocerebrosides; (▲,△), sulfatides.

breast-fed group (Table 2). In the SMA-fed preterm infant group DHA concentrations appeared consistent with those of the term-born SMA-fed infants (Fig. 3) although as previously stated no statistical between group analysis was possible. In the youngest, 7-wk-old SMA- and mixed-fed preterm infants, the lowest nervonic acid concentrations of 0.7 and 0.65%, respectively, were found (Fig. 4). Although not shown, a similar pattern was present in cerebellar white matter lignoceric acid. Subsequent high-performance thin-layer chromatographic analysis of lipid extracts failed to reveal a concomitant reduction in the VLCFA-rich galactocerebrosides and sulfatides in these premature infants (Fig. 5).

DISCUSSION

Researchers who have examined neonatal brains by magnetic resonance imaging have identified maturation of the cerebellum in advance of the cerebrum, with significant cerebellar myelination before birth (13). We found a very similar pattern of cerebellar cortex fatty acid concentrations in term-born infants to those previously observed in cerebral cortex (6), with a DHA "deficiency" associated with formula feeding and in conjunction with an increase in n-6 series PUFA, particularly DPA. In addition, limited dietary provision of α-linolenic acid (0.4% of total fatty acids) to the CGOST-fed infants was probably responsible for their low cortex DHA concentrations. Our findings, with respect to feeding groups, must be somewhat tempered by the relatively low mean birthweights in the cerebellar cortex SMA and CGOST groups at 3.0 kg, as compared with that of the breast-fed group at 3.35 kg.

It has been thought that preterm infants might be uniquely vulnerable to deficits in fatty acid accretion if they are born before the cerebellar growth spurt *in utero*. Although no exclusively human milk-fed preterm infants were included in this study, there were no differences in cerebellar cortex fatty acid concentrations between the term and preterm SMA fed groups in spite of mean gestational ages of 38.6 and 33.4 wk, respectively. It is known, however, that when precursor α-linolenic acid is provided, some DHA can be synthesized even in preterm infants (14). The supply of α-linolenic acid at 1.5% of total fatty acids in the SMA formula may have been adequate for that purpose.

The DHA concentration in cerebellar white matter was also significantly lower in the SMA-fed than the age-matched breast-fed group. This outcome, similar to that in the cerebellar cortex, may seem surprising as the cerebellum constitutes only 6–8% of total brain weight. Moreover, as cerebral and cerebellar DHA concentrations are equivalent at birth and decline in the white matter thereafter, cerebellar incorporation of DHA should amount to less than 1 g in the first year of life. If dietary DHA is not provided, endogenous synthesis of DHA may be sub-optimal or turnover of PUFA may be greater in the cerebellum than cerebrum. The relatively low cerebellar white matter lignoceric and nervonic acid concentrations in the 7-wk-old, preterm (32- and 36-wk gestation) infants (Fig. 4) seem to correlate more closely with postgestational than chronological age and may be as a result of them missing the late fetal accretion phase. It has been observed that "normal" concentrations of cerebellar glycosphingolipids (galactocerebrosides and sulfatides) are maintained even in undernourished infants (11); and the relative percentages of phospho- and sphingolipids, as measured by scanning densitometry after charring, do not appear to be compromised by preterm delivery or early milk feeding regimens devoid of both long-chain PUFA and VLCFA (Fig. 5). Whether the precise process of conversion of sphingosine to ceramide in the infant cerebellum, predominantly by incorporation of VLCFA, is disrupted cannot be assessed in this study.

Animal research has shown that nutritional deprivation in early postnatal life may affect motor coordination (15,16); however it is not known if the dietary-related structural differences in human cerebellar cortex and white matter DHA in this work could also affect functional neurodevelopment. Any alteration in cerebellar function might be expected to manifest in subtle modification in neurological activity associated specifically with fine motor coordination. Perhaps a parallel may be drawn between the increasing rates of formula feeding and the emergence of relatively benign, proprioception-related conditions, such as dyspraxia. An "epidemic" of uncoordinated, dyspraxic individuals has been identified in upward of 6% of school-age children (17), with a higher prevalence (14%) in children of low (<2.5 kg) birthweight (18). In conclusion, the authors assert that formula milk compositions should more closely resemble those of human milk, especially with respect to PUFA content.

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Influence of Diet on Fatty Acids of Three Subtropical Fish, Subfamily Caesioninae (*Caesio diagramma* and *C. tile*) and Family Siganidae (*Siganus canaliculatus*)

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ABSTRACT: The total lipid and fatty acid compositions of tissues and the stomach contents of three subtropical marine fish species, subfamily Caesioninae, *Caesio diagramma* and *C. tile,* and family Siganidae *Siganus canaliculatus,* were investigated to clarify the differences between these species. Triacylglycerols (TAG) were the dominant depot lipids of the three species, whereas wax esters were found as a minor component. In particular, muscle lipids were found to contain mainly glycerol derivatives such as TAG and phospholipids. The major fatty acids identified in the three species were 16:0, 18:0, 18:1n-9, and 22:6n-3 (docosahexaenoic acid, DHA). In addition, noticeable levels of 16:1n-7, 18:1n-7, 20:4n-6 (arachidonic acid, AA), and 20:5n-3 (eicosapentaenoic acid) were found. DHA was the most abundant polyunsaturated fatty acid (PUFA) in the muscle and viscera lipids of the three species. The high DHA levels in the lipids of all the organs were found to be higher than those of the lipid extracted from the stomach contents of the three fishes. In addition, the specimens of *S. canaliculatus* contained significantly higher levels of AA in its tissues than did the other two species. A high AA content is unusual since such high levels of n-6 PUFA are rarely found in higher marine organisms. These levels may be due to its characteristic feeding pattern, because *S. canaliculatus* prefer and mainly feed on seaweed, which often contains high amounts of n-6 PUFA, such as linoleic acid (18:2n-6) and AA.

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Reportedly marine pelagic fishes selectively store various fatty acids from their prey in the form of triacylglycerols (TAG) which are used as depot lipids. These lipids generally contain high levels of long-chain n-3 polyunsaturated fatty acids (PUFA), which predominate in phytoplankton lipids over α-linolenic acid (1–7). In particular, the tissue lipids of tuna species, which are highly migratory fishes, are markedly high in docosahexaenoic acid (DHA) (8–11). In the marine food chain, tuna species are at the highest trophic level, and their high DHA levels may be a result of their trophic position in the food chain and accumulation of planktonic DHA through feeding on small fishes. Marine fishes, with the exception of turbot, are generally unable to synthesize DHA (2,12–18).

On the other hand, it has been suggested that environmental conditions, such as temperature variations, have an influence on the n-3 PUFA content of lipids in small marine fish species (2,5,19–25). For example, the DHA content of lipids in tropical and subtropical marine fish species are reportedly lower than those of arctic and subarctic ones (26-29). Fishes living in warm-water seas do not appear to require as much DHA in their cell membranes as cold-water species (19–25). The lipid content of tropical fish species is generally influenced by high ambient temperature, and the membrane lipids of these fishes are easily fluidized even if the major fatty acids in their lipids are composed of saturated and monoenoic fatty acids. The n-3 PUFA ratios of the total fatty acids (TFA) in tropical and subtropical fish lipids are usually comparatively low, and this is a general tendency in the lipids of marine organisms living in warm seas. However, this trend in lipid utilization has been confirmed for only a few fish species (26–29).

In the present study, we assess the relationship between the lipids of the two subtropical Caesioninae species (*Caesio diagramma* and *C. tile*) and one Siganidae species (*Siganus canaliculatus*), and those of their prey, to determine the position of these fishes in the marine food chain. In this study, the lipid and TFA compositions of their tissues and stomach contents were analyzed. These species are abundant in the sea off the coast of the main island of Okinawa, Japan, and are an important marine resource that is consumed by the inhabitants of the southern islands of Japan.

MATERIALS AND METHODS

Materials. Details of the specimens of two subtropical fish species used, *C. diagramma* and *C. tile* are given in Table 1.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FFA, free fatty acid; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acids; TAG, triacylglycerols; TFA, total fatty acids; TL, total lipids; TLC, thin-layer chromatography.

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TABLE 1
Locality of Capture and Biological Data of the Three Subtropical Fish Species[a]

Entry	Scientific name	Date	Locality	Length (cm)	Weight (g)
	Caesio diagramma				
1	(n = 4)	August 29, 1996	26°15′N 127°30′E	22.8 ± 0.1	261.9 ± 5.1
2	(n = 7)	May 6, 1997	24°30′N 124°30′E	18.8 ± 0.1	139.1 ± 2.4
	C. tile				
3	(n = 4)	September 9, 1996	24°30′N 124°30′E	22.1 ± 0.1	225.6 ± 2.4
4	(n = 6)	May 6, 1997	24°30′N 124°30′E	19.7 ± 0.2	167.8 ± 5.2
	Siganus canaliculatus				
5	(n = 6)	November 22, 1993	24°30′N 127°55′E	19.7 ± 0.1	227.5 ± 3.2
6	(n = 8)	August 18, 1997	24°30′N 127°55′E	22.0 ± 0.2	252.9 ± 7.3

[a]Results are expressed as the mean ± standard error (n = 4–8) for each entry.

The *C. diagramma* specimens were caught in the East China Sea near Okinawa (four specimens: 26°15′ N, 127°30′ E, August 29, 1996; and seven specimens: 24°30′ N, 124°30′ E, May 6, 1997) in the subtropical Pacific Ocean. Specimens of *C. tile* were also caught in the same sea (24°30′ N, 124°30′ E) on September 9, 1996, and May 6, 1997. Specimens of *S. canaliculatus* were caught in the vicinity of Katsurenzaki Peninsula in Nakagusuku Bay (24°30′ N, 127°55′ E) in two different seasons: on November 22, 1992 (six specimens), and on August 18, 1997 (eight specimens). All samples were immediately frozen and kept at –40°C for 3 wk prior to analysis. All specimens were dissected and separated into respective tissues (muscle, liver, and other viscera) and stomach contents. Other viscera, containing abdominal lipids, and the stomach contents were separated from the stomach by scraping its inner wall.

Lipid extraction and the analysis of lipid classes. After the measurement of biological data, each tissue was minced and homogenized in a mixture of chloroform/methanol (2:1, vol/vol). A portion of the homogenized sample was extracted according to the procedure of Folch *et al.* (30). The crude total lipids (TL) were separated into classes on silicic acid columns, and the constituent lipids were quantified by gravimetric analysis of column chromatographic fractions. The first eluate (dichloromethane and *n*-hexane, 2:3, vol/vol) was used to collect the fraction containing wax esters and steryl esters. The second eluate (dichloromethane) from the column contained the TAG fraction. This was followed with dichloromethane/ether (9:1, vol/vol), eluting sterols; dichloromethane/methanol (9:1, vol/vol), eluting free fatty acids (FFA); dichloromethane/methanol (1:1, vol/vol), eluting phosphatidylethanolamine (PE), and dichloromethane/methanol (1:20, vol/vol), eluting phosphatidylcholine (PC). Individual lipids, separated from each lipid class, were identified by comparison with standard samples using thin-layer chromatography (TLC; thickness of 0.25 mm for analysis, Kieselgel 60, Merck and Co. Ltd., Darmstadt, Germany) and nuclear magnetic resonance (NMR). All sample lipids were dried under argon at room temperature and stored at –40°C.

Preparation of methyl esters and gas–liquid chromatographic analysis. Individual components of the TAG and phospholipid fractions were converted to fatty acid methyl esters by direct transesterification, refluxing each component with methanol containing 1% concentrated hydrochloric acid for 3 h (31). The methyl esters thus obtained were purified by column chromatography with silica gel by elution with dichloromethane.

The composition of the fatty acid methyl esters was determined by gas–liquid chromatography using a Shimadzu GC-8A gas chromatograph (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) equipped with two fused-silica capillary columns (TC-WAX, 30 m × 0.25 mm i.d.; 0.25 µm film, GL Science Co. Ltd., Tokyo, Japan; Omegawax-250, 30 m × 0.25 mm i.d.; 0.25 µm film, Supelco Japan Co. Ltd., Tokyo, Japan), and an HP 5890 series II gas chromatograph (Hewlett-Packard Co., Yokogawa Electric Corporation, Tokyo, Japan) equipped with an Omegawax-250 fused-silica capillary column. The injector port and column temperature were maintained at 250 and 200°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 area percentages of fatty acids from analyses performed on the capillary columns were provided by means of a Shimadzu Model C-R5A (Shimadzu Seisakusho Co. Ltd., Kyoto, Japan) electronic integrator.

Peak identification. Fatty acid methyl esters were identified using (i) marine lipid methyl esters as standards (Omegawax test mixture No. 4-8476, Supelco Japan Ltd.) and (ii) comparison of semilogarithmic plots of the relative retention time (RRT) against carbon chain lengths of known fatty acids from fish oil using the method of Ackman (32).

NMR spectrometry. Spectra were recorded on a GSX-270 NMR spectrometer (JEOL Co. Ltd., Tokyo, Japan) in the pulsed Fourier transform mode at 270 MHz in deuterochloroform solution using tetramethylsilane as an internal standard.

Statistical analyses. For all samples and treatments, more than three replications were made. The significant differences of the means were determined using Student's *t*-test at a significance level of $P < 0.05$.

RESULTS AND DISCUSSION

Lipid contents of the three subtropical species. The TL content of the three species, *C. diagramma*, *C. tile,* and *S. canaliculatus* are given in Table 2. The muscle lipids of these species contained low levels of lipid (Table 2) in comparison with those of other fish species (1–4). However, all the specimens examined accumulated most of their deposit lipids in the viscera, because the amount of visceral lipids were comparatively high (3.3–24.8%). These three species, in subtropical seawater, could hardly acquire these depot lipids directly from their prey, because the mean lipid levels of their stomach contents were 1.7% (1.7 ± 0.0%). This is low even when it is compared to that of their muscle. Very small amounts of partly digested zooplankton were found in the stomachs of the Caesioninae species, while only a moderate amount of partly digested seaweed was found in the stomachs of *S. canaliculatus*. The feeding behavior of *S. canaliculatus*, which is an herbivorous fish, differs greatly from those of the Caesioninae species, which are carnivorous fishes.

Lipid classes in the TL. The lipid classes in the tissues (muscle, liver, and other viscera) of *C. diagramma*, *C. tile*, and *S. canaliculatus* are shown in Table 2. In this study, the ratios of these components in the lipid classes in the respective individuals of the same species did not differ significantly ($P > 0.05$). The lipids of the tissues of these three species mainly contained glycerol derivatives similar to those of other epipelagic fish species. For example, the total amounts of TAG (a major neutral depot lipid) and phospholipids (major polar tissue lipids) in the muscle lipids were 67.8–83.5% of the TL. The high proportions of phospholipids (47.8–61.6%) in muscle lipids of the Caesioninae species differ from that of the Siganidae species. The mean numerical values of the phospholipids in the Caesioninae muscles were 47.8% for *C. diagramma* and 61.6% for *C. tile*, but that in *S. canaliculatus* was 7.8%. Therefore, only *S. canaliculatus*, among these three species, accumulated neutral deposit lipid in both muscle and viscera. The phospholipids in these fish species contained mainly PC and PE, which were identified by comparing them with authentic sample R_f values using TLC and NMR. FFA, which are presumably derived from the glycerides such as TAG and phospholipids through hydrolysis by enzymes during storage, were found in the lipids of all tissues—in particular, in the liver because these enzymes may be predominant and active there. Therefore, the total amount of glycerol derivatives such as TAG, FFA, and phospholipids may be more than 60% of the TL in all tissues of the three fish species. The lipids of these fishes contained only a small quantity of wax esters (0.6–5.4%).

Sterols (Table 2) were found at moderate levels (5.5–16.2%) in all samples. The purified sterols were immediately crystallized after isolation, and cholesterol was found to be predominant in the sterol fraction. It was identified with authentic cholesterol using TLC and NMR.

Fatty acid composition in the respective classes of each tissue. The fatty acid composition of the major components (TAG and phospholipids) separated from the lipids of the muscle, liver, and other viscera of the two Caesioninae species are shown in Tables 3 and 4. At least 50 compounds were detected and identified, of which more than 30 major compounds are shown. In TAG (the major neutral lipids), the fatty acid composition varied slightly between tissues, and the major components (>5%) of both species were 16:0 (23.7–30.4%), 18:0 (7.6–10.2%), 18:1n-9 (8.6–11.6%), and 22:6n-3 (DHA, 10.7–20.6%). Noticeable amounts (>2%) of other highly unsaturated fatty acids such as saturated fatty acid 14:0 (3.7–5.7%), monoenoic acids 16:1n-7 (2.3–4.2%),

TABLE 2
Lipid Contents[a] and Lipid Classes[a] of the Three Subtropical Fishes[b]

	Lipid contents[c]	WE[d]	TAG	Sterols	FFA	PE	PC
Caesio diagramma							
Muscle	0.8 ± 0.1	0.6 ± 0.0	35.7 ± 2.0	9.2 ± 1.3	6.5 ± 0.6	20.1 ± 0.6	27.7 ± 1.0
Liver	5.9 ± 0.5	3.0 ± 0.2	37.4 ± 2.4	11.0 ± 0.7	30.6 ± 2.4	13.9 ± 1.6	3.0 ± 0.4
Other viscera	24.8 ± 2.5	0.6 ± 0.1	84.1 ± 1.5	5.5 ± 0.5	7.2 ± 0.9	2.5 ± 0.2	0.1 ± 0.0
Stomach contents	1.7 ± 0.0						
C. tile							
Muscle	0.6 ± 0.0	1.0 ± 0.1	19.9 ± 3.2	11.0 ± 0.6	6.5 ± 0.3	28.6 ± 1.1	33.0 ± 2.1
Liver	3.9 ± 0.2	4.8 ± 0.4	20.2 ± 2.8	15.9 ± 1.0	29.9 ± 1.8	18.9 ± 1.2	10.3 ± 1.4
Other viscera	3.3 ± 0.1	5.4 ± 0.6	24.7 ± 1.8	16.2 ± 0.3	29.6 ± 1.0	14.2 ± 0.9	9.9 ± 1.5
Stomach contents	1.7 ± 0.1						
Siganus canaliculatus							
Muscle	0.7 ± 0.0	0.6 ± 0.0	60.0 ± 3.1	7.7 ± 0.2	22.1 ± 2.4	6.8 ± 0.9	1.0 ± 0.1
Liver	3.3 ± 0.1	3.7 ± 0.2	30.2 ± 2.1	12.6 ± 1.0	49.1 ± 2.8	3.4 ± 0.4	1.0 ± 0.1
Other viscera	5.1 ± 0.4	0.9 ± 0.1	68.6 ± 3.0	8.2 ± 0.6	18.2 ± 2.0	2.6 ± 0.5	0.4 ± 0.1
Stomach contents	1.7 ± 0.2	3.1 ± 0.5	45.5 ± 2.5	12.6 ± 0.3	34.1 ± 2.2	3.7 ± 0.2	0.5 ± 0.1

[a]Results are expressed as weight percentage of total lipids.
[b]Data are mean ± standard error ($n = 6$).
[c]Results are expressed as weight percentage of wet tissues.
[d]Both WE and SE were contained as major components in fraction 1. WE, wax esters; TAG, triaclglycerols; FFA, free fatty acids; PE, phosphatidylethanolamine; PC, phosphatidylcholine.

TABLE 3
Fatty Acid Composition of the Lipids in the Respective Organs of *Caesio diagramma*[a]

	Muscle				Liver				Other viscera[b]		
	TAG	FFA	PE	PC	TAG	FFA	PE	PC	TAG	FFA	PE
Total saturated	39.2	38.7	28.6	20.6	47.3	42.7	32.3	33.4	41.0	46.0	37.7
14:0	3.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	4.0 ± 0.1	2.3 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	4.7 ± 0.1	4.5 ± 0.8	2.9 ± 0.1
15:0	1.2 ± 0.0	0.3 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	1.4 ± 0.0	1.3 ± 0.0	0.5 ± 0.0	0.7 ± 0.1	1.4 ± 0.0	1.3 ± 0.1	0.8 ± 0.0
16:0	23.7 ± 0.2	11.4 ± 3.1	6.5 ± 0.3	15.9 ± 0.2	30.4 ± 0.7	26.7 ± 1.2	15.1 ± 0.3	19.3 ± 1.0	24.3 ± 0.2	26.8 ± 0.9	19.2 ± 0.5
17:0	1.6 ± 0.1	1.2 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	1.8 ± 0.0	1.6 ± 0.0	1.4 ± 0.1	1.3 ± 0.1	1.7 ± 0.0	1.6 ± 0.1	1.4 ± 0.0
18:0	7.7 ± 0.1	24.3 ± 3.1	20.2 ± 0.2	3.3 ± 0.2	8.3 ± 0.3	10.1 ± 0.3	13.9 ± 0.5	10.5 ± 0.5	7.6 ± 0.1	10.3 ± 0.6	12.2 ± 0.3
20:0	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.5 ± 0.0
22:0	0.4 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
24:0	0.3 ± 0.0	0.4 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
Total monoenoic	20.7	15.9	9.9	9.8	23.4	28.2	12.4	12.2	22.3	20.9	19.7
14:1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.9 ± 0.2
15:1	0.1 ± 0.0	0.9 ± 0.2	2.4 ± 0.4	2.2 ± 0.2	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.7 ± 0.1
16:1n-9	1.8 ± 0.4	0.2 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.7 ± 0.1	0.3 ± 0.0	0.5 ± 0.0	0.6 ± 0.1	0.3 ± 0.0	0.4 ± 0.0
16:1n-7	2.3 ± 0.3	1.9 ± 0.2	2.2 ± 0.3	0.5 ± 0.0	3.8 ± 0.2	5.0 ± 0.5	1.0 ± 0.1	2.4 ± 0.6	4.2 ± 0.2	4.1 ± 0.2	2.6 ± 0.2
16:1n-5	0.3 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
17:1	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
18:1n-9	10.1 ± 0.3	6.1 ± 0.5	2.8 ± 0.2	5.4 ± 0.2	11.6 ± 0.3	15.1 ± 1.0	6.6 ± 0.4	5.8 ± 0.1	11.0 ± 0.4	10.0 ± 0.3	8.8 ± 0.4
18:1n-7	2.4 ± 0.0	4.6 ± 0.4	1.2 ± 0.2	0.8 ± 0.0	3.1 ± 0.1	3.8 ± 0.1	2.4 ± 0.1	1.7 ± 0.1	2.4 ± 0.0	2.4 ± 0.1	2.9 ± 0.1
18:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:1n-11	0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1
20:1n-9	0.8 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	1.3 ± 0.1	1.1 ± 0.1	0.5 ± 0.0	0.4 ± 0.0	0.9 ± 0.0	0.6 ± 0.0	1.0 ± 0.1
20:1n-7	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:1n-11	0.5 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:1n-9	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
22:1n-7	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24:1n-9	0.8 ± 0.1	1.0 ± 0.2	0.2 ± 0.0	0.1 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.1	0.1 ± 0.0	0.6 ± 0.1	1.3 ± 0.0	1.1 ± 0.1
Total polyenoic	37.3	42.7	57.8	68.1	26.8	22.0	49.4	47.4	34.2	27.6	38.1
n-6 series	4.6	7.9	5.9	4.1	4.4	5.4	6.2	5.9	4.8	5.5	6.2
16:2n-6	1.1 ± 0.0	1.5 ± 0.3	1.1 ± 0.1	0.4 ± 0.0	1.2 ± 0.0	1.2 ± 0.1	0.7 ± 0.1	1.0 ± 0.1	1.2 ± 0.0	1.0 ± 0.1	0.9 ± 0.0
18:2n-6	1.0 ± 0.1	0.7 ± 0.1	0.6 ± 0.1	0.6 ± 0.0	1.0 ± 0.0	1.2 ± 0.1	1.1 ± 0.1	0.6 ± 0.0	1.2 ± 0.0	1.8 ± 0.1	1.5 ± 0.1
18:3n-6	0.6 ± 0.0	0.5 ± 0.1	0.9 ± 0.1	0.2 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.0	0.7 ± 0.0
20:3n-6	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.1
20:4n-6	1.5 ± 0.1	4.9 ± 0.9	3.2 ± 0.1	2.7 ± 0.1	1.2 ± 0.1	2.4 ± 0.1	3.6 ± 0.2	3.5 ± 0.4	1.3 ± 0.1	1.7 ± 0.2	2.8 ± 0.1
n-3 series	32.7	34.8	51.9	64.0	22.4	16.6	43.2	41.5	29.4	22.1	31.9
16:3n-3	0.3 ± 0.0	0.1 ± 0.0	5.5 ± 0.2	0.4 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	0.5 ± 0.1	1.5 ± 0.3
16:4n-3	0.1 ± 0.0	0.1 ± 0.0	0.8 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
18:3n-3	0.8 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.9 ± 0.0	1.3 ± 0.1	0.5 ± 0.0
18:4n-3	0.7 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.9 ± 0.0	0.9 ± 0.1	0.3 ± 0.0
20:3n-3	0.1 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0
20:4n-3	0.7 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	0.4 ± 0.1	0.5 ± 0.0
20:5n-3	4.3 ± 0.1	4.1 ± 0.8	1.9 ± 0.1	3.5 ± 0.1	2.8 ± 0.1	2.3 ± 0.1	3.8 ± 0.2	3.7 ± 0.3	4.1 ± 0.1	3.3 ± 0.3	2.7 ± 0.1
22:4n-3	1.8 ± 0.1	3.5 ± 0.6	4.7 ± 0.1	6.6 ± 0.1	1.3 ± 0.1	1.2 ± 0.1	2.9 ± 0.2	2.9 ± 0.2	1.6 ± 0.1	1.2 ± 0.1	3.1 ± 0.2
22:5n-3	2.1 ± 0.0	1.7 ± 0.2	1.3 ± 0.0	1.7 ± 0.0	1.8 ± 0.1	1.3 ± 0.1	1.3 ± 0.1	1.7 ± 0.1	2.2 ± 0.0	1.0 ± 0.1	1.2 ± 0.0
22:6n-3	20.6 ± 0.4	23.8 ± 3.1	35.7 ± 0.6	50.0 ± 0.6	12.6 ± 0.4	9.9 ± 1.3	32.4 ± 1.1	31.1 ± 1.8	17.2 ± 0.3	12.1 ± 1.1	20.8 ± 0.6
Unknown	1.2 ± 0.1	0.9 ± 0.1	1.4 ± 0.1	0.9 ± 0.0	1.5 ± 0.1	0.3 ± 0.1	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	1.1 ± 0.0

[a]Results are expressed as weight percentage of the total fatty acids. Data are mean ± standard error (n = 6–8).
[b]The levels of PC in the other viscera of *C. diagramma* were very low, and its fatty acid composition could not be analyzed. For abbreviations see Table 2.

18:1n-7 (2.3–3.0%), and 20:5n-3, eicosapentaenoic acid (EPA) (2.8–4.3%) were detected.

In polar lipids, the major components in both the PE and PC of the Caesioninae species, were 16:0 (4.4–19.2% for PE and 15.9–19.3% for PC), 18:0 (10.3–20.2% for PE and 3.3–10.5% for PC), 18:1n-9 (2.8–8.8% for PE and 4.7–8.4% for PC), and DHA (20.8–41.2% for PE and 27.5–50.0% for PC). Noticeable amounts of other highly unsaturated fatty acids such as 20:4n-6 (arachidonic acid, AA; 2.8–6.8% for PE and 2.7–6.2% for PC), EPA (1.9–4.0% for PE and 3.5–7.1% for PC), and 22:4n-3 (3.1–5.6% for PE and 2.9–6.6% for PC) were detected. Among all these lipids, saturated fatty acids 16:0 and 18:0, monoenoic acids 16:1 and 18:1, and n-3 PUFA EPA and DHA were generally the dominant fatty acids.

In general, the n-3 PUFA levels in muscle lipids (32.1–63.7% for *C. diagramma* and 24.8–63.6% for *C. tile*) were higher than those in visceral lipids (16.8–40.9% for *C. diagramma* and 19.6–49.8% for *C. tile*). Furthermore, high levels of n-3 PUFA (51.2–55.3% in PE of muscles and 63.6–63.7% in PC of muscles) in the polar lipids were detected, compared with those in the neutral lipids (24.8–32.1% in TAG of muscles and 34.8–45.0% in FFA of muscles). Among the PUFA, DHA is predominant (Tables 3 and 4). Consequently, the n-3 PUFA levels in PE and PC of the muscles were the highest, and this confirms that n-3 PUFA is an

TABLE 4
Fatty Acid Composition of the Lipids in the Respective Organs of *Caesio tile*[a]

	Muscle				Liver				Other viscera			
	TAG	FFA	PE	PC	TAG	FFA	PE	PC	TAG	FFA	PE	PC
Total saturated	43.5	35.5	26.0	20.7	47.9	49.4	32.6	29.8	45.5	40.4	33.0	31.3
14:0	4.9 ± 0.1	0.6 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	4.2 ± 0.2	2.6 ± 0.2	0.7 ± 0.1	0.4 ± 0.1	5.7 ± 0.2	5.4 ± 0.4	0.8 ± 0.1	0.6 ± 0.0
15:0	1.4 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	1.7 ± 0.0	1.4 ± 0.0	0.4 ± 0.0	0.7 ± 0.1	1.7 ± 0.0	1.3 ± 0.1	0.4 ± 0.0	0.6 ± 0.0
16:0	24.1 ± 0.3	15.2 ± 2.0	4.4 ± 0.1	15.8 ± 0.4	28.1 ± 0.7	27.6 ± 0.9	13.2 ± 0.4	16.4 ± 0.7	24.8 ± 0.3	23.7 ± 0.7	12.5 ± 0.9	19.1 ± 0.6
17:0	1.9 ± 0.1	1.2 ± 0.0	0.8 ± 0.1	0.5 ± 0.0	2.1 ± 0.1	2.1 ± 0.2	1.7 ± 0.0	1.3 ± 0.0	2.1 ± 0.0	1.6 ± 0.0	1.7 ± 0.1	1.3 ± 0.0
18:0	9.2 ± 0.3	17.1 ± 1.3	20.2 ± 0.5	3.7 ± 0.1	10.2 ± 0.3	14.5 ± 0.8	16.2 ± 0.6	10.6 ± 0.7	9.2 ± 0.2	7.2 ± 0.2	16.8 ± 0.8	9.0 ± 0.4
20:0	1.1 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.8 ± 0.1	0.6 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	1.0 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.3 ± 0.0
22:0	0.6 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.6 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
24:0	0.3 ± 0.0	0.4 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Total monoenoic	22.0	11.1	9.9	9.2	0.5	17.1	9.5	8.3	18.7	17.6	11.6	14.6
14:1	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.6 ± 0.1	0.1 ± 0.1	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.1 ± 0.0
15:1	0.2 ± 0.0	0.2 ± 0.1	4.1 ± 0.2	2.0 ± 0.1	0.5 ± 0.1	0.1 ± 0.0	0.7 ± 0.1	0.4 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	1.0 ± 0.2	0.9 ± 0.1
16:1n-9	1.0 ± 0.1	0.2 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	1.8 ± 0.3	1.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.4 ± 0.1	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.0
16:1n-7	3.3 ± 0.1	1.2 ± 0.1	0.3 ± 0.0	0.4 ± 0.0	2.8 ± 0.2	2.2 ± 0.2	0.9 ± 0.1	0.4 ± 0.0	3.4 ± 0.1	3.7 ± 0.2	0.8 ± 0.1	0.9 ± 0.0
16:1n-5	0.2 ± 0.0	0.1 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
17:1	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0
18:1n-9	10.7 ± 0.4	5.0 ± 0.2	3.1 ± 0.0	4.9 ± 0.1	8.7 ± 0.4	7.5 ± 0.4	4.0 ± 0.3	4.7 ± 0.2	8.6 ± 0.2	8.7 ± 0.2	5.2 ± 0.4	8.4 ± 0.3
18:1n-7	2.3 ± 0.1	2.4 ± 0.2	1.2 ± 0.1	1.0 ± 0.0	2.6 ± 0.2	2.8 ± 0.1	2.3 ± 0.1	1.2 ± 0.0	2.5 ± 0.0	2.2 ± 0.0	2.1 ± 0.1	2.4 ± 0.1
18:1n-5	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:1n-11	0.4 ± 0.1	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:1n-9	1.2 ± 0.0	0.4 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.8 ± 0.0	0.7 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.8 ± 0.0	0.5 ± 0.0	0.5 ± 0.1	0.4 ± 0.0
20:1n-7	0.3 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:1n-11	0.6 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.1	0.0 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:1n-9	0.4 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0
22:1n-7	0.2 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-9	0.6 ± 0.1	1.1 ± 0.1	0.1 ± 0.0	0.2 ± 0.1	0.2 ± 0.1	0.7 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	0.6 ± 0.1	0.6 ± 0.1	0.1 ± 0.0
Total polyenoic	31.1	52.0	61.6	68.5	26.3	30.0	55.5	59.2	32.8	38.9	52.7	52.3
n-6 series	5.6	6.5	5.8	4.4	6.0	8.1	7.3	8.2	5.1	6.3	10.1	9.2
16:2n-6	1.5 ± 0.1	0.5 ± 0.0	0.8 ± 0.0	0.4 ± 0.0	1.4 ± 0.0	1.5 ± 0.2	0.7 ± 0.1	0.8 ± 0.0	1.4 ± 0.0	1.1 ± 0.0	1.3 ± 0.0	1.1 ± 0.0
18:2n-6	1.1 ± 0.0	1.6 ± 0.1	0.6 ± 0.0	0.7 ± 0.0	1.2 ± 0.0	1.3 ± 0.1	1.4 ± 0.1	0.8 ± 0.0	1.1 ± 0.0	2.0 ± 0.0	0.9 ± 0.0	1.1 ± 0.0
18:3n-6	1.2 ± 0.1	0.8 ± 0.1	1.0 ± 0.0	0.2 ± 0.0	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.5 ± 0.0	0.7 ± 0.0	0.5 ± 0.0	0.9 ± 0.0	0.5 ± 0.0
20:3n-6	0.5 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
20:4n-6	1.3 ± 0.0	3.4 ± 0.2	3.3 ± 0.1	2.9 ± 0.2	2.5 ± 0.2	4.3 ± 0.4	4.5 ± 0.1	5.9 ± 0.2	1.4 ± 0.1	2.4 ± 0.2	6.8 ± 0.1	6.2 ± 0.2
n-3 series	25.5	45.5	55.8	64.1	20.3	21.9	48.2	51.0	27.7	32.6	42.6	43.1
16:3n-3	0.3 ± 0.0	0.5 ± 0.1	3.4 ± 0.1	0.2 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	1.0 ± 0.1	0.4 ± 0.1	0.3 ± 0.0	0.3 ± 0.0	1.7 ± 0.3	0.4 ± 0.0
16:4n-3	0.2 ± 0.0	0.0 ± 0.0	0.3 ± 0.0	0.0 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
18:3n-3	0.7 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.6 ± 0.1	0.5 ± 0.1	0.5 ± 0.0	0.3 ± 0.0	0.9 ± 0.1	1.8 ± 0.1	0.3 ± 0.0	0.4 ± 0.0
18:4n-3	0.6 ± 0.0	0.2 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.7 ± 0.0	1.4 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:3n-3	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.5 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
20:4n-3	0.6 ± 0.0	0.5 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.6 ± 0.1	0.6 ± 0.1	0.7 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	0.3 ± 0.0	0.4 ± 0.0
20:5n-3	3.2 ± 0.2	3.0 ± 0.3	2.2 ± 0.1	3.8 ± 0.1	3.3 ± 0.0	3.2 ± 0.4	3.2 ± 0.1	7.1 ± 0.3	3.7 ± 0.1	6.6 ± 0.4	4.0 ± 0.2	6.5 ± 0.1
22:4n-3	1.4 ± 0.0	3.5 ± 0.2	5.6 ± 0.2	6.3 ± 0.1	1.1 ± 0.1	1.6 ± 0.1	3.1 ± 0.1	3.5 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	4.5 ± 0.1	4.4 ± 0.0
22:5n-3	1.7 ± 0.1	1.3 ± 0.1	1.5 ± 0.0	1.8 ± 0.0	1.6 ± 0.1	1.2 ± 0.1	2.0 ± 0.1	2.1 ± 0.1	1.4 ± 0.0	1.3 ± 0.0	1.7 ± 0.1	1.7 ± 0.1
22:6n-3	15.5 ± 0.6	34.9 ± 1.1	41.2 ± 0.3	50.1 ± 0.7	10.7 ± 0.2	12.1 ± 0.9	34.3 ± 0.9	34.8 ± 0.8	16.8 ± 0.5	17.5 ± 1.1	28.0 ± 0.9	27.5 ± 0.8
Unknown	1.2 ± 0.1	0.8 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.1 ± 0.2	1.7 ± 0.3	2.7 ± 0.3	1.9 ± 0.2	1.4 ± 0.1	1.0 ± 0.0	1.8 ± 0.2	1.5 ± 0.1

[a]Results are expressed as weight percentage of the total fatty acids. Data are mean ± standard error (n = 6–8). For abbreviations see Table 2.

important muscle membrane component. The fatty acid composition of all tissues of the two species were similar, having high DHA contents, especially with the DHA content of the PC of the muscle exceeding 50% (50.0% for *C. diagramma* and 50.1% for *C. tile*) of the TFA. Although various saturated, monoenoic, and polyunsaturated fatty acids were observed in the Caesioninae species (similar to those of other marine fish species), the high levels of n-3 PUFA in the fatty acid composition of tissue lipids of the Caesioninae were significantly different from those of the other tropical fish species ($P <$ 0.05) (26–29). Even the mean DHA content levels (15.5–20.6%) of the TAG reached more than 10% of the TFA, while those in the TL of tropical and subtropical fish are reported to be only several percentage. For example, the mean DHA content of TFA of 15 species of fishes caught in tropical waters is about 4% of TFA, and in some cases the DHA content is less than 2% of the TFA, whereas for those caught in temperate and subarctic zones the contents vary between 5 and 20% (2,26,27).

The fatty acid composition of the same major lipid classes of the *S. canaliculatus* are shown in Table 5. In neutral lipids, the major components in the TAG of *S. canaliculatus* were 14:0 (3.3–6.3%), 16:0 (30.1–33.6%), 18:0 (5.1–8.4%), 16:1n-7 (4.5–7.9%), 18:1n-9 (9.0–9.7%), and DHA (8.5–14.1%)—similar to those in the Caesioninae species. Noticeable amounts of other fatty acids, such as 18:1n-7

TABLE 5
The Fatty Acid Composition of the Lipids in the Respective Organs of *Siganus canaliculatus*[a]

	Muscle				Liver			Other viscera[b]		
	TAG	FFA	PE	PC	TAG	FFA	PE	TAG	FFA	PE
Total saturated	43.7	32.5	28.5	28.0	47.6	33.8	34.4	46.0	37.3	35.6
14:0	5.9 ± 0.2	2.1 ± 0.2	1.6 ± 0.3	0.4 ± 0.0	3.3 ± 0.3	1.1 ± 0.1	1.2 ± 0.2	6.3 ± 0.1	3.3 ± 0.2	3.9 ± 0.2
15:0	1.1 ± 0.0	0.6 ± 0.0	0.4 ± 0.1	0.1 ± 0.0	0.9 ± 0.1	0.4 ± 0.0	0.6 ± 0.1	1.0 ± 0.0	0.7 ± 0.0	1.1 ± 0.1
16:0	30.1 ± 0.4	22.7 ± 0.9	14.8 ± 1.5	9.7 ± 0.4	33.7 ± 0.6	23.3 ± 1.5	14.7 ± 1.2	31.6 ± 0.4	22.6 ± 1.0	18.2 ± 0.7
17:0	1.1 ± 0.1	0.7 ± 0.0	0.7 ± 0.0	0.7 ± 0.0	0.9 ± 0.1	0.6 ± 0.0	0.9 ± 0.1	1.3 ± 0.1	1.0 ± 0.0	0.9 ± 0.1
18:0	5.1 ± 0.2	6.1 ± 0.1	10.7 ± 1.2	16.9 ± 0.8	8.4 ± 0.3	8.2 ± 1.0	16.7 ± 0.7	5.2 ± 0.2	9.4 ± 0.6	11.0 ± 0.3
20:0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
22:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
24:0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Total monoenoic	23.3	18.4	14.8	8.6	17.6	15.6	10.6	22.0	17.7	16.6
14:1	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.8 ± 0.1
15:1	0.1 ± 0.0	0.1 ± 0.0	2.2 ± 0.6	1.1 ± 0.2	0.1 ± 0.0	0.7 ± 0.3	1.2 ± 0.3	0.1 ± 0.0	0.1 ± 0.0	2.1 ± 0.3
16:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.1	0.5 ± 0.1
16:1n-7	7.9 ± 0.2	4.1 ± 0.3	2.5 ± 0.4	0.7 ± 0.0	4.5 ± 0.3	3.0 ± 0.3	2.1 ± 0.3	6.4 ± 0.5	4.8 ± 0.2	3.7 ± 0.1
16:1n-5	0.5 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.1	1.4 ± 0.3	0.6 ± 0.0	0.4 ± 0.0
17:1	0.5 ± 0.0	0.6 ± 0.1	0.4 ± 0.0	0.2 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.5 ± 0.0	0.6 ± 0.0
18:1n-9	9.7 ± 0.2	8.8 ± 0.2	6.1 ± 0.5	4.2 ± 0.2	8.5 ± 0.3	7.6 ± 0.6	3.9 ± 0.1	9.0 ± 0.3	7.6 ± 0.2	6.1 ± 0.1
18:1n-7	3.4 ± 0.2	3.3 ± 0.2	2.3 ± 0.1	1.6 ± 0.1	2.6 ± 0.1	2.1 ± 0.1	1.9 ± 0.1	3.3 ± 0.1	2.9 ± 0.1	2.5 ± 0.1
18:1n-5	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:1n-11	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1n-9	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0
20:1n-7	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-11	0.3 ± 0.0	0.4 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0
22:1n-9	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-7	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
24:1n-9	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.2 ± 0.1	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
Total polyenoic	29.0	46.4	54.7	61.4	31.7	48.2	51.2	28.7	42.1	40.3
n-6 series	6.8	15.0	13.6	13.0	8.9	12.1	18.4	7.8	15.6	14.1
16:2n-6	0.6 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	0.5 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	1.5 ± 0.2	0.6 ± 0.0	0.4 ± 0.0	1.0 ± 0.1
18:2n-6	2.1 ± 0.1	2.3 ± 0.1	1.5 ± 0.1	0.9 ± 0.0	1.3 ± 0.1	1.0 ± 0.1	1.3 ± 0.1	2.2 ± 0.1	2.4 ± 0.1	2.3 ± 0.1
18:3n-6	0.7 ± 0.1	0.6 ± 0.1	0.5 ± 0.0	0.8 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	0.6 ± 0.0	0.8 ± 0.1	0.6 ± 0.0	0.4 ± 0.0
20:3n-6	0.7 ± 0.0	1.1 ± 0.1	0.9 ± 0.0	0.5 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	1.0 ± 0.1	0.7 ± 0.0	1.1 ± 0.1	1.1 ± 0.0
20:4n-6	2.7 ± 0.2	10.6 ± 0.8	10.3 ± 0.8	10.3 ± 0.6	6.3 ± 0.6	9.9 ± 0.2	14.0 ± 0.7	3.5 ± 0.3	11.1 ± 0.8	9.3 ± 0.6
n-3 series	22.2	31.4	41.1	48.4	22.8	36.1	32.8	20.9	26.5	26.2
16:3n-3	0.1 ± 0.0	0.1 ± 0.0	1.8 ± 0.5	1.5 ± 0.3	0.1 ± 0.0	0.8 ± 0.3	0.7 ± 0.2	0.2 ± 0.0	0.2 ± 0.0	0.7 ± 0.2
16:4n-3	0.1 ± 0.0	0.3 ± 0.1	0.3 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.7 ± 0.2	0.3 ± 0.1	0.1 ± 0.0	0.4 ± 0.1
18:3n-3	1.4 ± 0.2	1.1 ± 0.1	0.6 ± 0.1	0.2 ± 0.0	0.7 ± 0.1	1.2 ± 0.3	0.3 ± 0.0	1.3 ± 0.1	1.1 ± 0.1	0.7 ± 0.0
18:4n-3	1.1 ± 0.2	0.8 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	0.5 ± 0.1	0.3 ± 0.0	0.1 ± 0.0	1.0 ± 0.1	0.8 ± 0.1	0.4 ± 0.0
20:3n-3	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4n-3	1.4 ± 0.1	1.3 ± 0.2	0.8 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	0.5 ± 0.0	0.6 ± 0.1	1.2 ± 0.1	1.1 ± 0.1	0.8 ± 0.1
20:5n-3	1.3 ± 0.1	2.6 ± 0.2	1.8 ± 0.1	1.4 ± 0.1	0.8 ± 0.0	1.2 ± 0.1	1.1 ± 0.2	1.6 ± 0.0	2.3 ± 0.2	1.3 ± 0.1
22:4n-3	1.1 ± 0.1	2.6 ± 0.2	3.3 ± 0.3	4.6 ± 0.3	1.9 ± 0.2	3.1 ± 0.2	2.7 ± 0.3	1.0 ± 0.0	2.1 ± 0.1	2.9 ± 0.2
22:5n-3	4.6 ± 0.3	5.7 ± 0.3	5.4 ± 0.1	5.2 ± 0.1	2.4 ± 0.1	3.8 ± 0.3	3.2 ± 0.3	4.0 ± 0.2	4.1 ± 0.3	2.4 ± 0.1
22:6n-3	9.1 ± 0.4	14.7 ± 0.6	24.8 ± 1.5	32.5 ± 0.6	14.1 ± 1.1	22.5 ± 0.8	20.9 ± 0.9	8.5 ± 0.4	12.3 ± 0.9	13.5 ± 0.7
Unknown	1.8 ± 0.1	2.0 ± 0.1	1.8 ± 0.1	1.9 ± 0.1	1.4 ± 0.1	2.4 ± 0.2	2.3 ± 0.2	1.6 ± 0.1	2.1 ± 0.2	2.8 ± 0.1

[a]Results are expressed as weight percentage of the total fatty acids. Data are mean ± standard error (*n* = 6–8).
[b]The levels of PC in the liver and other viscera of *S. canaliculatus* were very low, and its fatty acid composition could not be analyzed.

(2.6–3.4%), AA (2.7–6.3%), and 22:5n-3 (2.4–4.6%), were found in the TAG. In polar lipids, the major components, both in the PE and PC of this species, were 16:0 (14.8–23.3% for PE and 9.7% for PC), 18:0 (8.2–10.7% for PE and 16.9% for PC), 18:1n-9 (3.9–6.1% for PE and 4.2% for PC), AA (9.3–14.0% for PE and 10.3% for PC), and DHA (13.5–24.8% for PE and 32.5% for PC). Noticeable amounts of other fatty acids 16:1n-7 (2.5–4.8% for PE and 0.7% for PC) and 18:1n-7 (1.9–2.5% for PE and 1.6% for PC), 22:4n-3 (2.7–3.3% for PE and 4.6% for PC), and 22:5n-3 (2.4–5.4% for PE and 5.2% for PC) were found. In the lipids of *S. canaliculatus*, comparatively high levels of n-3 PUFA (40.8% for PE of muscle lipids and 48.1% for PC of muscle lipids) were also found in polar lipids of muscles, similar to those of the Caesioninae species.

In addition, high levels of n-6 PUFA were also present in the fatty acid composition of the *S. canaliculatus* lipid, and this high n-6 PUFA level differs from those of other marine fish species. In particular, the high levels of AA (9.3–14.0% in PE, and 10.3% in PC) in the fatty acid composition of the

TABLE 6
Fatty Acid Composition of the Lipids of the Stomach Contents of the Three Subtropical Fish Species[a]

	C. diagramma	*C. tile*	*S. canaliculatus*[b]		
	TL[b]	TL	TAG	FFA	PE
Total saturated	44.3	50.5	45.7	37.4	42.9
14:0	4.3 ± 0.2	3.2 ± 0.1	6.1 ± 0.1	3.7 ± 0.4	3.9 ± 0.3
15:0	1.5 ± 0.0	1.4 ± 0.0	1.1 ± 0.0	0.9 ± 0.1	2.5 ± 0.2
16:0	25.5 ± 0.2	29.4 ± 0.5	31.2 ± 0.4	21.5 ± 1.0	21.2 ± 1.0
17:0	1.1 ± 0.1	2.3 ± 0.0	1.2 ± 0.1	1.2 ± 0.0	1.4 ± 0.1
18:0	10.3 ± 0.3	12.7 ± 0.3	5.5 ± 0.2	9.7 ± 0.5	13.2 ± 0.9
20:0	0.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.3 ± 0.0
22:0	0.6 ± 0.0	0.6 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
24:0	0.4 ± 0.0	0.3 ± 0.0	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
Total monoenoic	20.0	19.9	22.3	16.2	19.5
14:1	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	2.4 ± 0.3
15:1n-6	0.4 ± 0.0	0.7 ± 0.1	0.1 ± 0.0	0.7 ± 0.2	1.2 ± 0.1
16:1n-9	0.4 ± 0.1	0.6 ± 0.1	1.4 ± 0.5	0.2 ± 0.0	0.1 ± 0.1
16:1n-7	3.3 ± 0.0	3.1 ± 0.1	6.2 ± 0.5	4.1 ± 0.4	4.2 ± 0.3
16:1n-5	0.2 ± 0.4	0.2 ± 0.0	0.5 ± 0.1	0.6 ± 0.0	0.4 ± 0.0
17:1	1.1 ± 0.2	0.5 ± 0.0	0.6 ± 0.0	0.7 ± 0.1	0.9 ± 0.0
18:1n-9	9.5 ± 0.0	9.5 ± 0.4	9.9 ± 0.3	6.2 ± 0.3	7.7 ± 0.3
18:1n-7	2.6 ± 0.0	2.9 ± 0.0	2.6 ± 0.2	2.7 ± 0.1	3.5 ± 0.2
18:1n-5	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.2 ± 0.0
20:1n-11	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.0
20:1n-9	0.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
20:1n-7	0.3 ± 0.0	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-11	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.5 ± 0.1	0.3 ± 0.0
22:1n-9	0.2 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
22:1n-7	0.1 ± 0.0	0.1 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.1 ± 0.0
24:1n-9	0.8 ± 0.0	0.8 ± 0.1	0.0 ± 0.0	0.0 ± 0.0	0.3 ± 0.0
Total polyenoic	24.4	25.1	27.8	41.4	25.7
n-6 series	0.8	5.8	7.0	16.4	10.6
16:2n-6	1.3 ± 0.0	1.0 ± 0.1	0.6 ± 0.0	0.6 ± 0.1	1.1 ± 0.1
18:2n-6	2.0 ± 0.2	1.4 ± 0.0	2.2 ± 0.1	2.4 ± 0.1	2.6 ± 0.1
18:3n-6	0.6 ± 0.0	0.7 ± 0.0	0.6 ± 0.1	0.6 ± 0.0	0.6 ± 0.1
20:3n-6	0.4 ± 0.0	0.4 ± 0.0	0.6 ± 0.0	1.1 ± 0.1	1.2 ± 0.1
20:4n-6	2.2 ± 0.1	2.3 ± 0.1	3.0 ± 0.2	11.7 ± 1.3	5.1 ± 0.5
n-3 series	23.6	19.3	20.8	25.0	15.1
16:3n-3	0.7 ± 0.1	0.8 ± 0.1	0.1 ± 0.0	0.4 ± 0.1	0.2 ± 0.0
16:4n-3	0.1 ± 0.0	0.2 ± 0.1	0.3 ± 0.1	0.1 ± 0.0	0.1 ± 0.0
18:3n-3	1.6 ± 0.2	0.9 ± 0.1	1.3 ± 0.1	1.0 ± 0.1	0.8 ± 0.1
18:4n-3	1.1 ± 0.1	0.6 ± 0.1	0.9 ± 0.1	0.8 ± 0.1	0.4 ± 0.1
20:3n-3	0.0 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.3 ± 0.0	0.3 ± 0.0
20:4n-3	0.3 ± 0.0	0.3 ± 0.0	1.2 ± 0.0	1.0 ± 0.0	0.7 ± 0.1
20:5n-3	4.5 ± 0.3	4.1 ± 0.2	1.4 ± 0.1	2.0 ± 0.1	0.8 ± 0.1
22:4n-3	1.6 ± 0.1	1.4 ± 0.1	1.0 ± 0.0	2.5 ± 0.2	2.2 ± 0.2
22:5n-3	0.8 ± 0.0	0.5 ± 0.0	3.7 ± 0.2	3.5 ± 0.2	1.5 ± 0.3
22:6n-3	12.4 ± 0.6	9.9 ± 0.2	9.3 ± 0.4	11.2 ± 0.4	5.5 ± 0.2
Unknown	1.3 ± 0.1	1.5 ± 0.2	1.5 ± 0.1	2.7 ± 0.1	2.9 ± 0.3

[a]Results are expressed as weight percentage of the total fatty acids. Data are mean ± standard error (n = 6–8).
[b]The levels of PC in the stomach contents of *S. canaliculatus* were very low, and its fatty acid composition could not be analyzed. TL, total lipids; for other abbreviations see Table 2.

phospholipids in the tissues of *S. canaliculatus* were markedly different from those of the other fish species (Table 5), which contain very low levels of n-6 PUFA in their lipids (1–7).

Fatty acid composition of stomach contents. The fatty acid composition of the stomach contents of the three subtropical species is shown in Table 6. The lipid levels of stomach contents from the two Caesioninae species were very low, and their respective classes could not be analyzed. Only the fatty acid composition of total lipids could be analyzed. In the stomach contents of the two Caesioninae species examined, the fatty acid compositions of these species were very similar, and the major fatty acids were 16:0 (25.5–29.4%), 18:0 (10.3–12.7%), 18:1n-9 (9.5%) and DHA (9.9–12.4%). In most cases, 16:0 was the dominant fatty acid. The lipids in the stomach contents of both Caesioninae species contained comparatively low levels of n-3 PUFA (18.2–22.8%), similar to the levels of the TAG of the tissues of the species. This sug-

gests that the fatty acid composition of the deposit lipids was directly influenced by those of stomach content lipids.

On the other hand, the stomach content lipids of *S. canaliculatus* mainly contained 14:0 (3.7–6.1%), 16:0 (21.2–31.2%), 18:0 (5.5–13.2%), 16:1n-7 (4.1–6.2%), 18:1n-9 (6.2–9.9%), and DHA (5.5–11.2%); in addition, noticeable amounts of 18:1n-7 (2.6–3.5%), 18:2 n-6 (2.2–2.6%), AA (3.0–11.7%), 22:4n-3 (1.0–2.2%), and 22:5n-3 (1.5–3.7%) were measured. The n-3 PUFA level in its prey lipids was comparatively low, similar to those of the prey of Caesioninae. The comparatively low PUFA levels in the lipids of their prey were considered to be reasonable because these organisms, which live in warm seawater, do not require the high levels of n-3 PUFA for their cell membranes (19–25). In addition, in the stomach contents lipids of *S. canaliculatus*, noticeable levels of AA were found, similar to that in its tissue lipids.

Food chain of the subtropical fish species and accumulations of PUFA in their tissue lipids. It is generally known that n-3 PUFA are required by all marine fishes including subtropical fish species (12–18). In particular, DHA is an essential fatty acid and is important for growth and survival—for example, for the development of juvenile striped jack (*Pseudocaranx dentex*) and red sea bream (*Pagrus major*) (13,33). Watanabe *et al.* (33) and Kaneko *et al.* (36) reported that when fishes are starved they selectively use saturated and monoenoic fatty acids as an energy source and use very little n-3 PUFA, which are conservatively stored in the tissues of marine fish. Moreover, it has recently been found that marine fishes are largely unable to synthesize DHA because they may lack the necessary Δ4- and Δ5-desaturases, and therefore the DHA in the bodies of these fishes must originate from dietary lipid (12–18). The DHA content of total lipid of marine fish may gradually increase during predatory feeding, and high DHA contents in the lipid of highly migratory fish species, which are the final predators, have often been observed. This may have resulted from the continuous accumulation of DHA which originates from their prey in the marine food chain (8–11,35,36).

DHA may be also necessary for polar lipids in the three subtropical fishes that we examined, resembling the compositions found in other marine fishes. The fatty acid compositions of the Caesioninae and Siganidae tissue lipids were nearly constant within each species ($P > 0.05$). In particular, the polar lipids in their muscles contained very high levels of n-3 PUFA. These fatty acid compositions were different from those of their prey, which is consistent with the low levels of n-3 PUFA generally found in the lipids of other small fish species in the subtropical and tropical zones. This suggests that the three species might not simply incorporate these fatty acids from their prey in the same ratios, and the comparatively high DHA levels in their tissue lipids must be due to selective accumulation. This is because the high DHA levels were found only in their polar lipids while the visceral TAG were very similar to the total lipids of the stomach contents. Furthermore, the three fish species examined consumed very little food, and conditions were similar to fasting. They may also utilize mainly saturated and monoenoic acids for energy and store n-3 PUFA, similar to migratory fish species. Even in species living in the coral seas in the subtropical zone, DHA levels in their tissue lipids were higher than those of their prey. Although n-3 PUFA levels in the lipids of tropical and subtropical fish species are generally low, all marine fish species may have a tendency to accumulate n-3 PUFA, and this tendency may be a general characteristic not only of the highly migratory fishes but also of other small predacious and active marine fish species.

Accumulation of AA by characteristic feeding of S. canaliculatus. With respect to the accumulation of PUFA, freshwater fish generally consume terrestrial prey with lipids rich in n-6 PUFA and consequently have high levels of n-6 PUFA in their lipids (4,37,38). In contrast, marine fish species generally have only n-3 PUFA which originate from marine phytoplankton. Therefore, AA levels were generally undetectable or negligible in the TFA of lipid of marine fish species. However, there are several marine species whose lipids contain comparatively high levels of n-6 PUFA. For example, some kinds of seaweed contain n-6 PUFA and some marine animals, such as abalone, which may consume seaweeds, have very high levels of n-6 PUFA (39–43).

The mean levels of AA in tissue phospholipids of the muscle and viscera of *S. canaliculatus* consistently reached 10% or more of the TFA (Table 5). Therefore, this species may have a vegetarian feeding habit similar to that of abalone, and its lipids consequently differ from those of carnivorous marine fish species. The noticeable levels of AA (3.0–11.7%) in the stomach content lipids of *S. canaliculatus* would likely result in the high AA levels in its tissues. High levels of AA (more than 10% of the TFA) in the tissues of *S. canaliculatus* were accompanied by high levels of n-3 PUFA, while those in deposit lipids and stomach contents lipids were lower than in the polar tissue lipids. We suggest that *S. canaliculatus* prefer and consume seaweed with lipids containing noticeable levels of AA and may therefore accumulate AA in its tissue lipids throughout its life feeding on the marine food chain in the coral seas.

As for *S. canaliculatus*, this phenomenon of high tissue contents of both n-3 and n-6 PUFA were similar to the high n-3 PUFA levels in Caesioninae tissues. Therefore, the tropical species of marine fish may have a tendency to accumulate both n-3 and n-6 PUFA in their tissues if their diet contains substantial amounts of these fatty acids. Therefore, high levels of AA may be useful as a lipid biomarker of herbivorous fishes, which prefer seaweed (44), because most marine fish do not have very high levels of AA.

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Arachidonic, Eicosapentaenoic, and Biosynthetically Related Fatty Acids in the Seed Lipids from a Primitive Gymnosperm, *Agathis robusta*

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ABSTRACT: The fatty acid composition of the seeds from *Agathis robusta*, an Australian gymnosperm (Araucariaceae), was determined by a combination of chromatographic and spectrometric techniques. These enabled the identification of small amounts of arachidonic (5,8,11,14-20:4) and eicosapentaenoic (5,8,11,14,17-20:5) acids for the first time in the seed oil of a higher plant. They were apparently derived from γ-linolenic (6,9,12-18:3) and stearidonic (6,9,12,15-18:4) acids, which were also present, *via* chain elongation and desaturation, together with other expected biosynthetic intermediates [bishomo-γ-linolenic (8,11,14-20:3) and bishomo-stearidonic (8,11,14,17-20:4) acids]. Also present were a number of C_{20} fatty acids, known to occur in most gymnosperm families, i.e., 5,11-20:2, 11,14-20:2 (bishomo-linoleic), 5,11,14-20:3 (sciadonic), 11,14,17-20:3 (bishomo-α-linolenic), and 5,11,14,17-20:4 (juniperonic) acids. In contrast to most other gymnosperm seed lipids analyzed so far, *A. robusta* seed lipids did not contain C_{18} Δ5-desaturated acids [i.e., 5,9-18:2 (taxoleic), 5,9,12-18:3 (pinolenic), or 5,9,12,15-18:4 (coniferonic)]. These structures support the simultaneous existence of Δ6- and Δ5-desaturase activities in *A. robusta* seeds. The Δ6-ethylenic bond is apparently introduced into C_{18} polyunsaturated acids, whereas the Δ5-ethylenic bond is introduced into C_{20} polyunsaturated acids. A general metabolic pathway for the biosynthesis of unsaturated fatty acids in gymnosperm seeds is proposed. When compared to Bryophytes, Pteridophytes (known to contain arachidonic and eicosapentaenoic acids), and species from other gymnosperm families (without such acids), *A. robusta* appears as an "intermediate," with the C_{18} Δ6-desaturase/$C_{18} \rightarrow C_{20}$ elongase/C_{20} Δ5-desaturase system in common with the former subphyla, and the unsaturated $C_{18} \rightarrow C_{20}$ elongase/C_{20} Δ5-desaturase system specific to gymnosperms. The following hypothetical evolutionary sequence for the C_{18} Δ6/Δ5-desaturase class in gymnosperm seeds is suggested: Δ6 (initial) → Δ6/Δ5 (intermediate) → Δ5 (final).

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Abbreviations: DMOX, dimethyloxazoline, ECL, equivalent chain length; FAME, fatty acid methyl ester; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NMR, nuclear magnetic resonance; TAG, triacylglycerol; TLC, thin-layer chromatography; UPIFA, unsaturated polymethylene-interrupted fatty acid.

In the course of systematic studies on gymnosperm seed fatty acid compositions, *ca.* 170 species have been analyzed in our Institutes, which represent approximately one-fourth of all extant species from this plant group. The species analyzed belong to the families Taxaceae, Cephalotaxaceae, Podocarpaceae, Pinaceae, Taxodiaceae, Cupressaceae, Sciadopityaceae, Ginkgoaceae, Cycadaceae, and Ephedraceae (1–18). Although the peculiar Δ5-unsaturated polymethylene-interrupted fatty acids (Δ5-UPIFA) found in gymnosperms were structurally characterized almost 40 yr ago (19–22), and have been repeatedly reported to occur in a few species since then, their ubiquity in the seeds of practically all gymnosperm species was not recognized until recently (23,24). However, exceptions are known [Gnetaceae (25) and Welwitschiaceae (26)].

Δ5-UPIFA may belong to the n-9, n-7, n-6, or n-3 metabolic series, being derived from oleic, palmitoleic (*via cis*-vaccenic), linoleic, or α-linolenic acids. They have the structures of 5,9-18:2 (taxoleic), 5,11-18:2 (ephedrenic), 5,9,12-18:3 (pinolenic), 5,9,12,15-18:4 (coniferonic), 5,11-20:2, 5,11,14-20:3 (sciadonic), and 5,11,14,17-20:4 (juniperonic) acids (for the suggested trivial names, first occurrences are to be found in Refs. 4,18,27,28). Based on the preceding structures and on those of putative intermediary metabolites also characterized in gymnosperm seeds, a complete sequence of reactions for the biosynthesis of Δ5-UPIFA in gymnosperm seeds has been proposed. However, there are some uncertainties regarding the number and the specificity of the Δ5-desaturase(s) (1,2,18). Owing to the great antiquity, on a geological time scale, of most gymnosperm families, and to the ill-defined and tenuous inter-family evolutionary links, it was necessary to repeatedly confirm the occurrence of Δ5-UPIFA in these families, and this was done for most of them by mass spectrometry (MS) coupled to gas–liquid chromatography (GLC) (12,18,29) and by ^{13}C nuclear magnetic resonance (NMR) spectrometry (13,14,18,30,31).

In addition to their peculiar structures, Δ5-UPIFA also show a strong tendency to be almost exclusively esterified to the α positions of triacylglycerols (TAG), likely the *sn*-3 position, which leads to high proportions of TAG with only one Δ5-UPIFA per molecule (13,14,18,30–35). Besides these aspects relevant to chemistry and biochemistry, Δ5-UPIFA in combination with other common fatty acids have been used as interesting chemometric markers for the taxonomy of conifers (3–6).

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Also, there has been increasing interest in the biochemical, physiological, and nutritional properties of Δ5-UPIFA in rodents, particularly of pinolenic, sciadonic, and juniperonic acids (36–51). With regard to the latter two acids, their structures closely resemble those of arachidonic (5,8,11,14-20:4) and eicosapentaenoic (5,8,11,14,17-20:5) acids, with only the Δ8-ethylenic bond lacking.

Thus, we had many reasons to continue our systematic study of gymnosperm seed fatty acids. However, it should be emphasized that Δ5-UPIFA are not exclusively found in gymnosperms, though they are characteristic of plants of that subphylum, as they also occur in a few angiosperm species (52–54). We report here for the first time on the seed fatty acids prepared from a species of the Araucariaceae family (which was apparently not explored until this study), *Agathis robusta*. Araucariaceae encompass two principal genera, *Araucaria* and *Agathis*, totaling 32 to 40 species (and the newly discovered monotypic genus *Wollemia*) of tropical-subtropical Old World distribution (absent from Africa) (55,56). This family is considered especially ancient, and for some authors (57), "the most primitive of living conifers," with "the longest fossil history of any of the Coniferales, overlapping that of the Cordaitales," *ca.* 250–300 million years ago. *Agathis robusta* is a tall tree (50 m) native to the coastal zone of South Queensland in Australia, where it is known as "Southern Queensland Kauri." This nonthreatened species is sometimes planted elsewhere as an ornamental tree, e.g., in France, in the most temperate zone of the Côte d'Azur (Riviera) (58).

MATERIALS AND METHODS

Seeds, oil extraction, and fatty acid methyl ester (FAME) preparation. *Agathis robusta* seeds were purchased from Sandeman Seeds (Pulborough, England). The extraction of lipids from dehulled seeds and the preparation of FAME were performed as described in detail elsewhere for other gymnosperm seeds (2–4). For quantitative purposes, FAME preparations were made in duplicate and analyzed twice on a DB-Wax column and once on a CP-Sil 88 column.

Analytical GLC. FAME were analyzed with a Carlo Erba 4130 chromatograph (Carlo Erba, Milano, Italy) equipped with a DB-Wax column (30 m × 0.32 mm i.d., 0.5 µm film; J&W Scientific, Folsom, CA). The oven temperature was 190°C and the inlet pressure of the carrier gas (helium) was 140 kPa. Alternately, a CP-Sil 88 column (50 m × 0.25 mm i.d., 0.2 µm film; Chrom-pack) was operated with temperature programming in a Carlo Erba HRGC chromatograph from 150 to 185°C at 4°C/min with H_2 at 100 kPa. The injector (split mode) and the flame-ionization detector were maintained at 250°C for both columns. Quantitative data were calculated with a SP 4290 integrator (Spectra Physics, San Jose, CA).

Tentative identification of FAME peaks by their equivalent chain lengths (ECL). The seed lipids from selected conifer species (29) and from meadowfoam were used as a source of Δ5-olefinic acid methyl esters with known structures to identify fatty acids from *A. robusta* seed lipids by GLC, either by coinjection or by comparison of their ECL (DB-Wax column) or retention times (CP-Sil 88). Borage oil was the source of γ-linolenic acid. Liver lipids of adult rats fed a standard chow diet or a fat-free diet for 10 wk from the time they were weaned were used as a source of Mead's, bishomo-γ-linolenic, arachidonic, and eicosapentaenoic acids.

Preparation of picolinyl esters. Picolinyl esters were prepared from the free acids (2-mg scale) *via* imidazolide intermediates by the method of Balazy and Nies (59). Reagents were dried over anhydrous sodium sulfate or molecular sieve before use. Products were dissolved in isohexane containing butylated hydroxytoluene (50 ppm) for analysis by gas chromatography–MS.

Preparation of dimethyloxazoline (DMOX) derivatives. DMOX derivatives were prepared either by heating the oil directly with 2-amino-2-methyl-1-propanol according to Garrido and Medina (60) or *via* the free acids, acid chlorides, and cyclization with trifluoroacetic anhydride (61).

High-performance liquid chromatography (HPLC). In order to obtain better-quality mass spectra from minor components, the latter were concentrated in the form of either picolinyl esters or DMOX derivatives by reversed-phase HPLC (62). A Gynkotek model 480 HPLC pump was utilized with a column of Hichrom RPB™ (250 × 10 mm; Hichrom Ltd., Reading, United Kingdom) and acetonitrile as mobile phase, with the flow rate programmed from 1 to 3 mL/min over 60 min. The temperature of the column was maintained at 20 ± 0.1°C by means of a column oven/cooler (HPLC Technology model TC-955; Macclesfield, United Kingdom). The sample (1 mg) was injected in a solution (10 µL) of acetone/acetonitrile (1:9, vol/vol). An evaporative light-scattering detector (Varex model III, Alltech Ltd., Deerfield, IL) was used in test runs, but timed fractions were collected in micropreparative applications in the absence of a detector.

GLC–MS. The derivatives were subjected to GLC–MS with a Hewlett-Packard 5890 Series II plus gas chromatograph attached to an HP model 5989 MS engine (Hewlett-Packard Ltd., Stockport, United Kingdom). The latter was used in the electron impact mode at 70 eV with a source temperature of 250°C. The GLC was fitted with on-column injection. For picolinyl ester and DMOX derivatives, a capillary column of fused silica coated with Supelcowax 10™ (0.25 mm × 25 m, 0.25 µm film; Supelco UK, Poole, United Kingdom) was employed. After holding the temperature at 80°C for 3 min, the column was temperature-programmed at 20°C/min to 180°C, then at 2°C/min to 280°C, where it was held for 15 min. Helium was the carrier gas at a constant flow rate of 1 mL/min.

RESULTS AND DISCUSSION

Fatty acid characterization and composition. The first distinctive feature of *A. robusta* seed oil (46% on a weight basis of the dehulled seeds) is its solid consistency at ambient temperature. This is explained by the high proportion of saturated acids with long and very long hydrocarbon chains (16:0 to 24:0, mostly even-numbered: *ca.* 37% of total fatty acids) (Table 1). This is a

TABLE 1
Fatty Acid Composition of *Agathis robusta* Seed Lipids

Fatty acid	Peak[a]	ECL[b]	Weight %[c]
16:0	1	16.00	3.35
7-16:1	2	16.23	0.04
9-16:1	3	16.30	0.02
?-16:2[d]	4	16.75	0.01
17:0	5	17.01	0.04
18:0	6	18.00	9.33
9-18:1	7	18.23	12.28
11-18:1	8	18.31	0.11
5,9-18:2	—[e]	18.44	ND[e]
9,12-18:2	9	18.70	36.88
5,9,12-18:3	—	18.92	ND
19:0	10	19.02	0.02
6,9,12-18:3	A	19.05	0.44
9,12,15-18:3	11	19.36	10.97
5,9,12,15-18:4	—	19.58	ND
6,9,12,15-18:4	B	19.72 (19.71)	0.23
20:0	12	20.00	12.46
11-20:1	13	20.22	0.72
5,11-20:2	14	20.37	0.05
?-20:2	15	20.54	0.04
?-20:2	16	20.61	0.03
11,14-20:2	17	20.69	0.32
5,11,14-20:3	18	20.83 (20.84)	0.06
8,11,14-20:3	C	20.98	0.19
21:0	19	21.00	0.11
5,8,11,14-20:4	D	21.19	0.20
11,14,17-20:3	20	21.36	0.04
5,11,14,17-20:4	21	21.50 (21.50)	0.17
8,11,14,17-20:4	E	21.63 (21.65)	0.06
5,8,11,14,17-20:5	F	21.85 (21.84)	0.05
22:0	22	22.00	11.01
23:0	—	23.01	0.06
24:0	—	24.00	0.61

[a]Numbering and lettering refer to peaks in Figure 1 (except for 23:0 and 24:0 acids).
[b]Equivalent chain lengths (ECL) determined on the DB-Wax capillary column (J&W Scientific, Folsom, CA) at 190°C, except for 23:0 and 24:0 acids (210°C). Calculated values in parentheses (according to Ref. 64).
[c]Mean of analyzes of three fatty acid methyl ester preparations.
[d]A question mark means that the positions of double bonds are unknown.
[e]—, chromatographic position not indicated on chromatograms; ND, not detected.

unique case among the 170 gymnosperm seed oils we have examined so far, which are liquid, though sometimes highly viscous. Among the unsaturated fatty acids commonly encountered in plant seed oils, the most abundant were C_{18} acids, i.e., oleic acid (and its isomer, *cis*-vaccenic acid, totaling 12.4%), linoleic acid (36.9%), and α-linolenic acid (11.0%). Surprisingly, no peaks corresponding to C_{18} Δ5-UPIFA, i.e., 5,9-18:2, 5,11-18:2, 5,9,12-18:3, and 5,9,12,15-18:4 acids, could be observed, even in trace amounts, in contrast to several other gymnosperm species, particularly from the Pinaceae family. On the other hand, two significant peaks (A and B in Table 1 and Fig. 1) not previouly observed in gymnosperm seed fatty acids were present in the C_{18} acid chromatographic zone. Neither of these two components coeluted with any of the former C_{18} Δ5-UPIFA when coinjected. Among "habitual" unsaturated C_{20} acids, eicosenoic acids included the main gondoic (11-20:1) acid, with possibly the 9-20:1 isomer visible as a small shoulder at the base of the 11-20:1 peak leading edge. Two C_{20} polyunsaturated acids frequently present in gymnosperm seeds, the 11,14-20:2 (bishomolinoleic) and 11,14,17-20:3 (bishomo-α-linolenic) acids, were also present in minor amounts. All C_{20} Δ5-UPIFA characterized in other gymnosperm species were present (or more rigorously speaking, peaks of the same ECL were observed) in *A. robusta* seed fatty acids; however, 5,11-20:2, 5,11,14-20:3, and 5,11,14,17-20:4 acids were present in abnormally low amounts. Four other peaks, C to F in Figure 1, were more or less intermingled with the preceding C_{20} Δ5-UPIFA and were tentatively attributed to C_{20} polyunsaturated acids owing to their elution in the C_{20} chromatographic zone, with some uncertainty for peak C, which partially overlapped a possible 21:0 acid.

The extra peaks (A to F) were not artifacts, as they remained unchanged when FAME were prepared with sodium methoxide at ambient temperature, or prepared with TAG purified by thin-layer chromatography (TLC). Moreover, the chromatographic profiles did not change whether FAME were purified by TLC or not (results not shown). This excluded any contamination by unsaponifiable components. Because cyclopropenic acids were shown to occur in some other Australian gymnosperm species (63), we performed the Halphen test, which produced negative results.

Fractionation of FAME by argentation TLC (Ag-TLC), followed by GLC analysis of individual fractions, allowed confirmation of the structures indicated above. In particular, the tentatively identified odd-numbered fatty acids, 17:0, 19:0, 21:0 and 23:0, were found in the saturated acid fraction. No *anteiso*-17:0 acid, such as was found in Pinaceae seed lipids (8), could be detected in that fraction. In addition to 9,12-18:2 acid, the dienoic acid fraction contained 5,11-20:2 and 11,14-20:2 acids, two other unknown minor eicosadienoic acids, and possibly a dienoic C_{16} acid. Components A to F had the following behavior: Peaks A and C coeluted with α-linolenic acid following Ag-TLC and had the same retention times as 6,9,12-18:3 (γ-linolenic; component A) and 8,11,14-20:3 (bishomo-γ-linolenic; component C) acids on two capillary columns, under isothermal or temperature-programming conditions. The 5,11,14-20:3 and 11,14,17-20:3 acids migrated along with the former trienoic acids. Peaks B, D, E, and F (not visible on the Ag-TLC plate, owing to their low concentration and the absence of a carrier component), not present in the 0, 1, 2, or 3Δ fractions, had probably migrated behind these fractions and were believed to contain at least four double bonds. This supposition was confirmed by comparison of the retention times of peaks D and F, which were identical to those of 5,8,11,14-20:4 and 5,8,11,14,14,17-20:5 acids, respectively, which were present in FAME prepared from rat liver lipids, here too on two columns with different polarities.

Calculation of the theoretical ECL for peaks B and E (no standards available), probably containing four ethylenic bonds, is based on the summation of ECL of related fatty acids with one less double bond, either on the methyl or on the carboxylic side, followed by subtraction of the ECL of the fatty acid having double bonds common to the fatty acids used in summation

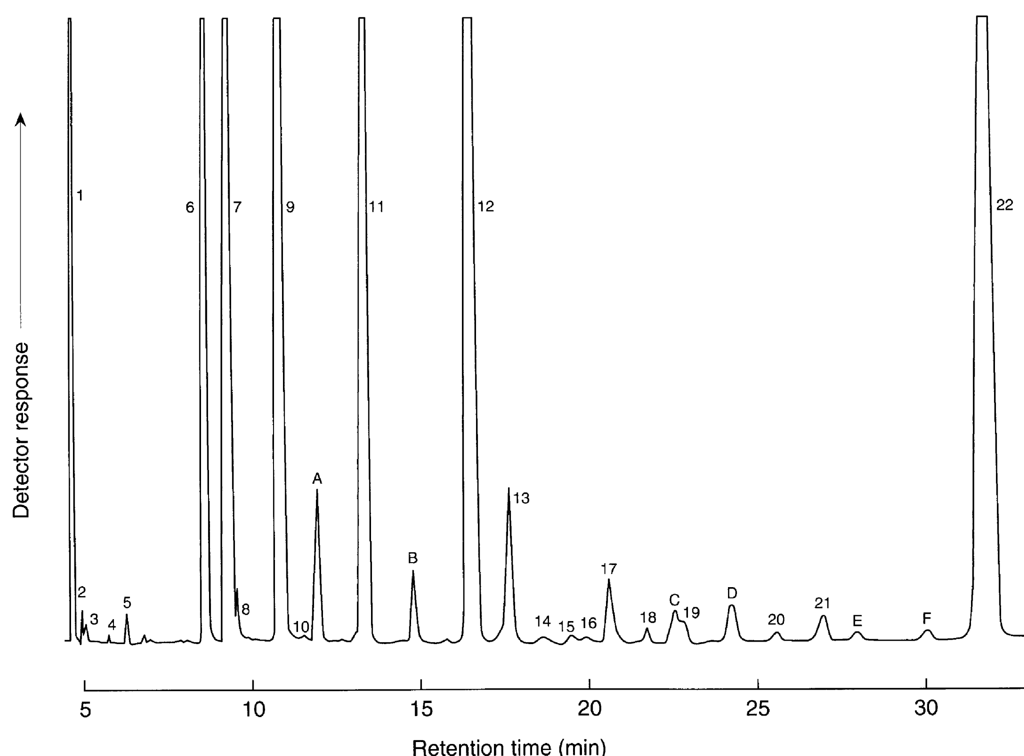


FIG. 1. Gas–liquid chromatogram of fatty acid methyl esters prepared from total lipids extracted from *Agathis robusta* seeds and analyzed on a polyethylene-glycol coated capillary column. Peak numbers and letters (corresponding to peaks not encountered in other gymnosperm species) are as in Table 1.

(64). The theoretical ECL of 6,9,12,15-18:4 acid, if corresponding to peak B, would be ECL (6,9,12-18:3) + ECL (9,12,15-18:3) – ECL (9,12-18:2) = 19.05 + 19.36 – 18.70 = 19.71 (experimental ECL, 19.72). Similarly, the theoretical ECL for peak E, possibly the 8,11,14,17-20:4 acid, would be ECL (8,11,14-20:3) + ECL (11,14,17-20:3) – ECL (11,14-20:2) = 20.98 + 21.36 – 20.69 = 21.65 (experimental ECL, 21.63). Calculations based on the same principle give theoretical ECL that are practically identical to experimental ECL when applied to sciadonic and juniperonic acids (Table 1). Consequently, theoretical ECL of peaks B and E support their tentative identification as 6,9,12,15-18:4 and 8,11,14,17-20:4 acids, respectively.

This accumulation of presumptions was quite indicative of the presence of arachidonic and eicosapentaenoic acids, as well as of their metabolic precursors, in the seed lipids of *A. robusta*, in addition to C_{20} Δ5-UPIFA. However, the two former polyunsaturated fatty acids had never previously been reported to occur in gymnosperm seeds, nor indeed in any other seed oil, and these tentative identifications obviously deserved further structural confirmations, i.e., by MS of appropriate derivatives.

With samples containing a complex mixture of fatty acids, experience has shown that it is often advisable to separate them into simpler fractions before proceeding to GLC–MS analysis, as picolinyl ester or DMOX derivatives of fatty acids may be less well resolved on GLC columns than methyl esters. Minor components that are incompletely resolved may give mixed mass spectra that are uninterpretable. Silver ion HPLC may be favored with such complicated samples as fatty acids from marine sources (65), but reversed-phase HPLC is easier to use with seed oils with a relatively limited range of chain lengths (62). The latter can be used with both picolinyl esters and DMOX derivatives *per se*, under identical conditions. It is our experience that both types of derivatives are of value for locating double bonds and other structural features, and they afford complementary structural information. In the discussion below for illustrative purposes, picolinyl ester derivatives form the basis of most descriptions, but in each instance, confirmation was obtained *via* the DMOX derivatives. A base-deactivated stationary phase was used for reversed-phase HPLC with a flow gradient of acetonitrile as mobile phase [this solvent is more easily removed from collected fractions than the water-containing mobile phases often recommended (66)]. With each derivative, five fractions were collected as illustrated for the picolinyl esters in Figure 2, and each fraction was subjected to GLC–MS.

Mass spectral data for key ions of both types of derivative of many of the fatty acids identified are listed in Table 2. In HPLC fraction 1, the main component was 6,9,12,15-18:4, as determined by the mass spectrum of each derivative. Fatty acids with Δ6 desaturation were not reported frequently in gymnosperms, except for an incorrectly identified γ-linolenic acid in *Pinus koraiensis* seed oil (67) [in fact, pinolenic acid (10)], and for the related 20:3n-6 acid [possibly confused with 7,11,14-20:3 (9) or 20:3n-3 acid] in conifer leaf lipids (68). Therefore the spectra are reproduced here. The spectrum of the picolinyl ester is pre-

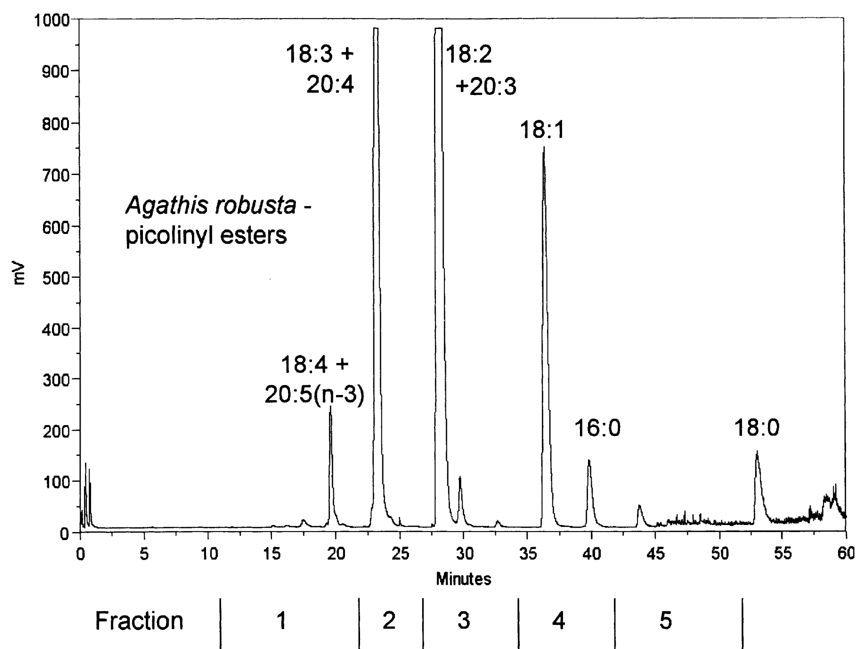


FIG. 2. Reversed-phase high-performance liquid chromatographic separation of picolinyl esters prepared from *A. robusta*, showing where fractions 1 to 5 were collected.

TABLE 2
Diagnostic Ions in the Mass Spectra of Picolinyl Esters and DMOX Derivatives of Some of the Unusual Fatty Acids Found in *Agathis robusta* Seed Oil[a]

Fatty acid	Diagnostic ions
Picolinyl esters	
6,9,12-18:3	151 (18%), 232 (6%), 258 (18%), 272 (16%), 298 (11%), M = 369 (39%)
6,9,12,15-18:4	151 (14%), 232 (5%), 258 (8%), 272 (6%), 298 (19%), 312 (5%), 338 (8%), M = 367 (14%)
5,11,14-20:3	151 (24%), 219 (15%), 232 (20%), 260 (8%), 286 (35%), 300 (11%), 326 (8%), 340 (17%), M = 397 (46%)
8,11,14-20:3	151 (16%), 220 (7%), 246 (8%), 260 (9%), 286 (35%), 300 (17%), 326 (12%), M = 397 (58%)
11,14,17-20:3	151 (22%), 262 (5%), 288 (21%), 302 (8%), 328 (37%), 342 (11%), 368 (16%), M = 397 (57%)
5,8,11,14-20:4	151 (12%), 218 (5%), 244 (8%), 258 (6%), 284 (19%), 298 (5%), 324 (6%), M = 395 (20%)
5,11,14,17-20:4	151 (13%), 219 (10%), 232 (13%), 260 (3%), 286 (14%), 300 (4%), 326 (18%), 340 (5%), 366 (7%), M = 395 (23%)
8,11,14,17-20:4	151 (10%), 220 (6%), 246 (5%), 260 (6%), 286 (12%), 300 (4%), 326 (15%), 340 (4%), 366 (6%), M = 395 (14%)
5,8,11,14,17-20:5	151 (9%), 218 (4%), 244 (6%), 258 (7%), 284 (10%), 298 (5%), 324 (10%), 338 (4%), 364 (5%), M = 393 (8%)
Vernolic acid	151 (13%), 274 (90%), 288 (27%), 316 (64%), 330 (37%), M = 387 (5%)
DMOX Derivatives	
6,9,12-18:3	126 (100%), 194 (10%), 206 (9%), 234 (6%), 246 (5%), M = 331 (10%)
6,9,12,15-18:4	126 (100%), 194 (10%), 206 (9%), 234 (10%), 246 (7%), 274 (4%), 286 (4%), M = 329 (6%)
5,11,14-20:3	126 (25%), 153 (13%), 180 (16%), 192 (1%), 220 (1%), 232 (1%), M = 359 (4%)
8,11,14-20:3	126 (100%), 182 (13%), 194 (4%), 222 (16%), 234 (6%), 262 (15%), 274 (6%), M = 359 (19%)
11,14,17-20:3	126 (100%), 224 (5%), 236 (3%), 264 (18%), 276 (9%), 304 (20%), 316 (13%), M = 359 (40%)
5,8,11,14-20:4	126 (35%), 153 (31%), 180 (6%), 192 (7%), 220 (8%), 232 (3%), 260 (2%), 272 (2%), M = 357 (5%)
5,11,14,17-20:4	126 (33%), 153 (14%), 222 (2%), 234 (4%), 262 (3%), 274 (3%), 302 (3%), 314 (2%), M = 357 (5%)
8,11,14,17-20:4	126 (100%), 182 (10%), 194 (5%), 222 (10%), 234 (6%), 262 (16%), 274 (7%), 302 (9%), 314 (4%), M = 357 (11%)
5,8,11,14,17-20:5	126 (41%), 153 (29%), 180 (5%), 192 (6%), 220 (8%), 232 (4%), 260 (4%), 272 (3%), 200 (2%), 312 (1%), M = 355 (3%)

[a]Data expressed as *m/z* value with percentage of base peak in brackets. DMOX, dimethyloxazoline.

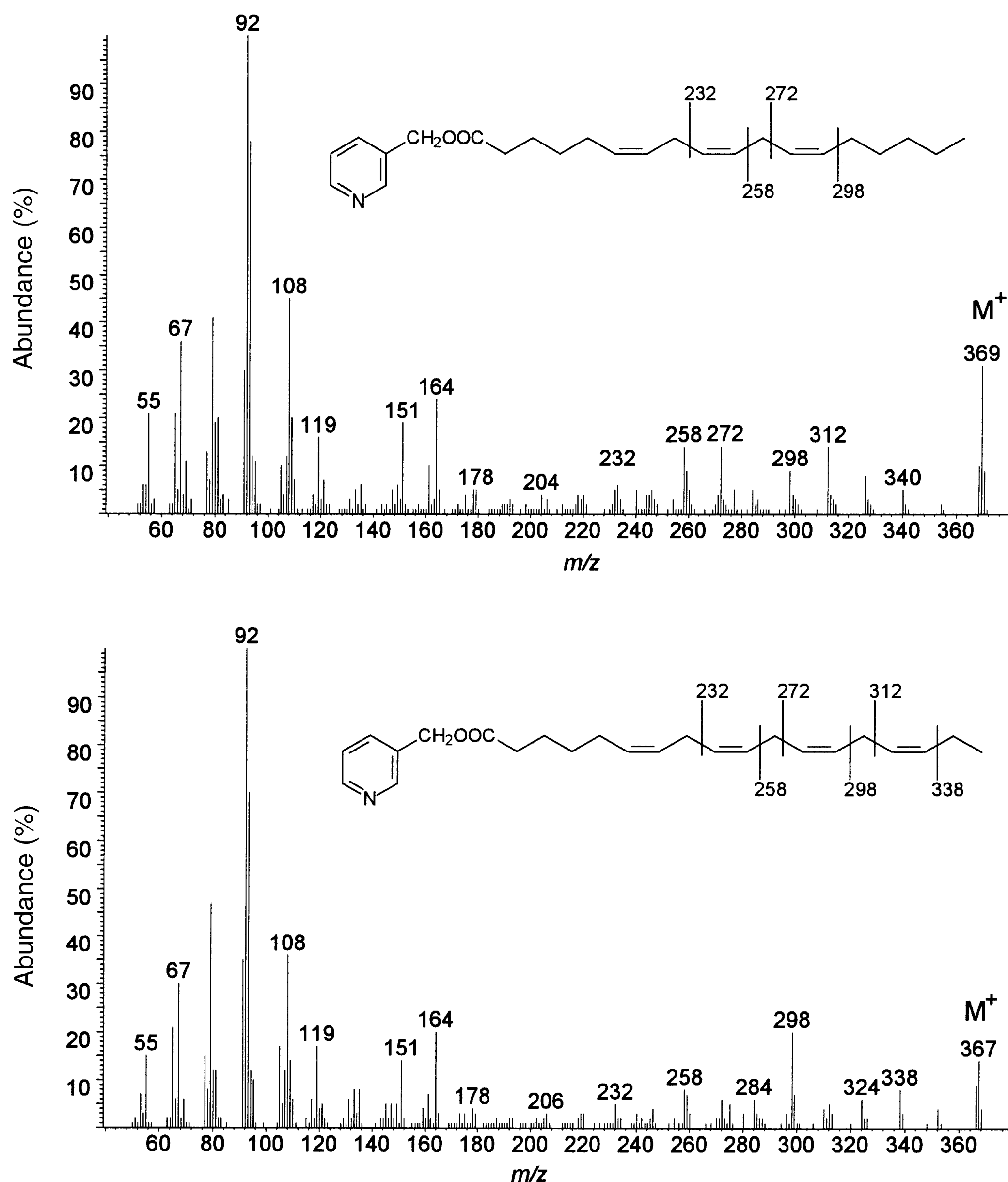


FIG. 3. Mass spectra of picolinyl esters of 6,9,12-octadecatrienoate (upper) and 6,9,12,15-octadecatetraenoate (lower).

sented in Figure 3. Key ions reflecting gaps of 26 amu for each double bond are at *m/z* = 232 and 258, 272 and 298, and 312 and 338 for double bonds in positions 9, 12, and 15, respectively. Diagnostic ions for the double bond in position 6 are less easily discerned, but experience has shown that other features would appear in the spectrum if the innermost double bond was in any other position. The spectrum is identical to that of the authentic fatty acid in borage oil (69). Similarly the spectrum of the DMOX derivative is identical to that of the authentic standard.

In addition, in HPLC fraction 1, 5,8,11,14,17-eicosapentaenoic acid was recognized for the first time in a seed oil. The GLC retention times and mass spectra of the picolinyl ester (70) and DMOX derivative (71) were identical to those of an authentic standard, and they are illustrated in Figure 4. The picolinyl ester had a molecular ion at *m/z* = 393, then a gap of 26 amu between *m/z* = 338 and 364 defined the double bond in position 17, while gaps of 26 amu between *m/z* = 298 and 324, 258 and 284, and 218 and 244 located the double bonds in positions 14, 11, and 8, respectively. The double bond in position 5 cannot be located unambiguously in this way, but the McLafferty ion at *m/z* = 151 is characteristically low in this instance (although this is not an absolute guide) (72). Again, other features would appear in the spectrum if the innermost double bond was in any other position. Interpretation of the spectrum of the DMOX derivative is somewhat different, but the result is equally clear.

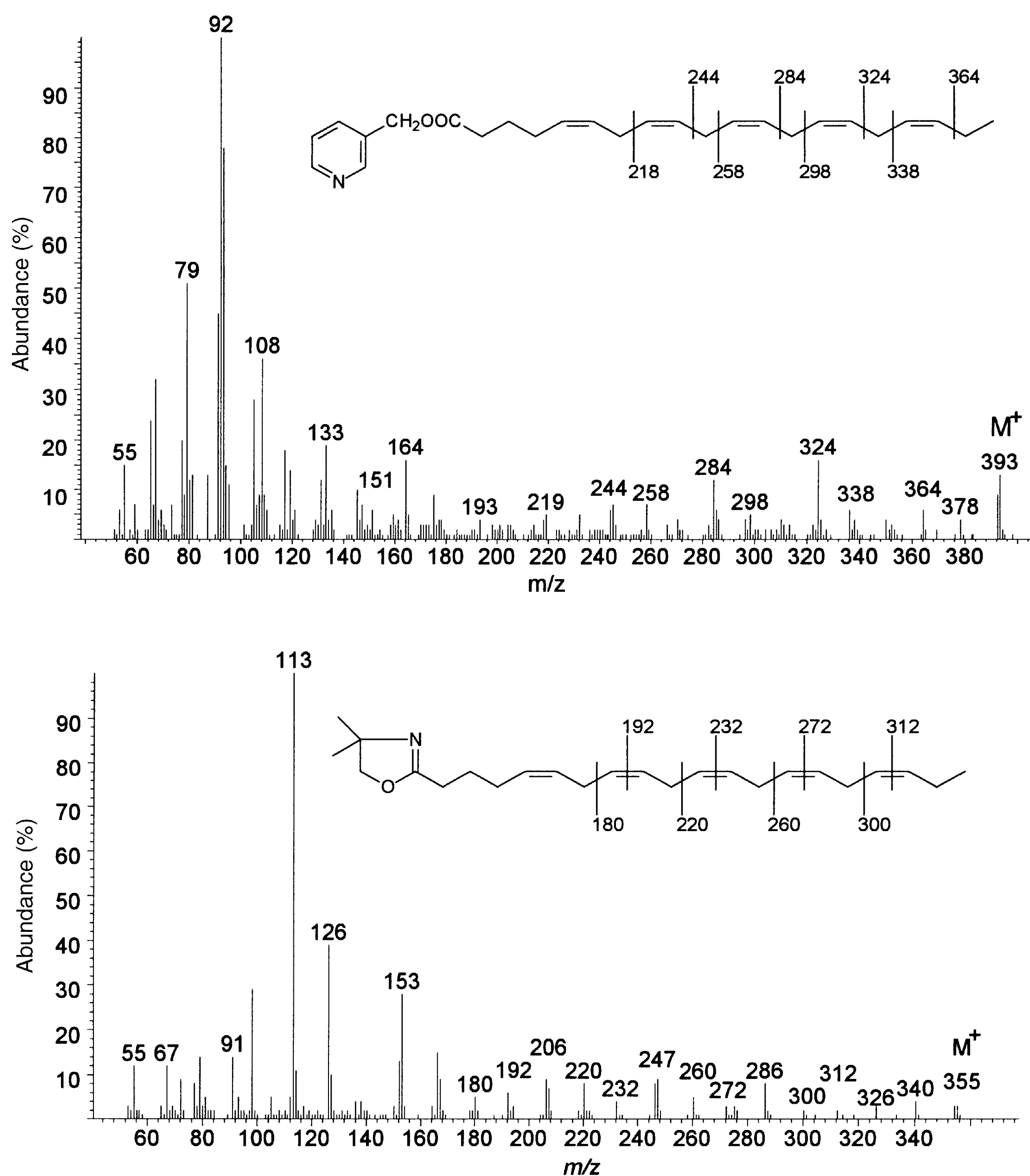


FIG. 4. Mass spectra of picolinyl ester (upper) and dimethyloxazoline (DMOX) derivative (lower) of 5,8,11,14,17-eicosapentaenoate.

Here, gaps of 12 amu, between ions at m/z = 180 and 192, 220 and 232, 260 and 272, and 300 and 312, locate the double bonds in positions 8, 11, 14, and 17, respectively. As with the picolinyl ester derivatives, the location of the innermost double bond is best confirmed by comparison with spectra of defined compounds.

A further minor component in this fraction was vernolic acid; the mass spectrum of its picolinyl ester derivative was identical to that of the true compound in the seed oil of *Vernonia galamensis*, and has been described elsewhere (59).

Fraction 2 from reversed-phase HPLC contained mainly α-linolenic acid, but rather unusually, γ-linolenic acid also was present, and its mass spectrum is illustrated in Figure 3. It is identical to that of the same compound in borage oil (59), and differs in significant details from the spectrum of picolinyl 5,9,12-octadecatrienoate (73), a fatty acid encountered more frequently in the seed oils of gymnosperms. However, a striking finding was that a small amount of arachidonic acid was also present in this fraction. As this is the first time this has been found in the seed oil of a higher plant, the spectrum of its picolinyl ester and DMOX derivative are recorded in Figure 5. In the lower molecular mass region, the spectra are analogous to those of eicosapentaenoate, but after the diagnostic ions for the double bond in position 14, there is a series of ions 14 amu apart for loss of successive methylene groups (and the terminal methyl group) only. A mass spectrum of picolinyl arachidonate has been published by Harvey (74), and of the DMOX derivative was reported by Zhang *et al.* (75). Two further tetraenoic

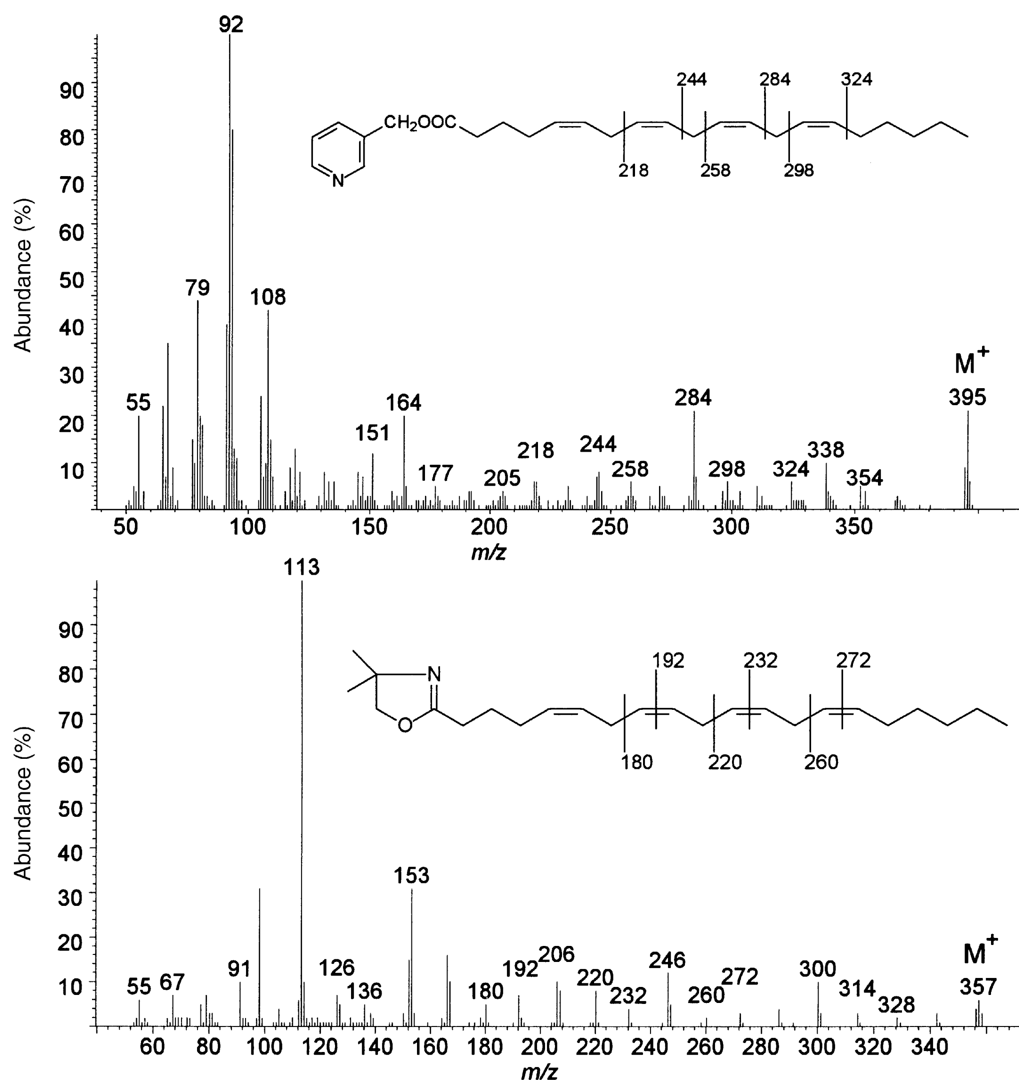


FIG. 5. Mass spectra of picolinyl ester (upper) and DMOX derivative (lower) of 5,8,11,14-eicosatetraenoate. See Figure 4 for abbreviation.

isomers were present, i.e., 5,11,14,17-20:4 and 8,11,14,17-20:4. The spectrum of the former as the picolinyl ester is illustrated in Figure 6. It can be seen that it differs appreciably from that of arachidonate and of eicosapentaenoate. In particular, gaps of 26 amu between *m/z* = 260 and 286, 300 and 326, and 340 and 366 define the double bonds in positions 11, 14, and 17, respectively; the fact that the ion at *m/z* = 151 is of low abundance and the presence of distinctive ions at *m/z* = 219 and 232 are characteristic of an isolated double bond in position 5 (76). Spectra for the DMOX derivatives of fatty acids with double bonds in position 5 have been described by Berdeaux and Wolff (29).

Linoleic acid was the main component of HPLC fraction 3, and it was accompanied by three eicosatrienoate isomers, i.e., 5,11,14-20:3, 8,11,14-20:3, and 11,14,17-20:3 acids. Mass spectra of picolinyl esters of the first and third of these are also illustrated in Figure 6, to illustrate the relationships of the spectra to those of the tetraenoate isomers. For example, that of 5,11,14-20:3 resembles the spectrum of 5,11,14,17-20:4 in the lower molecular mass region, and differs mainly in that the diagnostic ions for the double bond in position 17 are replaced by ions representing cleavage in successive methylene groups. The spectrum of picolinyl 8,11,14-20:3 has diagnostic ions at *m/z* = 262 and 288, 302 and 328, and 342 and 368 for double bonds in positions 11, 14, and 17, respectively (77).

The remaining reversed-phase HPLC fractions contained mainly saturated and monoenoic compounds with some C_{20} dienes, most of which were identified by GC–MS.

Biosynthetic pathways in gymnosperm seeds. To our knowledge, this is the first study describing the simultaneous presence of arachidonic *and* eicosapentaenoic *and all* their metabolic intermediates in a plant *seed*. With regard to the end products, arachidonic acid has not yet been reported to occur in gymnosperm seed lipids, though reports by Russian researchers (78) mention its presence in the cambium zone of *Larix sibirica*

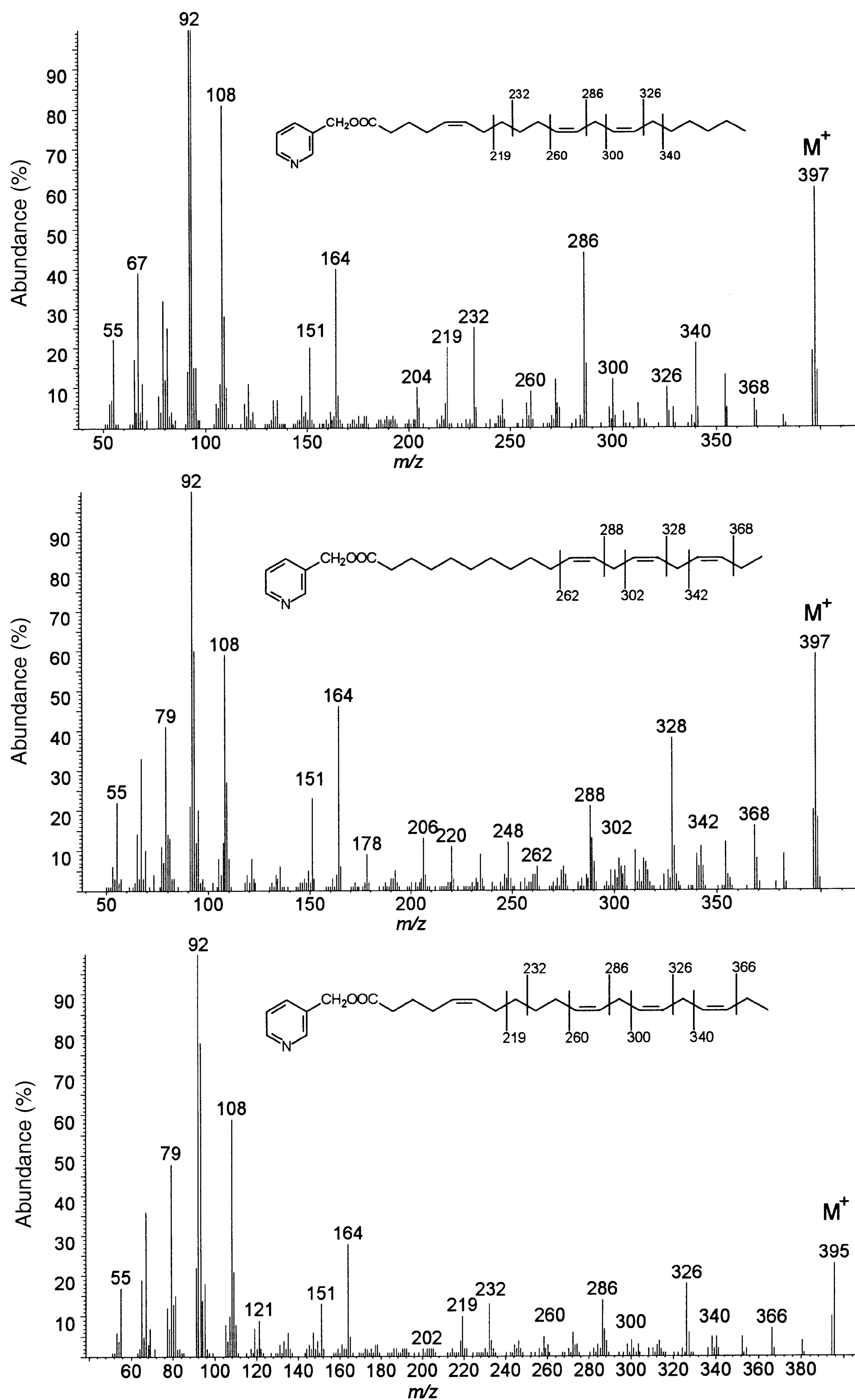


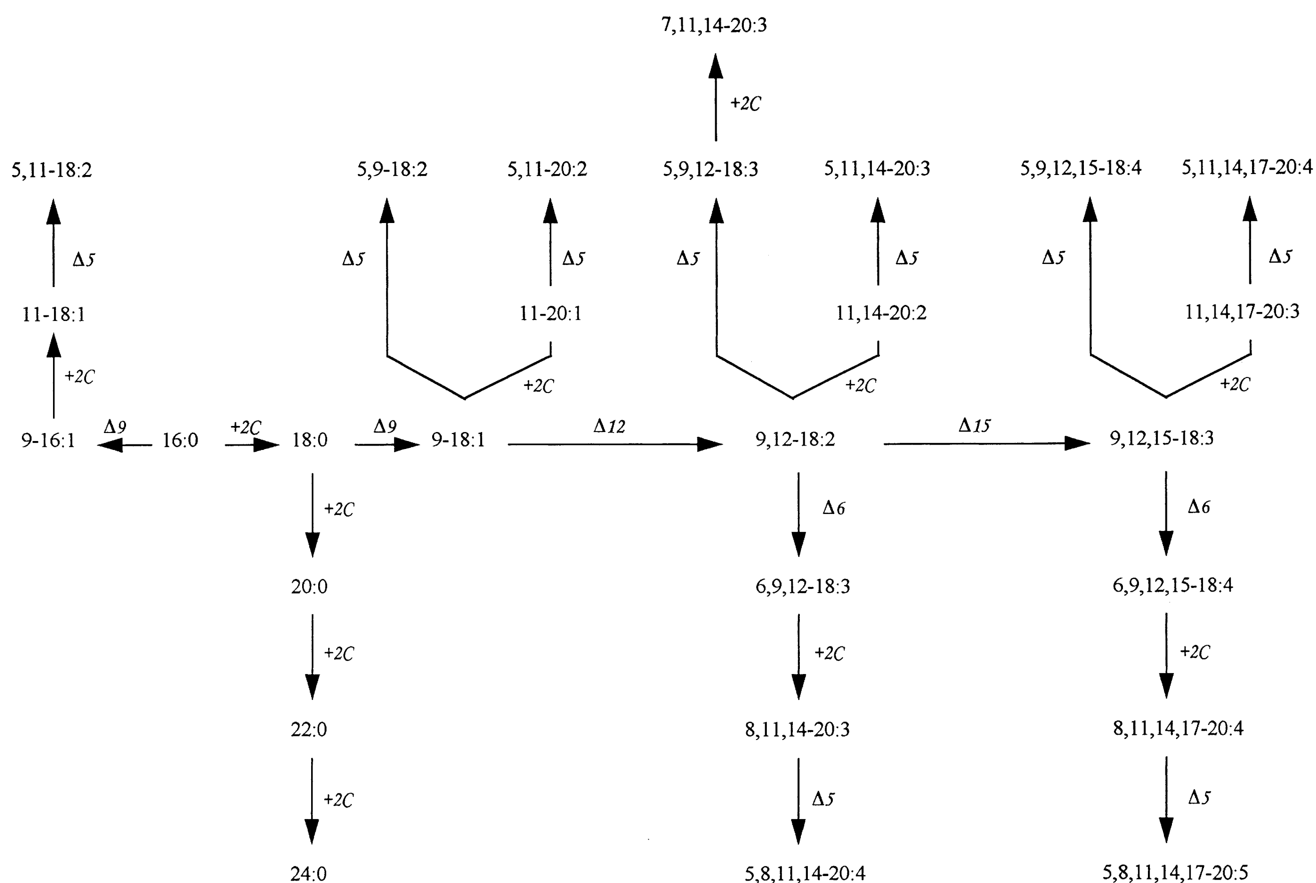
FIG. 6. Mass spectra of picolinyl esters of 5,11,14-eicosatrienoate (upper), 11,14,17-eicosatrienoate (middle), and 5,11,14,17-eicosatetraenoate (lower).

(Siberian larch), but not of intermediaries between linoleic acid and arachidonic acid. Similar observations were made by the same group on *Populus balsamifera* (balsamic poplar) buds (79). Though structural evidence was given, such as ^{1}H NMR data, iodine number and refractive index, that need relatively large amounts of material, details on the isolation procedure of arachidonic acid for these measurements were unfortunately lacking. It should be added, at least for *L. sibirica* wood, that other thorough and detailed GLC–MS studies of pine (*Pinus sylvestris*) and spruce (*Picea abies*) wood extracts did not mention arachidonic acid (80,81). Arachidonic acid also has been found in different parts of a few angiosperm species, e.g., garlic (*Allium sativum*) cloves (82) and *Aloe vera* above-ground parts (83). In the latter studies, arachidonic acid was identified by GLC only, and the presence of 41.7% γ-linolenic acid relative to total fatty acids was reported for *A. vera* (83). More convincing and intriguing is the characterization of arachidonic acid in virgin wheat-germ oil by GLC–MS of its methyl ester after fractionation by distillation (84). No intermediates were reported in that study. Eicosapentaenoic acid has been reported to occur in *A. sativum* cloves as well (82). To our knowledge, until now, these findings have not been not confirmed, though several reports (reviewed in Refs. 85 and 86) describe the prostaglandin-like effects of some plant extracts.

When considering angiosperm species that contain γ-linolenic acid or stearidonic acid in their seed lipids [e.g., borage (*Borago officinalis*), evening primrose (*Oenothera biennis*), or black currant and other *Ribes* spp.], the initial Δ6-unsaturated intermediates are present, but neither their higher homologs nor the potential end products, arachidonic and eicosapentaenoic acids, are found. Consequently, this is the first report on a Spermaphyte species whose seeds contain all the precursors and intermediaries needed for the simultaneous biosynthesis of arachidonic and eicosapentaenoic acids, and the latter acids themselves. With regard to this biosynthetic activity, *A. robusta* is close to more primitive plants, such as Bryophytes and Pteridophytes, with extant species (mosses, liverworts, ferns) still able to synthesize arachidonic and/or eicosapentaenoic acids in several parts of the plant (22,87–97). Horsetail (*Equisetum*), however, does not contain the latter polyunsaturated acids, though Δ5-UPIFA have been characterized in these plants (22).

In a previous paper (18), we suggested that gymnosperms might contain two Δ5-desaturases, one specific for C_{18}-unsaturated acids, and the other for C_{20}-unsaturated acids. It is necessary to define "unsaturated" because no 5-18:1 or 5-20:1 acids have been detected in gymnosperm seeds (our columns allow a clear-cut resolution of these monoenoic acids from their Δ9 isomers), indicating that 18:0 and 20:0 acids are not substrates of the Δ5-desaturases. An alternative designation of the C_{18}-specific and C_{20}-specific Δ5-desaturases could be "Δ9-specific" and "Δ11-specific," respectively, because the C_{18} and C_{20} substrates coincide with fatty acids containing a Δ9- and a Δ11-ethylenic bond, respectively. Owing to the existence in some species of a 5,11-18:2 acid [i.e., in *Ginkgo biloba* and *Ephedra* spp. (12,18)], which is both a C_{18} and a Δ11-unsaturated acid, a decision cannot be made regarding the true specificities, chain length, or position of the first double bond. From the absence of C_{18} Δ5-UPIFA in *A. robusta* seeds, it can be inferred that this species does not contain the C_{18}-specific Δ5-desaturase, or more rigorously, the corresponding activity. In lieu of this enzyme, a C_{18}-specific Δ6-desaturase would be present. However, *A. robusta* has the C_{20} Δ5-desaturase in common with other gymnosperm species, and also the unsaturated-$C_{18} \rightarrow C_{20}$ elongase. We have recapitulated in Scheme 1 the biosynthetic pathways that are likely to take place in gymnosperm seeds. All pathways seldom occur together in species of a given family, and the figure is only intended to show the variety of unsaturated fatty acids that is to be found in gymnosperm seeds. For gymnosperm leaves, C_{20} Δ5-UPIFA are present in all species analyzed so far (68). However, C_{18} Δ5-UPIFA would only occur in species from the Pinaceae family, which would indicate that the C_{18} Δ5-desaturase activity observed in the seeds is not forcefully expressed in the leaves. Thus, it cannot be inferred from the present study whether arachidonic and eicosapentaenoic acids are present or not in *A. robusta* leaves, as for example, in green parts of mosses and ferns. This deserves further investigation.

Considerations on the evolution of the Δ6- and Δ5-desaturases. The trivial term "higher plants," used to describe Bryophytes, Pteridophytes, and Spermaphytes (that include gymnosperms and angiosperms), is not generally accepted, and the name Cormophytes better applies to these vegetal phyla taken as a whole. "Higher plants" may also have a restricted definition, being limited to Spermaphytes (seed plants), and we will adopt the former definition. Among higher plants, the succession on a geological time scale of the three phyla is certain, though (extinct?) intermediates are difficult to identify. As regards the C_{18} Δ6- and C_{20} Δ5-desaturase activities, the former appears common to many microalgae, algae, fungi, Bryophytes, and Pteridophytes (at least to several species from these groups) and to some Araucariaceae, at least to the species studied here, *A. robusta*. On the other hand, the C_{18} Δ5-desaturase activity is characteristic of gymnosperms: Δ5-UPIFA with 18 carbon atoms have been found in the seeds of all Coniferophyte (Pinatae and Gingkoatae) families (in more or less high amounts, their absence being most probably linked to secondary events) and Cycadophytes, with the exception of Welwitschiaceae and Gnetataceae, the taxonomic positions for which are uncertain (they are regarded as members of either the gymnosperm or the chlamydosperm subphyla). *Agathis robusta*, undoubtedly a gymnosperm, would thus show an intermediate archaic state between Pteridophytes, or more likely some related extinct plants (e.g., the fossil Pteridospermaphytes, considered earlier as Pteridophytes and later as gymnosperms), and other gymnosperms, being characterized by C_{18} Δ6-desaturase activity. *Agathis robusta* would not be an isolated case among Araucariaceae, as γ-linolenic and arachidonic acids were also characterized in *Araucaria bidwillii* and *A. araucana* seeds, which additionally show typical C_{18} Δ5-UPIFA, and consequently "mixed" C_{18} Δ5/Δ6 activities (Wolff, R.L., Christie, W.W., Pédrono, F., and Marpeau, A.M., manuscript in preparation). Although complementary studies of other Araucariaceae species are obviously needed, the evolutionary sequence



SCHEME 1

Bryophytes, Pteridophytes → Araucariaceae → Coniferophytes can be suggested as a working hypothesis. Finally, Araucariaceae and all other Coniferophyte families share a common C_{20} Δ5-desaturase activity, demonstrated by the systematic presence of sciadonic acid in the seed lipids (they also are present in *Cycas* and *Ephedra* spp. among Cycadophytes). Because in Pinatae families other than the Araucariaceae the C_{18} Δ6-desaturase activity has evolved into a C_{18} Δ5-desaturase activity, though the C_{20} Δ5-desaturase activity in addition to the elongase activity were kept unchanged, arachidonic and eicosapentaenoic acids are no longer synthesized in these plants. Our study also shows that the C_{20} Δ5-desaturase does not need Δ8-desaturated substrates (i.e., 20:2n-6 and 20:3n-3 acids are substrates) to exert its activity in plants. The C_{20} Δ5-desaturase activity thus appears a rather constant feature throughout the entire plant kingdom, except in "modern" angiosperms, where this activity is apparently limited to a few species of the Ranunculaceae family (52,53).

A C_{18} Δ6-desaturase activity was probably the primeval status in higher plants as it occurs in Bryophytes and Pteridophytes, likely derived from some (micro) algae. This archaic enzymatic activity is still present in *A. robusta*. In *A. bidwillii*, the C_{18} Δ6-desaturase and Δ5-desaturase activities are simultaneously present. In other Coniferophytes, including *G. biloba* and some Cycadophytes, only the C_{18} Δ5-desaturase activity is present. The sequence Δ6 → Δ6/Δ5 → Δ5 C_{18} desaturation would parallel the phylogenetic series based on the evolution of the leaves in Araucariaceae from a dichotomous foliar nervation to a simple one (98,99). This hypothetical sequence would be: *Buriada heterophylla* (extinct) → *Agathis* → *Araucaria*, section *Columbea* → *Araucaria,* section *Eutacta*. In apparent disagreement with this point of view, fossil evidences indicate that *Araucaria* spp. (Jurassic) would have preceded *Agathis* spp. (Tertiary), though there is no certain way of distinguishing between *Agathis* spp. and *Araucaria* spp. permineralized wood (100–102). The fossilized wood of possible ancestors known as *Araucarioxylon* from the Lower Carboniferous is hardly distinguishable from that of extinct cordaites and glossopterids (100–102).

A recent study (103) has indicated that expression of a *Mortierella alpina* Δ5-desaturase cDNA in a *Saccharomyces cerevisiae* recombinant used either endogenous 9-18:1 or added 8,11,14-20:3 acids as substrates to give taxoleic and arachidonic acids, respectively. Expression of the *M. alpina* cDNA in transgenic canola seeds resulted in the production of taxoleic and pinolenic acids (103). Thus, in that experiment, the *M. alpina* Δ5-desaturase cDNA would allow expression of an enzymatic activity that may use both C_{20} and C_{18} unsaturated acids as sub-

strates, which would be at variance with our hypothesis of two distinct Δ5-desaturase activities in gymnosperm seeds. On the other hand, a mutant of *M. alpina* (1S-4 Mut 49) defective in the Δ6-desaturase activity is apparently able to accumulate sciadonic and juniperonic acids in TAG and phospholipids, but not taxoleic or pinolenic acids (103). The wild-type strain would not contain any Δ5-UPIFA, but rather large amounts of arachidonic acid, which needs a C_{18} Δ6-desaturase, an unsaturated $C_{18} \rightarrow C_{20}$ elongase system, and a C_{20} Δ5-desaturase. Finally, it has been demonstrated (105) that replacement of a few amino acids in a given desaturase is able to convert it into another enzyme with different specificities (chain length and position of desaturation). The picture that now emerges is that such mutational changes could have occurred at the very beginning of the history of gymnosperms, in conjunction with their emergence.

These experiments (103–105), as well as the present study, emphasize the molecular similitudes and possible evolutionary relationships between the Δ6- and Δ5-desaturases in primitive gymnosperms. *Agathis robusta*, and possibly other Araucariaceae species, may be a source for gene encoding for the complete enzymatic machinery that ensures the synthesis of arachidonic and eicosapentaenoic acids in seed lipids. On the other hand, γ-linolenic acid has, or is considered to have, great biomedical and economical promise, because it is a more immediate precursor of arachidonic acid than linoleic acid in humans, as it bypasses the crucial Δ6-desaturation step. The present findings indicate that it is conceivable, in principle, to directly produce arachidonic (instead of its precursor γ-linolenic acid only) and/or eicosapentaenoic acid in cultivatable seed plants in the future.

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Lipids of the Pawpaw Fruit: *Asimina triloba*

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ABSTRACT: The fatty acid composition and structure of pawpaw fruit *(Asimina triloba)* triglycerides were examined and found to contain fatty acids ranging from C_6 to C_{20}. Octanoate represented 20% of the fatty acids while other medium-chain fatty acids were present in low amounts. Analysis of the intact triglycerides by high-temperature gas–liquid chromatography gave an unusual three-cycle carbon number distribution. Analysis of triglyceride fractions separated according to degree of unsaturation suggested that one octanoate was paired with diglyceride species containing long-chain fatty acids. Determination of the double-bond positions of monoene fatty acids revealed *cis* Δ9 and *cis* Δ11 hexadecenoate and *cis* Δ9, *cis* Δ11, and *cis* Δ13 octadecenoate isomers were present in significant quantities. Octanoate and positional monoene fatty acid isomers were found only in the fruit lipids and not in the seed lipids. Phenacyl esters of fatty acids were found to be useful derivatives for structure determination using multiple types of analyses.

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The pawpaw [*Asimina triloba* (L.) Dunal] is a large tree fruit that grows wild in a region from East Texas to the Eastern Seaboard and northward to the Great Lakes (1). The pawpaw is the only temperate member of the Annonaceae family that grows in North America (1,2). Eight other species of *Asimina* are native to the U.S. mainland, but they are of little commercial value at this time (3). The pawpaw fruit can grow as a singlet, but normally grows in bunches of three or more. The fruit is cylindrical in shape, typically 5 to 15 cm (2 to 6 in.) long, 3 to 8 cm (1 to 3 in.) wide, and can weigh up to 450 g (1 lb). During the growing season, the pawpaw has a whitish to light-green color that turns yellow to brownish at maturity. The ripe fruit is highly aromatic and the banana-like flesh surrounds two rows of large bean-shaped brown seeds.

Research, primarily at Kentucky State University and Purdue University, on pawpaw horticulture has led to a commercial interest in this fruit (1,3). Interest is also being generated in the popular press (4).

The present study examines the fatty acids and triglyceride structure of the pawpaw.

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Abbreviations: Ag^+-TLC, silver ion thin-layer chromatography; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; TLC, thin-layer chromatography.

METHOD AND MATERIALS

Pawpaws. Immature pawpaws were collected in July and mature ones in September from a native stand near Palmer, Kentucky. Both the mature fruit flesh and the immature pawpaws were transported back to Texas in an ice chest. Seeds from immature pawpaws were removed and the lipids extracted without freezing. Flesh from mature pawpaws was stored at –20°C until extracted.

Lipid extraction and fractionation. Pawpaw fruit (flesh) and seeds (without seed coat) were homogenized with chloroform/methanol (2:1, vol/vol), filtered, washed twice with 2 vol of water, and the chloroform fraction was evaporated to dryness with a rotary evaporator at reduced pressure. The lipid samples were redissolved in chloroform, filtered through a fine porosity fritted-disc Buchner funnel, evaporated, held at high vacuum for 30 min, and weighed. Total lipids were separated into neutral lipid and polar lipid fractions by silicic acid chromatography (5) and weighed. The polar lipid fraction, predominantly phospholipids, was examined for fatty acid composition. The neutral lipid fraction was analyzed by thin-layer chromatography (TLC) on absorbent layers of Silica Gel G developed in hexane/diethyl ether/glacial acetic acid (80:20:1, by vol). A typical separation is shown in Figure 1A along with standards. Preparative TLC on 20 × 20 cm plates was used to isolate the triglyceride fraction for further analyses. Pawpaw fruit triglycerides were fractionated according to degree of unsaturation on 6% silver nitrate-impregnated Silica Gel G-60 chromatoplates developed in chloroform/methanol (99:1, vol/vol). Separated fractions were visualized by spraying with Rhodamine 6G and viewing under ultraviolet light (Fig. 1B). Triglycerides were released from the silver nitrate/Silica Gel G-60 absorbent with 1 mL of methanol and 1 mL of aqueous 20% sodium chloride after being scraped into a Teflon-lined screw-cap culture tube. Hexane was used to extract the triglycerides, which were filtered through a fine porosity fritted-disc Buchner funnel and stored at –20°C.

Derivative preparation and analyses. Methyl esters of the various lipids were prepared by the time-honored 2% sulfuric acid-catalyzed transesterification in methanol method (6). Butyl esters were prepared by the same transesterification method using *n*-butanol. Triglycerides were hydrolyzed with a mild saponification procedure (7) to yield free fatty acids after saponification. Phenacyl derivatives were prepared

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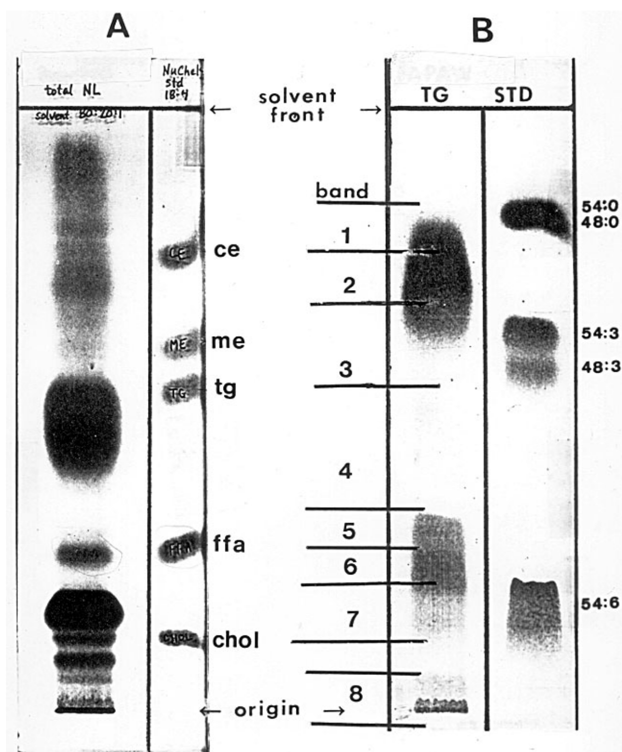

FIG. 1. (A) Thin-layer chromatoplates of total pawpaw fruit neutral lipids and (B) pawpaw fruit triglycerides separated on silver ion-impregnated adsorbent layers. Solvent systems were (A) hexane/diethyl ether/acetic acid (80:20:1, by vol) and (B) chloroform/methanol (99:1, vol/vol). Abbreviations: ce, cholesterol ester; me, methyl ester; tg, triglyceride; ffa, free fatty acid; and chol, cholesterol. Numbered bands (B) represent the area of fractions collected for additional analyses (see Tables 2 and 3).

using α-bromoacetophenone as described earlier (8). The ozonides used to determine the position of the double bonds in the monoenes were prepared as described previously (9). Hexadecenoate and octadecenoate phenacyl esters were isolated by high-performance liquid chromatography (HPLC) using a C_{18} reversed-phase column as previously described (8). Methyl esters and butyl esters were analyzed on a 30 m × 0.53 mm (i.d.) DB-225 (J&W Scientific, Folsom, CA) capillary column using a Varian 3700 gas chromatograph (Walnut Creek, CA). Column temperature was programmed from 140 to 225°C at 3°C/min and 60 to 225°C at 5°C/min for methyl and butyl esters, respectively. Intact triglycerides, phenacyl esters, and phenacyl ozonides were analyzed on a 15 m × 0.53 mm (i.d.) DB-PS1 (J&W Scientific) column. The phenacyl esters' column temperatures were programmed from 60 to 250°C at 8°C/min, while the injector temperature was programmed from 60 to 300°C at 150°C/min and held for 10 min. Column temperatures for triglyceride analyses were programmed from 100 to 225°C at 10°C/min and then to 350°C at 5°C/min. The injector temperature was programmed from 60 to 325°C at 150°C/min and held for 20 min. Phenacyl ozonide column temperatures, with and without triphenylphosphine, were programmed from 60 to 300°C at 8°C/min, and the injector temperature was programmed from 60 to 300°C at 150°C/min. Generally, the reproducibility for methyl ester analysis of fatty acids with C-12 or longer chain lengths and butyl esters of fatty acids of C-8 and longer was ±5% for major peaks (>10% total) and ±15% for minor peaks (<10% total). Quantities of positional isomers were determined from the gas–liquid chromatography (GLC) analysis of methyl esters. Reproducibility of results from the GLC analysis of intact triglycerides was equivalent to that of methyl ester analysis. The data were collected with an IBM model 9000 (Danbury, CT) laboratory computer. Peak identification was established by cochromatography with standard reference fatty acids. All the procedures used to extract, derivatize, and analyze the lipids have been described in detail (6).

RESULTS

The mature pawpaw fruit contained approximately 1% total lipid on a wet weight basis. The mature seed contained 9.0% total extractable lipid. The fruit lipids were composed of 51% neutral lipid and 49% polar lipid, whereas the mature seed consisted of 95% neutral lipid. The immature fruit and seed contained less than 25% of the lipids of mature fruit and seed lipids and had nearly equal levels of neutral and polar lipids.

Results from the analysis of the fatty acid methyl esters from the seeds and fruit neutral and polar lipids of mature and immature pawpaws by GLC are given in Table 1. The fatty acid composition of the mature and immature seeds was characterized by high levels of 18:2 and 18:1. Seed polar lipids contained more 16:0 than neutral lipids. Immature seeds contained more 16:0 and 18:3 than mature seeds. The 18:1 and 18:2 percentages that we observed in the seeds are almost the opposite of that reported by Hilditch (10); however, there are significant differences from the technology of 60 yr ago to that of today. The only other unusual feature of the seed fatty acid composition is the absence of the 14:0, hexadecenoate, and octadecenoate isomers found in the fruit (Table 1). The fatty acid compositions of the pawpaw fruit given in Table 1 are not accurate because, as we will show, a high percentage of octanoate methyl ester present was lost during solvent evaporation. The methyl ester percentages of the mature fruit triglycerides and polar lipids show the presence of 14:0 and elevated levels of octadecenoate isomers relative to the immature fruit lipids. Because of budgetary and time constraints, seed-, immature-, and polar-lipid analyses were not made beyond this point.

Analysis of fruit triglycerides. As shown in Figure 1A, the pawpaw fruit neutral lipids contained a variety of compounds of varying polarity. The large triglyceride band was isolated by preparative TLC for analysis by high-temperature GLC. The carbon-number distribution of the mature pawpaw fruit triglycerides is shown in Figure 2. The peak numbers represent the number of carbon atoms in the fatty acids of the glyceride; i.e., peak number 36 could be made up of 3 × 12:0 or any combination of fatty acids to yield 36. The chromatogram shows an atypical distribution. The low-molecular-weight

TABLE 1
Fatty Acid Composition of Pawpaw Triglycerides and Phospholipids from Mature and Immature Seeds and Fruit

Pawpaw tissue; lipid source	Fatty acid methyl ester percentage[a,b]												
	14:0	14:1	16:0	16:1A	16:1B	18:0	18:1A[c]	18:1B[c]	18:1C[c]	18:2	18:3	20:0	20:1
Mature seed; triglycerides			4.1	0.6		1.8	31.0			61.5	1.0		
Mature seed; polar lipids			15.3	0.6		4.9	33.0			41.6	1.9	1.0	0.9
Immature seed; triglycerides			10.4	1.0		1.6	21.1			58.4	7.3		0.4
Immature seed; polar lipids			30.4			1.6	2.4			50.8	15.0		
Mature fruit; triglycerides	6.9	0.6	15.1	6.6	2.2	2.6	23.2	16.5	7.2	5.8	11.1	0.3	
Mature fruit; polar lipids	1.2	0.1	17.4	5.6	1.5	2.0	23.0	18.6	6.0	6.6	14.8		
Immature fruit; triglycerides			18.3	9.8	1.9	6.2	19.2	2.1		34.0	8.6		
Immature fruit; polar lipids			19.7	1.2		1.3	11.3	2.7		2.1	44.3	17.4	

[a]The difference between the sum of any row and 100% represents minor amounts of other fatty acids not given.
[b]Values for fruit triglycerides are not accurate because lower-molecular-weight fatty acid methyl esters were lost during sample concentration. See Table 3 for butyl ester and phenacyl derivative values.
[c]Positional octadecenoate isomers.

triglycerides indicated that shorter-chain fatty acids than indicated in Table 1 were present. The three-cycle appearance of the carbon number distribution indicated either a preferential pairing of fatty acids or an unusual fatty acid distribution pattern.

Figure 1B shows the separation of pawpaw fruit triglycerides and standard by Ag^+-TLC. Tristearin and tripalmitin were not separated, but triolein and tripalmitolein were resolved in the standard mixture. Trilinolein with six double bonds was retarded on the TLC plate near the origin. Because of the effect of fatty acid chain length as noted in triolein and tripalmitolein separations, the numbered bands in the pawpaw triglycerides lane may not correspond to the number of double bonds in the standards. The pawpaw triglyceride sample gave two major bands and origin material, but even band 4 shows that no material contained triglycerides.

The carbon-number ditribution of the total and the eight Ag^+-TLC bands (Fig. 1B) of pawpaw triglycerides is given in Table 2 along with a 1,2,3-random calculated distribution. The lower-numbered bands with less unsaturation had higher

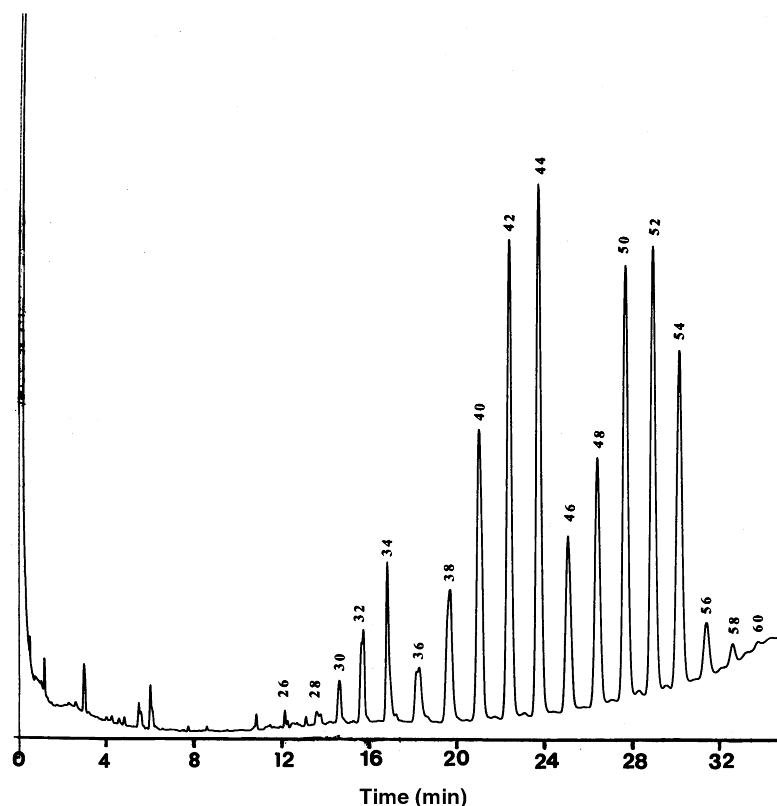

FIG. 2. High-temperature gas–liquid chromatogram of pawpaw fruit triglycerides. The numbered peaks represents the sum of the fatty acid carbon atoms. Analytical conditions are given in the Methods and Materials section.

TABLE 2
Distribution of Molecular Species of Mature Pawpaw Fruit Triglycerides and Triglyceride Fractions Separated by Silver Nitrate

Triglyceride fraction	Percentage of triglyceride species[b]																
	28	30	32	34	36	38	40	42	44	46	48	50	52	54	56	58	60
1,2,3-Rand[c]	1.3	1.5	5.8	6.2	4.8	7.9	12.1	12.7	9.8	4.0	8.5	8.8	6.2	2.4	0.1		
Total	0.2	0.9	1.5	2.9	1.3	4.5	9.1	13.3	14.8	5.4	7.3	12.6	13.0	10.5	1.8	0.6	0.1
Band 1	0.1	0.9	3.6	4.7	3.0	7.0	12.8	13.1	8.4	5.5	8.3	14.3	12.2	3.6	0.9	0.5	0.2
Band 2	0.2	1.4	5.1	1.3	3.9	10.5	13.8	10.7	7.4	6.1	9.2	12.6	8.9	2.8	0.9	0.5	0.1
Band 3			1.1	0.2	1.6	6.7	18.9	23.5	5.7	7.2	8.4	8.1	11.6	1.8	0.4		
Band 4			0.3	1.5	1.0	1.6	5.1	10.5	12.3	6.2	8.4	15.4	19.9	14.9	1.8	0.5	0.2
Band 5				6.1	1.8	3.1	9.8	11.1	11.1	4.5	4.4	13.7	17.6	8.4	2.9	1.0	0.3
Band 6						0.3	1.2	9.6	31.3	6.2	7.1	15.3	13.7	11.9	2.7	0.5	
Band 7						0.6	3.1	8.6	11.2	2.2	1.9	7.5	19.3	39.4	4.0	0.5	0.2
Band 8								3.8	16.1	2.8	1.8	12.0	18.5	34.6	6.4	1.0	0.4

[a]See Figure 1 for identity of bands separated by silver nitrate thin-layer chromatography.
[b]The difference between the sum of any row and 100 %represents minor amounts of other components not given in the table.
[c]1,2,3-Rand, a calculated 1,2,3-random distribution from fatty acid chain length percentages of total pawpaw fatty acid butyl esters.

percentages of the low-molecular-weight glycerides than the more unsaturated glycerides. Since diene and triene fatty acids were restricted to 18-carbon fatty acids, such a profile might be expected. The cyclic distribution generally followed all fractions showing lower percentages of carbon numbers 36 and 46. The calculated carbon number distribution did not

TABLE 3
Comparison of Coconut Oil Fatty Acid Percentages Determined by Three Methods and the Distribution of Fatty Acids in Pawpaw Triglyceride Fractions Separated by Silver Nitrate TLC[a]

Triglyceride fatty acid derivatives	Fatty acid percentages															
	6:0	8:0	10:0	12:0	14:0	14:1	16:0	16:1 Δ9	16:1 Δ11	18:0	18:1 Δ9	18:1 Δ11	18:1 Δ13	18:2	18:3	20s
Coconut phenacyl[b] HPLC		10.5	7.5	48.3	16.3		7.7			1.8	4.5			1.2		
Coconut phenacyl GLC, DB-PSI	0.6	7.7	6.0	48.0	20.4		8.9			2.3		6.0				
Coconut, FABE GLC, DB-225	0.9	9.7	7.5	48.4	18.9		7.5			1.5	3.5			0.7		
Pawpaw phenacyl[c] GLC, DB-225	0.8	10.2	1.6	1.8	8.4	0.4	14.0	5.7		0.4		53.0			2.9	
Pawpaw, FABE[d] GLC, DB-225	3.4	21.9	3.0	3.7	12.8	1.4	15.9	6.8	2.2	2.2	9.4	5.3	2.8	2.7	6.4	0.3
Pawpaw, FABE[e] TLC, band 1	3.1	21.1	3.4	4.0	15.8	0.7	20.1	6.5	2.1	2.7	11.1	4.6	2.3	0.6	0.3	0.4
Pawpaw, FABE TLC, band 2	3.5	27.0	4.0	4.6	17.7		23.7	3.4	1.0	2.4	5.4	1.9	0.9			
Pawpaw, FABE TLC, band 3	5.5	29.0	3.3	2.7	7.1	2.5	5.8	10.5	3.0	0.8	12.9	8.1	4.2	2.6	1.3	
Pawpaw, FABE TLC, band 4	1.9	13.2	2.0	2.7	10.1	2.1	12.8	6.2	2.4	2.3	7.2	5.7	3.2	8.2	9.4	1.5
Pawpaw, FABE TLC, band 5	3.1	23.4	3.1	3.9	12.5		13.1	2.0		2.1	3.5	4.6	2.0	4.1	15.9	
Pawpaw, FABE TLC, band 6	1.9	12.3	1.5	1.6	6.7	1.1	7.1	4.8	1.4	1.1	7.5	9.3	4.5	6.2	31.9	0.5
Pawpaw, FABE TLC, band 7	2.9	11.9	1.3	1.7	4.4	3.9	4.1	8.5	3.1	0.5	8.4	9.3	6.0	7.7	25.3	0.7
Pawpaw, FABE TLC, band 8	0.6	4.2	0.7	1.3	5.2	0.7	6.2	1.7	0.8	1.6	2.6	4.3	2.3	10.2	48.1	

[a]Abbreviations: HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography; TLC, thin-layer chromatography; FABE, fatty acid butyl ester.
[b]Reference 8.
[c]Mean of three triglyceride preparations.
[d]Mean of seven triglyceride preparations.
[e]All pawpaw butyl esters from TLC bands were analyzed by GLC. TLC band retention factors are shown in Figure 1.

agree completely with the percentages of the total, but the cyclic distribution was apparent.

Phenacyl and *n*-butyl ester derivatives were prepared to avoid the loss of low-molecular-weight fatty acids. Table 3 shows the fatty acid composition of coconut oil, pawpaw triglycerides, and pawpaw triglyceride fraction (Fig. 1B) derivatives. Coconut oil, which contains medium-chain fatty acids of known composition, was included for comparison. The unsaturated phenacyl esters of the fatty acids were not resolved on the DB-PS1 column, but even with this limitation the values were in good agreement with earlier values. Likewise, the butyl ester fatty acid composition of coconut oil was in good agreement with the earlier HPLC data (8). The composition of pawpaw triglyceride fatty acid phenacyl esters differed from the butyl ester profile in that the unsaturated octadecanoates eluting as a single peak represented a higher percentage of the total than the butyl ester values. Although these data do not appear to be quantitative, the utility of the phenacyl esters will become apparent later. The butyl ester derivatives of the pawpaw triglycerides show that octanoate represented more than 20% of the total fatty acids while hexanoate, decanoate, and dodecanoate values were low. The pattern of octanoate representing a significant percentage of the pawpaw triglycerides was prevalent in all fractions except number eight. The percentage of 18:3 increased as the degree of unsaturation in the triglyceride increased; i.e., band number eight contained nearly 50% 18:3.

Analysis of the hexadecenoates and octadecenoates. GLC analyses of the pawpaw fatty acid butyl esters or methyl esters on the DB-225 capillary column showed three peaks eluting near oleate. The identity of these fatty acids was established by two independent methods. Phenacyl esters of total pawpaw triglycerides as shown in Figure 3A were separated according to degree of unsaturation by Ag^+-TLC. The monoene phenacyl esters analyzed by GLC are shown in Figure 3B. This monoene fraction of phenacyl esters was used to isolate hexadecenoate and octadecenoate fractions by HPLC. The isolated 16:1 and 18:1 fractions of phenacyl esters as analyzed by GLC are shown in Figure 3C and 3D, respectively.

In Figure 4 the thermal degradation of phenacyl ozonides of pawpaw octadecenoates to yield phenacyl aldesters during GLC with (right) and without (left) triphenylphosphene is shown. The numbered peaks represent the position of the double bond with reference to the carbonyl carbon. The lettered peaks (A,B,C) are triphenylphosphine and degradation or rearrangement products. The hexadecenoate ozonide fractions gave rise to 9 and 11 phenacyl aldesters.

We found the phenacyl esters that we had previously used

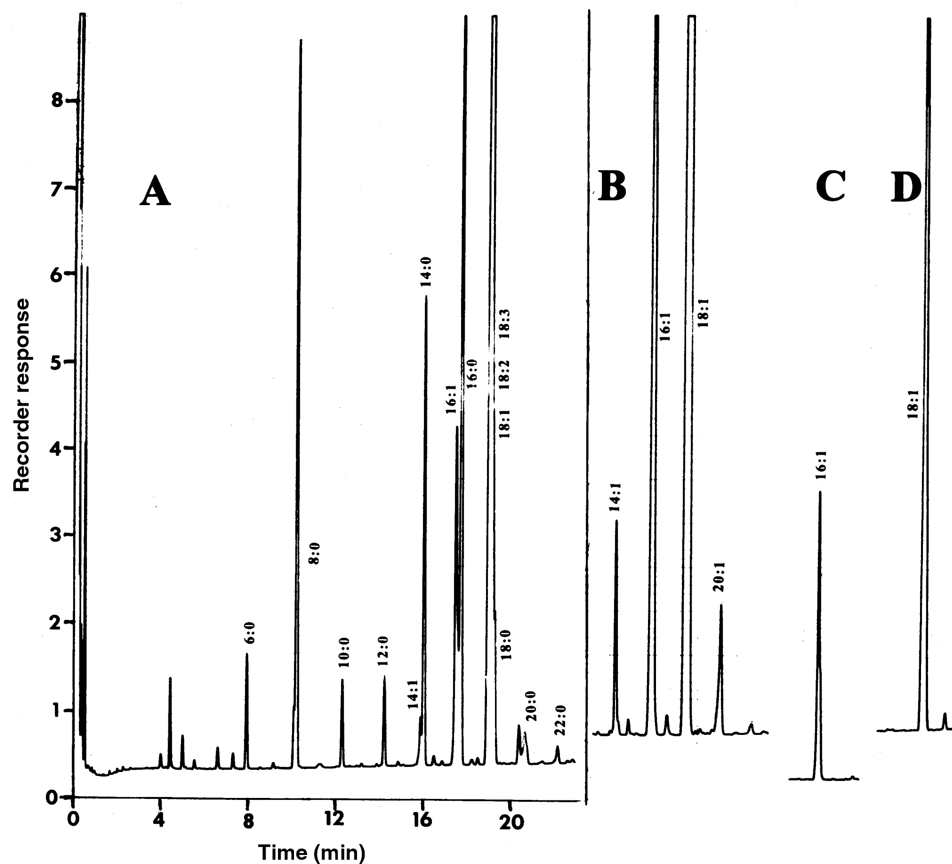

FIG. 3. (A) Typical gas–liquid chromatograms of pawpaw triglyceride fatty acid phenacyl esters; (B) monoene phenacyl esters isolated by silver ion-thin-layer chromatography; (C) phenacyl esters of hexadecenoates; and (D) octadecenoates isolated by high-performance liquid chromatography. Analytical conditions are described in the Methods and Materials section.

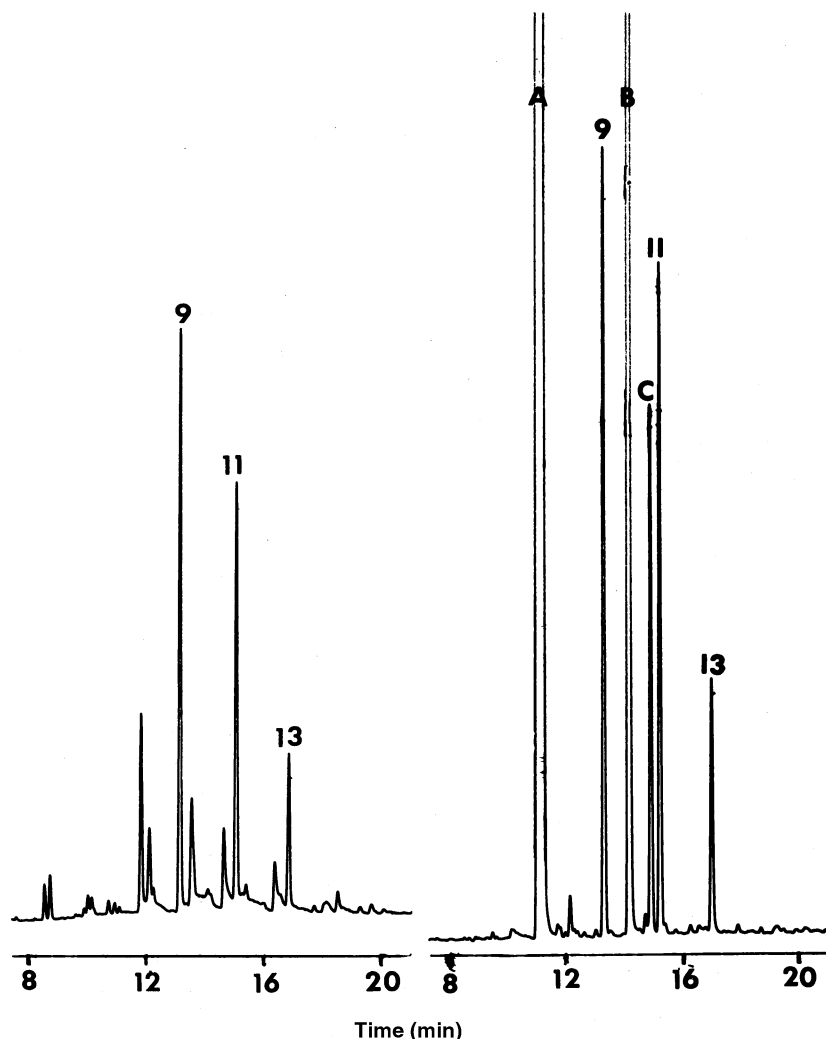

FIG. 4. These chromatograms show the thermal degradation products of pawpaw triglyceride octadecenoate ozonides of phenacyl esters in the presence (right) and absence (left) of triphenylphosphine. The numbered peaks of the aldesters represent the position of the double bonds relative to the carbonyl carbon. The lettered peaks represent triphenylphosphine and degradation or rearrangement products. Analytical conditions are described in the text.

as a chromophore for the analysis of fatty acids by HPLC very useful in determining structure. The phenacyl esters are stable to analysis by GLC with good peak symmetry (Fig. 3). The phenacyl derivatives can be separated by Ag^+-TLC and then analyzed by GLC (Fig. 3B) or further fractionated by HPLC and then checked for purity by GLC (Fig. 3C and D). Ozonides can be prepared from the monoene phenacyl esters and their thermal degradation products analyzed by GLC (Figs. 4 and 5). The stability of the phenacyl esters of fatty acids allows one to alternate from one type of analysis to another; and the derivatives are stable at reduced temperatures for long storage periods.

Analyses of the pawpaw triglyceride monoene methyl esters by GLC on a DB-225 capillary column and of an authentic mixture of Δ9 octadecenoate, Δ11 octadecenoate, and Δ13 octadecenoate methyl esters are shown in Figure 5A and 5B, respectively. Cochromatography of the pawpaw monoene methyl esters and the standard methyl esters showed that the octadecenoates coeluted.

DISCUSSION

This appears to be the first study to examine the lipids of the mature pawpaw fruit. It is quite clear (Table 1) that seed lipid fatty acid composition shows little relation to the fruit lipids. Both mature and immature seed triglyceride and polar lipids exhibited a simple fatty acid composition without medium-chain fatty acids or monoene fatty acid isomers. Although the fatty acid composition of the fruit and seed can be different (11), the marked contrast between the pawpaw fruit and seed is striking. Comparison of the triglyceride and polar lipid fatty acid compositions of the mature and immature pawpaw indicates that the medium-chain and isomer monoene fatty acid biosyntheses occur in the latter stages of development or dur-

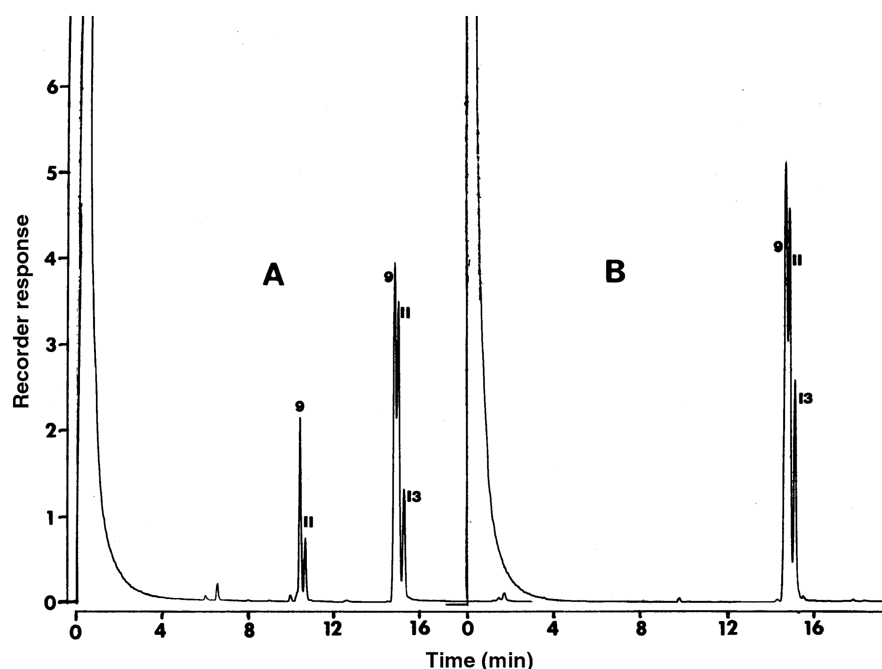

FIG. 5. These chromatograms show the separation of (A) pawpaw triglyceride monoene methyl esters and (B) authentic octadecenoate standards using a DB-225 capillary column. The first set of peaks in chromatograph A represent the hexadecenoates. Analytical conditions are described in the Methods and Materials section. Numbers beside the peaks represent the position of the double bonds relative to the carbonyl carbon.

ing ripening. Also, Table 1 indicates that 18:3, presumably linolenic acid, was more prominent in the immature seeds and fruit lipids. Elevated percentages of 18:3 in developing fibers of cotton were also observed (Wood, R., unpublished data). The involvement of 18:3 during the development of systems that have little or no 18:3 at maturity would seem to be an interesting area for investigation.

Analysis of the pawpaw fruit triglyceride intact and fatty acid derivatives by GLC on polar and nonpolar columns demonstrates that octanoate represents 20% of the total fatty acids. Methyl and ethyl octanoates, along with other volatiles, have been identified in the volatiles and head space volatiles of pawpaw fruit (12,13). Trace quantities of caprylic, capric, and lauric acids have been reported in the total lipids of the pawpaw fruit (14). The acids could have come from the volatile fraction (12,13). Our data appear to be the first to identify octanoate as a major component of the pawpaw fruit triglyceride. Presumably the triglyceride could be the source of the ethyl and methyl octanoates identified in the volatile fraction (12,13). This assumption is supported by TLC plate A in Figure 1, which shows a major band just above the standard cholesterol band, an area where diglycerides migrate. We did not examine this band to prove it was a diglyceride.

The carbon number distribution of the triglycerides fractionated according to degree of unsaturation (Table 2), and the fatty acid compositions of the fractions (Table 3) allow the triglyceride molecular species to be examined. Comparison of the calculated 1,2,3-random distribution with that of the determined total distribution shows a lack of agreement, but the reduced levels of carbon number 36 and 46 were apparent as observed in most fractions. The percentage of octanoate in all the fractions except band 8 (Table 3) indicates a selective pairing of octanoate with most diglyceride species. Examination of Figure 2 and Table 2, band 3, shows that carbon number 42 was one of the most abundant. Table 3 indicates that nearly one-third of the fatty acids was octanoate, or one for every triglyceride molecule. Figure 1B suggests that band 3 species probably contained two double bonds. Resolution 54:3 and 48:3 suggest that 42:2, of which one fatty acid is octanoate, would migrate in this region of the plate. The high percentage of 16:1 and 18:1 indicates the major triglyceride species in band 3 was 8:0-16:1-18:1. By using the same reasoning, carbon number 44 of band number 5 (Fig. 1B) contains 8:0-18:1-18:3 as the major species.

This study also appears to be the first to report the occurrence of significant quantities of three octadecenoate isomers in a single plant source. It can be assumed that there is one Δ9 desaturase and an active elongation system.

The occurrence of octanoate is not unique to the pawpaw fruit. Octanoate is found in edible oils (coconut and palm kernel oil), dairy products from milk goats, essential oils, and numerous other sources (11). Generally, the levels are relatively low and octanoate is flanked by shorter (6:0)- or longer (10:0)-chain fatty acids (or both), which are 25% or more of the concentration of octanoate. The high concentration of octanoate (20%) in the pawpaw fruit triglycerides, where the concentration of flanking fatty acids is about 3% of the total fatty acids, does appear to be unique. Clearly, there is an interesting metabolic process that leads to the accumulation of octanoate. It could be a unique fatty acid synthase or a unique

chain-shortening process. In the future, investigators may find the pawpaw a unique source of genes that can be used to improve the odor and flavor of other fruits and vegetables.

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I dedicate this manuscript to my dear departed mother, Tena Harris Wood (1918–1995), and my father, Ocie B. Wood, whose inspirations will last a lifetime. Thanks go to my sister, Linda Wood Sullivan, for collecting the pawpaws and to Shirley Edwards for manuscript preparations. Special thanks go to my loving wife, Arlene, for years of proofreading and being my best friend.

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Stability of Cyclopropane and Conjugated Linoleic Acids During Fatty Acid Quantification in Lactic Acid Bacteria

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ABSTRACT: Seven methods commonly used for fatty acid analysis of microrganisms and foods were compared to establish the best for the analysis of lyophilized lactic acid bacteria. One of these methods involves fat extraction followed by methylation of fatty acids, while the other methods use a direct methylation of the samples, under different operating conditions (e.g., reaction temperature and time, reagents, and pH). Fatty acid methyl esters were identified by gas chromatography–mass spectrometry and quantified by on-column capillary gas chromatography. Two reliable methods for the analysis of fatty acids in bacteria were selected and further improved. They guarantee high recovery of classes of fragile fatty acids, such as cyclopropane and conjugated acids, and a high degree of methylation for all types of fatty acid esters. These two direct methylation methods have already been successfully applied to the analysis of fatty acids in foods. They represent a rapid and highly reliable alternative to classical time- and solvent-consuming methods and they give the fatty acid profile and the amount of each fatty acid. Using these methods, conjugated linoleic acids were identified and quantified in lactic acid bacteria.

Paper no. L8208 in *Lipids 34*, 1107–1115 (October 1999).

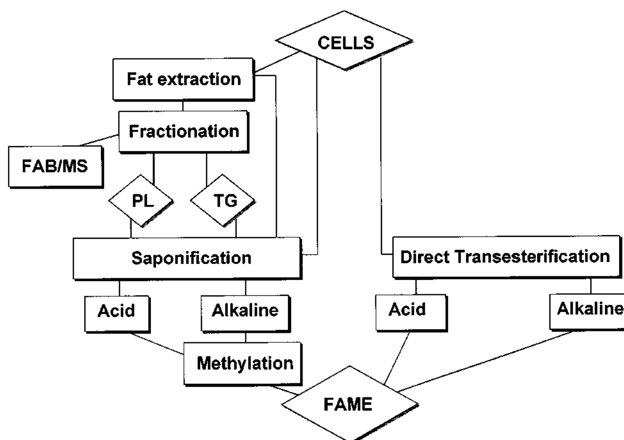
Fatty acid (FA) composition in microorganisms is assessed for several purposes in the field of food science. For many years, FA analysis has been recognized as a useful and rapid tool for the characterization of microorganisms in foods (1,2) and as a complementary analysis to taxonomic classification (1–12). In addition, knowledge of the FA composition may help to evaluate the nutritional quality of alternative microbiological sources of fats (9,13–17).

Many articles describe the influence of growth conditions, such as temperature (18–23), pH (23,24), medium (13–17,23), and oxidative stress (21) on FA composition. However, the main aim of our studies is to understand the influence of FA composition on membrane structure and functionality (in terms of fluidity and permeability). Knowing the FA composition of cell membranes can also help in understanding the mechanism of antioxidant and antimicrobial molecules (25).

Depending on the objective, it is possible to analyze the FA composition of complete cells or the FA distribution of membrane phospholipids (PL) (14,21). Of course, in this latter case, previous purification of PL is necessary (14). Several methods for FA analysis are described in the literature. Scheme 1 summarizes the possibilities for the analysis of fatty acids in bacteria. It shows that bacteria may be directly saponified in an acid (18) or alkaline environment (5,11,16, 19,25–29), or directly transesterified to fatty acid methyl esters (FAME) (30). The lipids may also be extracted and then saponified and esterified to FAME (3,6,12,13,22,24).

The most commonly applied procedure is based on direct saponification of the sample using methanolic NaOH followed by HCl- or BF_3-catalyzed methylation. Another widely used method involves lipid extraction (principally carried out according to Folch *et al.*, 31) and successive saponification/methylation of the total lipids or of the separated fractions of PL and triglycerides (TG). Another technique, developed by Aluyi *et al.* (21, cited in 32), consists of a fat extraction according to Folch *et al.* (31) and direct analysis by fast atom bombardment/mass spectrometry (FAB/MS). This method has the advantages of being very rapid and of identifying both PL and TG in a single analysis.

Recently, direct methylation of a sample using sodium methoxide as catalyst was successfully performed (30). This procedure is less time- and solvent-consuming. Other similar procedures have already been applied to the quantification of FA in oils and foods (33–35).



SCHEME 1

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Abbreviations: CLA, conjugated linoleic acid; DMF, dimethylformamide; FA, fatty acid; FAB/MS, fast atom bombardment/mass spectrometry; FAME, fatty acid methyl ester; GC–MS, gas chromatography–mass spectrometry; PL, phospholipid; TG, triglyceride.

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A critical point of a method for FA analysis in bacteria is the high reactivity of certain categories of FA under acidic conditions. Cyclopropenic acids and conjugated linoleic acid (CLA), especially, are easily decomposed. For this reason, several authors (1,26,30,36) strongly recommended avoiding acidic conditions. On the other hand, Mayberry and Lane (37) have stressed that alkaline methylation may not be strong enough to cleave all types of FA. The same authors therefore proposed a sequential alkaline/acidic hydrolysis.

The aim of the present work was to find an optimal procedure for the quantification of FA in whole cells of freeze-dried bacteria. Methods were compared according to their reliability and rapidity. Special attention was paid to the recovery of cyclopropanic acids and CLA. The most promising methods were tested and compared: (i) fat extraction with chloroform/meth-anol (31) followed by methylation in alkaline environment; (ii) acid-catalyzed direct methylation (35); (iii) alkali-catalyzed direct methylation (30,33,34).

EXPERIMENTAL PROCEDURES

Reagents. Quantitative 37-component FAME standard and methanolic hydrochloric acid were purchased from Supelco (Division of Fluka Chemie AG, Buchs, Switzerland). Methyl esters of cyclopropane fatty acids (*cis*-9,10- and *cis*-11,12-methyleneoctadecanoate) were purchased from Larodan Fine Chemicals AB Chemie Brunschwig (Basel, Switzerland). Triundecanin, 1-tetradecene, methylpelargonate, dimethylformamide (DMF) and disodium hydrogen citrate sesquihydrate were purchased from Fluka Chemie AG. Methanol, methanolic sodium methoxide solution, and water were purchased form Merck (Geneva, Switzerland). Hexane was purchased from Mallinckrodt Baker P.H. Stehelin (Basel, Switzerland). *Tert*-butyl-methyl ether and heptane were purchased from Sigma (Division of Fluka Chemie AG, Buchs, Switzerland). Octadecadienoic conjugated FAME (CLA standard) were purchased from Nu-Chek-Prep. Inc. (Elysian, MN).

Solutions. The internal standard solution 1 contains triundecanin, 1-tetradecene, and methylpelargonate in *tert*-butyl-methyl ether (1 mg/mL for each standard). The internal standard solution 2 contains triundecanin, 1-tetradecene, and methylpelargonate in hexane (1 mg/mL of each). The methylation solution contains sodium methoxide in methanol (1 M). Disodium hydrogen citrate in water (0.57 M) is used as neutralization solution.

Samples. Lyophilized lactic acid bacteria (*Lactobacillus*), obtained by fermentation, were used throughout the study. After fermentation the culture was centrifuged at 4500 rpm for 20 min at 10°C. Cell pellets were washed twice with sterile saline solution and freeze-dried using a LSL-Secfroid Lyolab-G lyophilizer (Aclens, Switzerland) for 24 h, with a final temperature of 20°C and a condenser temperature of 80°C. The dried vials, containing about 1 g dry matter for 10 mL suspension, were capped under air and stored at 4°C prior to analysis.

Methods 1.1, 1.2, and 1.3. These methods are based on the procedure developed by Suter and Grob (33,34) with minor modifications. Three different pretreatments were carried out to maximize recovery.

(i) Method 1.1 [with dimethylformamide (DMF) pretreatment]. One hundred milligrams of bacterial sample was treated with 2 mL of DMF at 100°C for 30 min. The subsequent steps were carried out according to "method 1.3 (without pre-treatment)."

(ii) Method 1.2 (with water pretreatment). One hundred milligrams of bacterial sample was treated with 2 mL of distilled water at room temperature for 5 min. The subsequent steps were carried out according to "method 1.3 (without pretreatment)".

(iii) Method 1.3 (without pretreatment). After the addition of 500 μg of internal standard (solution 1 in *tert*-butyl-methyl ether), 100 mg of bacterial sample was transesterified with 2 mL of 1 M methanolic sodium methoxide for 2 min at room temperature. FAME were extracted using 1 mL heptane and 4 mL of neutralization solution. After centrifugation at 2000 rpm for 5 min, the upper organic layer was diluted 10 times with hexane before gas chromatographic analysis.

Method 2. This method is based on the procedure developed by Ulberth and Henninger (35) with minor modifications. One hundred milligrams of bacterial sample, after the addition of 500 μg of internal standard (solution 2 in hexane), was transesterified using 1 mL of methanolic hydrochloric acid (1.5 M) and 1 mL methanol, at 80°C for 10 min. Two milliliters of distilled water was then added and an aliquot of the upper organic phase was diluted 25 times with hexane and used for gas chromatographic analysis.

Methods 3.1 and 3.2. Method 3.1 is based on a procedure developed by Rozès *et al.* (30). One hundred milligrams of bacterial sample spiked with 500 μg of internal standard (solution 2 in hexane) was transesterified with 2 mL of sodium methoxide (1 M in methanol) for 1 min at room temperature. FAME were then extracted using 1 mL of hexane. After centrifugation at 2000 rpm for 5 min, the organic upper phase was diluted 10 times with hexane before gas chromatographic analysis. The same method was also modified (method 3.2) using a water pretreatment. One milliliter of water was previously added to the bacterial sample and, after 5 min, the procedure was completed as described above.

Method 4. This method was developed by Folch *et al.* (31). Six hundred milligrams of bacterial sample spiked with 500 μg of internal standard (solution 2 in hexane) was extracted twice using 25 mL of chloroform/methanol solution (3:2, vol/vol) for the first extraction, and 15 mL of chloroform/methanol/water solution (10:10:1, by vol) for the second extraction. The liquid phase was filtered and washed with 10 mL of distilled water, and the aqueous phase was again extracted with 5 mL of the chloroform/methanol/water solution. The two organic phases were collected and evaporated under vacuum at 40°C. Extracted fat was transesterified using the procedure described in "Method 1.3 (without pretreatment)."

Method 5. This method is based on the procedure described by Muuse *et al.* (38). It comprises methylation of the sample (100 mg, spiked with methyl tridecanoate as internal

standard) using 2 M methanolic potassium hydroxide (reaction time: 2 min at room tempearture). After centrifugation for 5 min at 2000 rpm, the supernatant is diluted with hexane before gas chromatographic analysis.

Gas chromatography. A Carlo Erba HRGC 5300 gas chromatograph (Brechbühler SA, Geneva, Switzerland) equipped with a flame-ionization detector was used. Hydrogen was used as carrier gas (purity >99.9997 %). A CP-Sil 88 capillary column (50 m length, 0.32 mm i.d.) coated with 100% cyanopropyl/polysiloxane (0.25 µm film thickness) was purchased from Chrompack (P.H. Stehelin). The column was operated at 60°C for 2 min, then the temperature was increased at 15°C/min to 135°C, held 1 min, then increased at 3°C/min until the final temperature of 215°C was reached. Five microliters of each sample solution was injected. Response factors for each FA relative to the internal standard (triundecanin) were calculated using a Supelco 37 standard solution. The performance of the methylation reaction was checked according to Suter and Grob (33,34) by using the ratios between triundecanin, 1-tetradecene, and methyl-pelargonate internal standards.

Mass spectrometry. The FAME were identified on a Finnigan MAT 8430 double focusing mass spectrometer (Finnigan MAT, Bremen, Germany) connected to a HP 5890 gas chromatograph (HP, Geneva, Switzerland) equipped with a CP-Sil 88 capillary column (Chrompack); 50 m × 0.32 mm i.d.; film thickness, 0.25 µm. Helium was used as the carrier gas at a pressure of 10 psi. The samples were injected using an on-column injector. Oven program was 60°C for 2 min, then 15°C/min up to 135°C, held for 1 min, then 3°C/min up to 220°C for 10 min. The transfer line was held at 220°C and the source at 180°C. Electron impact mass spectra were acquired at 70 eV from 20 to 500 Da. The location of double bonds in unsaturated FA was determined after 2-alkenyl-4,4-dimethyloxazoline derivatization (39) by gas chromatography–mass spectrometry (GC–MS) on a Finnigan MAT SSQ-7000 quadrupole mass spectrometer connected to an HP 5890 gas chromatograph. All the GC–MS parameters were identical to those described above.

RESULTS AND DISCUSSION

Method comparison. The FA composition of lyophilized lactic acid bacteria (*Lactobacillus*) was determined using seven different methods to check their performance and to select the best. Six direct methylation methods (1.1, 1.2, 1.3, 2, 3.1, and 3.2; see the Experimental Procedures section) were compared with a method (4; see the Experimental Procedures section) involving a preliminary fat extraction before methylation. The direct methods differ in the type of pretreatment performed and the methylation reactives used.

All the analyses were performed in duplicate and the results were expressed as the means of the two values obtained. All methylated FA were identified by comparing their retention times and mass spectra with the one obtained from commercial standard FAME. The location of double bonds in unsaturated FA was established after 2-alkenyl-4,4-dimethyloxazoline derivatization and GC–MS analysis. For example, all the 18:2 FAME showed, after electron impact at 70 eV, a molecular ion at *m/z* 294, fragments at *m/z* 279 ($[M - CH_3]^+$), 263 ($[M - OCH_3]^+$), and the ion series 123, 109, 95, 81, 67,

TABLE 1
Fatty Acid (FA) Composition Obtained with Seven Different Methods[a]

	Method						
FA	1.1	1.2	1.3	2	3.1	3.2	4
8:0	0.07	0.12	1.09	0.06	0.34	0.82	0.18
10:0	0.07	0.13	0.29	0.09	0.28	0.44	0.21
12:0	0.51	0.73	1.64	0.47	0.80	2.35	0.79
13:0	0.06	0.10	0.29	0.05	0.43	<0.05	0.10
14:0	2.82	4.12	4.64	2.72	3.25	7.30	2.92
14:1n-5 + 15:0 *anteiso*	0.43	0.66	0.95	0.42	0.40	1.48	0.45
15:0	0.29	0.37	<0.05	0.28	<0.05	0.51	0.28
16:0	9.49	10.98	12.50	9.40	14.80	13.22	9.76
16:1n-7	4.73	6.41	6.97	4.62	4.56	10.11	4.49
17:0	0.21	0.24	<0.05	0.22	0.11	<0.05	0.24
17:1n-7	0.59	0.78	0.43	0.65	0.69	0.79	0.63
18:0	3.83	3.17	3.61	4.38	5.70	2.95	4.03
18:1 total *trans* isomers	1.80	1.71	1.48	1.80	3.00	1.85	1.98
18:1n-9, n-7, and other *cis*	34.74	35.99	35.30	35.69	35.22	33.98	34.03
18:2 total *trans* isomers	0.44	0.57	<0.05	0.45	<0.05	<0.05	0.49
18:2n-6 *cis*	1.39	1.60	2.07	1.29	1.83	2.16	1.46
18:3n-3α	0.23	0.29	<0.05	0.20	0.28	0.70	0.21
9,10-methylene-18:0	32.22	25.42	24.37	30.53	24.82	15.86	31.45
20:1n-9 + 18:2 conj.	4.46	4.92	4.37	4.53	3.45	4.29	4.29
Total other FA	1.63	1.70	0.00	2.14	0.06	1.18	2.01
Total FA	100.00	100.00	100.00	100.00	100.00	100.00	100.00
g Total FA/100 g bacteria	2.21	1.44	0.13	2.64	0.30	0.82	0.46

[a]Results expressed as g FA/100 g total FA and as g total FA/100 g bacteria. Methods are described in detail in the Experimental Procedures section.

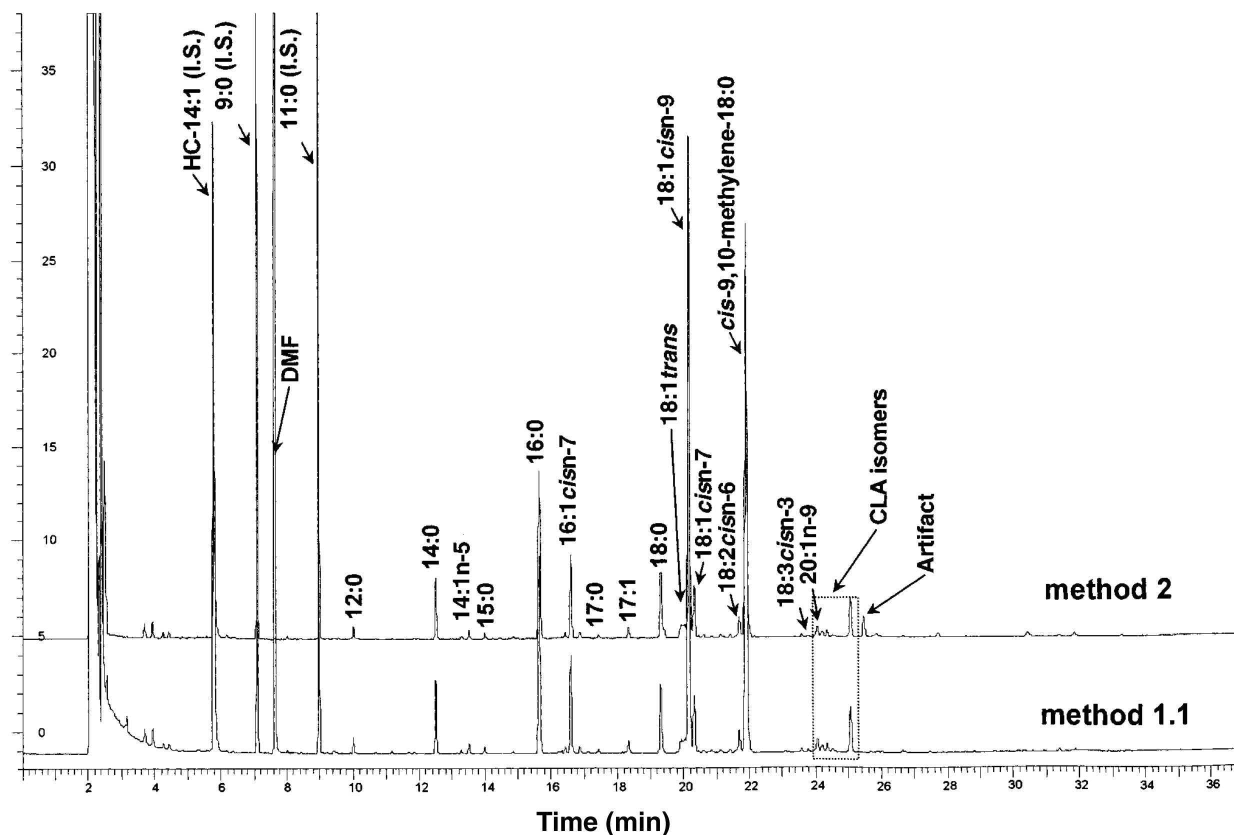

FIG. 1. Fatty acid (FA) profile of lyophilized bacteria obtained with methods 1.1 and 2 (see Experimental Procedures section). CLA, conjugated linoleic acid; DMF, dimethylformamide; I.S., internal standard; HC-14:1, 1-tetradecene.

55, and 41. After 2-alkenyl-4,4-dimethyloxazoline derivatization, the mass spectrum of a 18:2 Δ9,11 showed a molecular ion at *m/z* 333 and diagnostic fragments at *m/z* 234, 222, 208, and 196, whereas the spectrum of an 18:2 Δ10,12 isomer displayed a molecular ion at *m/z* 333 and diagnostic fragments at *m/z* 248, 236, 222, and 210 (39). Additional information about *cis/trans* isomerization was taken from Chardigny *et al.* (40), Shantha *et al.* (41), and Chin *et al.* (42). *cis*-9,10-Methyleneoctadecanoate methyl ester (dihydrosterculic acid methyl ester) was identified and is one of the major fatty acids found in the lactic acid bacteria analyzed.

In Table 1 the results are shown as FA composition (g FA/100 g total FA) and as total absolute quantity of FA recovered. The recovery of total FA varies widely between the different methods, being higher for methods 1.1 and 2 and lower for methods 1.2 and 3.1. Method 4 had a very low FA recovery.

Method 4 is widely used for the analysis of biological materials (3,6,12,13,22,24) and operates under mild conditions that avoid FA destruction (especially CLA and cyclopropane FA). Therefore, we have considered it as a reference for comparison of the other methods. From this comparison, we can immediately eliminate methods 1.2, 1.3, 3.1, and 3.2 which give a different FA composition and a decrease in fragile cyclopropane FA. Methods 1.1 and 2 gave the best results. Method 2 was also retained as a valuable procedure because of its high absolute total FA recovery. This method, in addition, involves acid methylation, which is effective on all the classes of lipids (43), is very rapid, and avoids pretreatment. On the other hand, alkaline-catalyzed methylation methods have been demonstrated in the literature not to be sufficiently effective on free FA or amide-linked FA (such as a part of hydroxylated FA) (37), while acting only on esterified FA (43).

Figure 1 shows the chromatograms obtained with methods 1.1 and 2. We retained methods 1.1 and 2 as valid alternatives to method 4 because of their high total FA recovery and accurate FA profile. In addition, these two methods are less solvent- and time-consuming than the other methods screened here.

Method repeatability. Repeatability was calculated for methods 1.1 and 2 by repeating the complete analysis four times. Repeatability of the two methods, as relative standard deviation (RSD%), was very similar and corresponded to 10% for values <1 g FA/100 g total FA; 5% for values between 1 and 5 g FA/100 g total FA; 2.5% for values >5 g FA/100 g total FA.

CLA analysis. Figure 2 shows the enlarged region of CLA isomers of the gas chromatogram (comparison of methods 1.1, 2, and 4). Because concerns were expressed in the literature (41,43) about the analysis of CLA FA in an acidic environment (as in method 2), several experiments were performed to check the stability of CLA during the analysis.

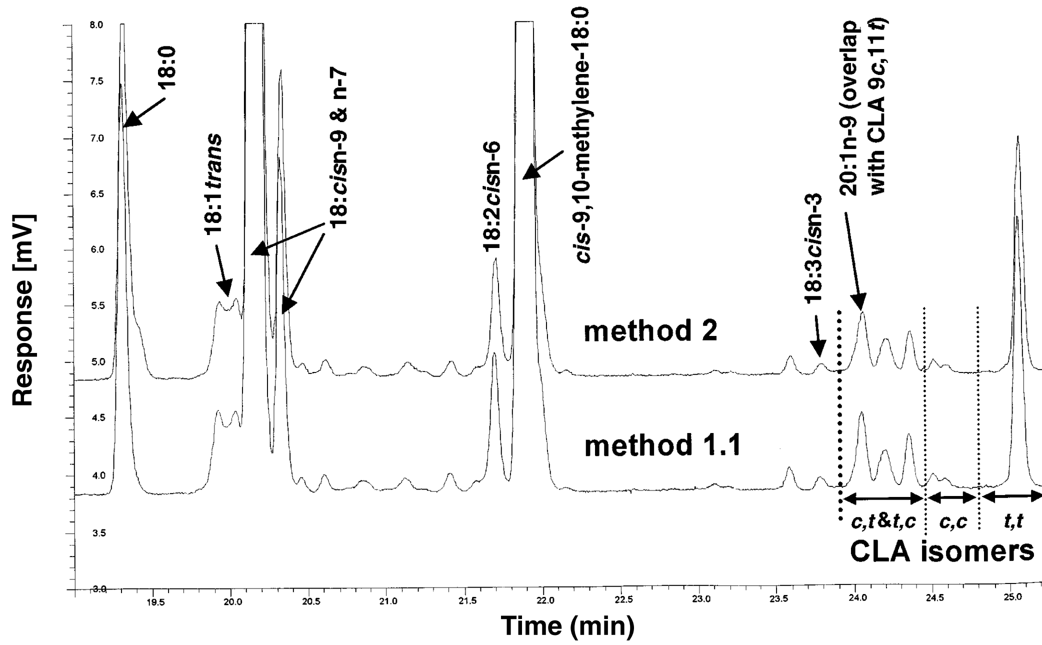

FIG. 2. Enlarged chromatogram of the region 18:0, 18:1, 18:2, and CLA isomers. See Figure 1 for abbreviation.

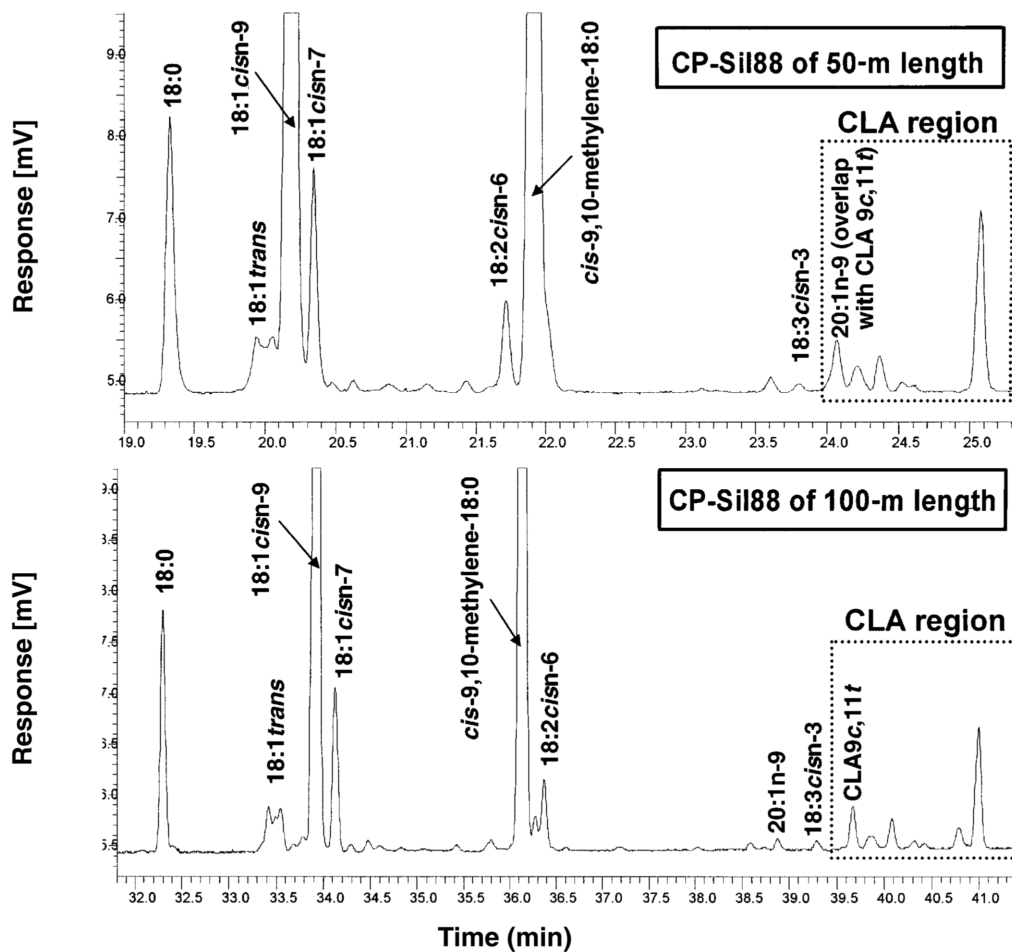

FIG. 3. Chromatogram of enlarged CLA region, obtained with 50- and 100-m CP-Sil 88 capillary columns. See Figure 1 for abbreviation.

TABLE 2
CLA Composition (as relative %) of a Butter Sample Analyzed by Methods 2 and 5[a]

	Method	
	2	5
Sum of *cis,trans* and *trans,cis* isomers	86	85
Sum of *cis,cis* isomers	3	5
Sum of *trans,trans* isomers	11	10
Total	100	100

[a]CLA, conjugated linoleic acid.

An experiment was carried out to check whether acidic pH and high temperatures during analysis lead to the formation of *trans,trans*-CLA isomers. A sample of butter (CLA < 1%) was analyzed using method 2 (acid-catalyzed methylation) and method 5 (alkaline cold methylation). For this experiment a 100-m CP-Sil 88 capillary column, with 0.25 mm i.d. and a film thickness of 0.2 μm, was used instead of the 50-m column used previously. With this column, better separation and quantification of the different CLA isomers were achieved due to separation of the 20:1n-9 and *c*9,11*t*-18:2 isomers. In Figure 3, the CLA profiles obtained with 50- and 100-m CP-Sil 88 columns are compared.

Table 2 summarizes the results obtained, expressed as normalized surfaces. They show that no transformation of *cis*-CLA forms into *trans*-CLA forms occurs. In order to assess the best analytical conditions (in terms of temperature, time, and acid concentration) for the analysis of lyophilized bacteria using method 2 (with methanolic HCl), a kinetic study was carried out. Four different temperatures (room temperature, 60, 80, and 100°C), four different reaction times (10, 30, 120 min, and 18 h), and three different acid concentrations (0.5,

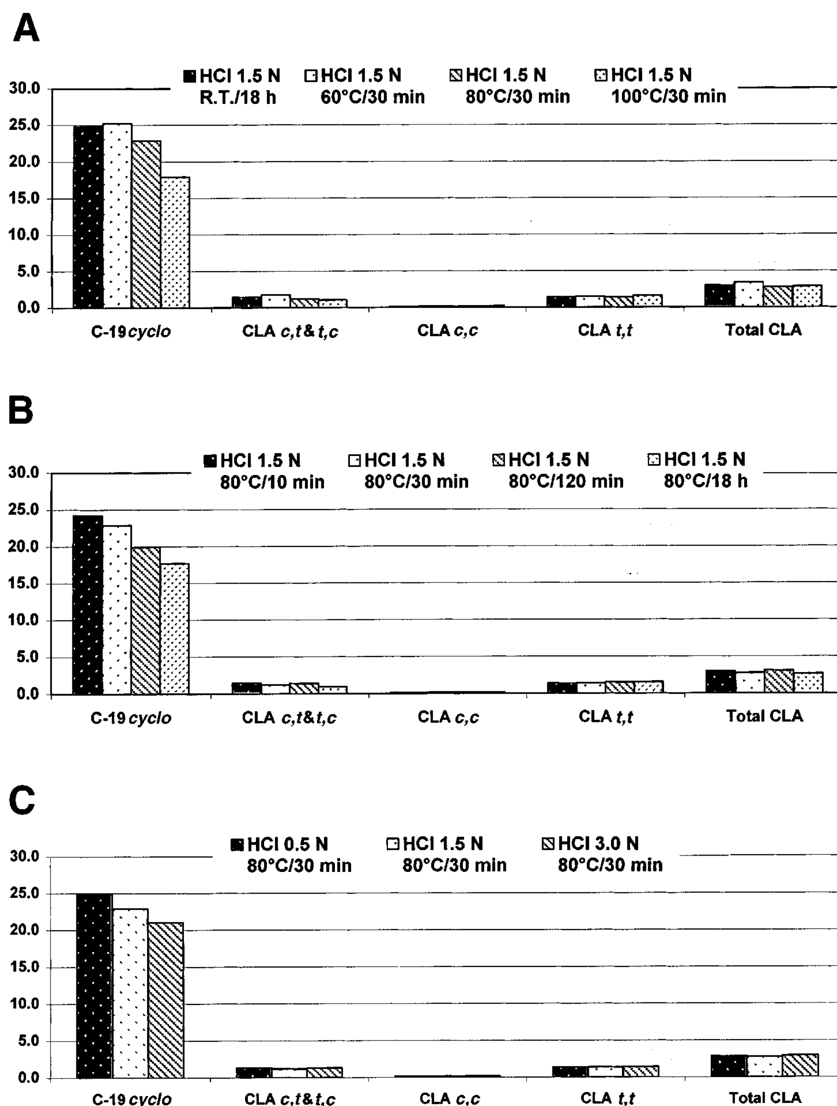

FIG. 4. Influence of temperature (A), time (B), and acid concentration (C) on the stability of cyclopropanic and CLA isomers in lyophilized lactic acid bacteria. Results expressed in g fatty acid/100 g total fatty acids. R.T., room temperature; for other abbreviation see Figure 1.

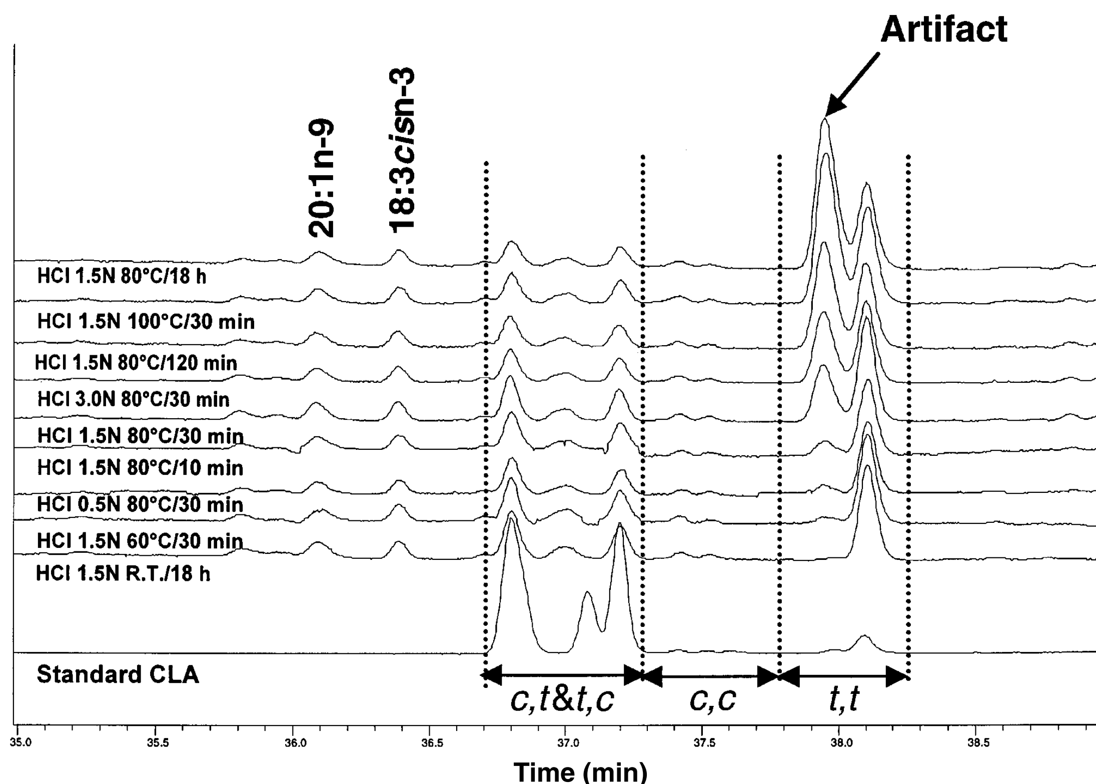

FIG. 5. Formation of degradation products during methylation of CLA isomers. The peak denoted "artifact" has been tentatively identified as 10-methyl-9-methoxy-octadecanoic acid methyl ester. See Figures 1 and 4 for abbreviations.

1.5, and 3.0 N) were compared. The results obtained for cyclopropane and CLA acids are shown in Figure 4, expressed as g FA/100 g of total FA. The results clearly demonstrate that a combination of a too-high acid concentration (higher than 1.5 N), at a too elevated temperature (higher than 60–80°C), and a too long reaction time (longer than 30 min) may degrade the FA. The formation of a degradation product is clearly visualized in Figure 5, where the chromatograms obtained under the different analytical conditions are compared. This artifact has been identified as 9-methoxy-10-methyl-octadecanoic acid methyl ester. Its mass spectrum showed a molecular ion at *m/z* 342 (<0.5%, confirmed after positive chemical ionization) and the diagnostic fragment ions at *m/z* 311 (2.9%), 279 (2.4%), 229 (3.4%), 201 (100%), 185 (12.4%), 169 (26.0%), 157 (71.9%), and 137 (36.9%). This compound is formed during derivatization by reaction between methanol and the cyclopropanic cycle of *cis*-9,10-methyleneoctadecanoic acid. In summary, the best combination of analytical parameters was (i) 80°C for 30 min with 0.5 N HCl; (ii) 60°C for 30 min with 1.5 N HCl; (iii) 80°C for 10 min with 1.5 N HCl; and (iv) room temperature for 18 h with 1.5 N HCl. These conditions lead to very similar profiles of CLA isomers with minimal formation of artifacts. Due to speed of analysis and minimization of artifact formation, the preferable conditions of analysis are 60°C for 30 min with 1.5 N HCl.

Evaluation of these results leads to the conclusion that method 2 (involving acid catalysis and heat treatment), when applied in the conditions specified, does not lead to substantial modification of CLA in lyophilized bacteria.

Analysis of lyophilized lactic acid bacteria. Method 2 was used to analyze five different types of lyophilized lactic acid bacteria to further confirm our findings. The results obtained are shown in Table 3 as duplicate values and means and confirm that method 2 is reliable and suitable for the analysis of FA composition in different types of lyophilized lactic acid bacteria.

A reliable and quantitative method for FA analysis in lyophilized lactic acid bacteria should respond to two principal criteria: first, to enable complete methylation and recovery of all types of FA esters (as well as amides and steryl esters) and, second, to avoid disruption of fragile FA (especially CLA and cyclopropane FA). Rapidity and facility of analysis are also prerequisites.

We have found two reliable methods (methods 1.1 and 2) for the quantification of FA in lyophilized lactic acid bacteria that guarantee high recovery of fragile classes of FA and a high degree of methylation for all the types of FA. These two direct methylation methods have already been successfully applied to the analysis of FA in foods. They represent rapid and reliable alternatives to classical time- and solvent-consuming methods. In addition, they not only give the FA composition but also allow the quantification of each FA. By

TABLE 3
FA Composition of Five Lyophilized Bacterial Samples (*Lactobacillus*)[a]

Bacterial strain	Sample 1			Sample 2			Sample 3			Sample 4			Sample 5		
sample number	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean	1	2	Mean
8:0	0.0	0.0	0.0	0.2	0.1	0.1	0.0	0.0	0.0	0.1	0.1	0.1	0.0	0.1	0.1
10:0	0.0	0.1	0.1	0.3	0.4	0.3	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
12:0	0.4	0.4	0.4	0.8	0.8	0.8	0.7	0.7	0.7	0.6	0.6	0.6	0.6	0.7	0.7
14:0	3.7	3.2	3.5	4.4	4.3	4.3	3.2	3.2	3.2	3.2	3.3	3.2	3.2	3.4	3.3
14:1n-5	0.3	0.3	0.3	0.5	0.6	0.5	0.5	0.4	0.5	0.4	0.4	0.4	0.3	0.4	0.3
15:0	0.3	0.3	0.3	0.4	0.5	0.4	0.3	0.3	0.3	0.4	0.3	0.3	0.4	0.4	0.4
16:0	10.1	9.7	9.9	13.7	13.4	13.6	8.5	8.9	8.7	9.0	9.4	9.2	10.3	10.5	10.4
16:1n-7	4.8	4.3	4.5	3.6	4.0	3.8	4.4	4.3	4.3	4.1	4.2	4.2	4.0	4.2	4.1
17:0	0.3	0.4	0.4	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3	0.3
17:1	0.9	0.8	0.8	0.6	0.7	0.6	0.8	0.8	0.8	0.8	0.8	0.8	0.8	0.7	0.7
18:0	3.7	4.2	3.9	2.9	3.0	2.9	4.5	4.5	4.5	4.8	5.0	4.9	5.5	5.7	5.6
18:1 total *trans* isomers	3.0	3.3	3.1	2.4	2.6	2.5	3.7	3.3	3.5	3.8	3.6	3.7	3.8	3.4	3.6
18:1n-9,n-7 and others	46.2	45.5	45.9	46.6	48.3	47.4	36.5	36.9	36.7	38.9	39.6	39.2	21.8	22.6	22.2
18:2 total *trans* isomers	0.5	0.6	0.5	0.4	0.4	0.4	0.5	0.4	0.5	0.4	0.5	0.5	0.4	0.2	0.3
18:2n-6 *cis*	2.0	1.6	1.8	0.9	1.6	1.3	2.4	1.5	1.9	2.0	1.4	1.7	2.9	1.4	2.1
18:2 conjugated (total CLA)	4.4	4.5	4.5	4.1	3.4	3.7	3.2	3.2	3.2	3.5	3.2	3.3	3.3	3.3	3.3
18:3n-3α	0.2	0.2	0.2	0.1	0.1	0.1	0.2	0.2	0.2	0.2	0.2	0.2	0.3	0.3	0.3
19-cyclopropane	16.1	17.4	16.8	14.3	13.3	13.8	27.2	28.1	27.7	24.5	25.3	24.9	38.6	40.2	39.4
20:1n-9	0.1	0.2	0.2	0.2	0.3	0.3	0.3	0.3	0.3	0.3	0.2	0.2	0.2	0.2	0.2
22:0	0.1	0.0	0.0	0.0	0.0	0.0	0.3	0.1	0.2	0.1	0.0	0.0	0.0	0.0	0.0
Total other FA	2.6	2.9	2.8	3.2	2.0	2.6	2.5	2.3	2.4	2.6	1.6	2.1	3.2	1.9	2.5
Total FA	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0

[a]Results expressed as g FA/100 g total FA. See Tables 1 and 2 for abbreviations.

using these two preparative methods and GC–MS analysis, it was possible to identify and quantify CLA in lactic acid bacteria.

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Synthesis of *trans*-4,5-Epoxy-(*E*)-2-decenal and Its Deuterated Analog Used for the Development of a Sensitive and Selective Quantification Method Based on Isotope Dilution Assay with Negative Chemical Ionization

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ABSTRACT: The volatile compound *trans*-4,5-epoxy-(*E*)-2-decenal (**1**) was synthesized in two steps with good overall yields. The newly developed method is based on *trans*-epoxidation of (*E*)-2-octenal with alkaline hydrogen peroxide followed by a Wittig-type chain elongation with the ylide formylmethylene triphenylphosphorane. For the synthesis of [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (***d*-1**), [2,3-$^2H_2$]-(*E*)-2-octenal was prepared by reduction of 2-octyn-1-ol with lithium aluminum deuteride and subsequent oxidation of [2,3-$^2H_2$]-(*E*)-2-octen-1-ol with manganese oxide. Compound ***d*-1** was used as internal standard for the quantification of **1** by isotope dilution assay. Among various mass spectrometry (MS) ionization techniques tested, negative chemical ionization with ammonia as reagent gas gave best results with respect to both sensitivity and selectivity. The detection limit was found to be at about 1 pg of the analyte introduced into the gas chromatography–MS system.

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trans-4,5-Epoxy-(*E*)-2-decenal (**1**) was first reported by Selke and coworkers (1) as a reaction product of autoxidized trilinolein. Model studies revealed 12,13-epoxy-9-hydroperoxy-10-octadecenoate, a degradation product of 13-hydroperoxy-9,11-octadecadienoic acid (13-HPOD), as a precursor of **1** (2). In addition, formation of **1** from 9-hydroperoxy-10,12-octadecadienoic acid (9-HPOD) and 2,4-decadienal as key intermediates has recently been reported in a systematic study (3). A reaction route was proposed for the formation of **1** from linoleic acid (4).

Compound **1** has been described in various food systems as a potent odorant eliciting metallic and green notes (5,6). It contributes to the development of off-flavors such as the green, hay-like flavor of soybean oil when stored in the dark (7) and "warmed-over" flavor of refrigerated cooked meat (8,9). Its sensorial relevance is due to low odor thresholds, i.e., 1.5 pg/L air (6), 15 ng/L water (10), and 1.3 [(μg/L oil (7)].

The role of **1** in biological systems has also been studied. Several α,β-unsaturated aldehydes formed upon lipid peroxidation have been shown to modify proteins, i.e., 4-hydroxy-2-alkenals (11) and 4,5-epoxy-2-alkenals (12). Compound **1** has been reported to be an intermediate in the formation of 4,5-dihydroxy-2-decenal (13), a cytotoxic aldehyde originating from the peroxidation of liver microsomal lipids (14). On the other hand, epoxyaldehydes such as *trans*-4,5-epoxy-(*E*)-2-heptenal and **1** may react with amino groups located in side chains of amino acids (15). Polymeric pyrrole derivatives identified in model systems containing such epoxyaldehydes and lysine are responsible for color formation and fluorescence (12,16). These reactions also generate 1-substituted pyrroles that have been proposed as indicators of oxidative stress in biological systems (12).

Compound **1** has recently been obtained by (salen)Mn(III)-catalyzed asymmetric epoxidation of (*E*,*Z*)-2,4-decenol with concomitant oxidation of the allylic alcohol to the aldehyde in 16% yield (17). However, most synthesis procedures reported so far are based on epoxidation of (*E*,*E*)-2,4-decadienal with 3-chloroperbenzoic acid (6,12). The deuterated analog (***d*-1**) was similarly prepared using labeled (*E*,*E*)-2,4-decadienal as starting material (4). Main limitations of the synthesis procedures are low yields, typically 5–10%, and time-consuming purification steps such as column chromatography (CC) and high-performance liquid chromatography.

In general, the amounts of **1** found in foods are low, i.e., up to ~11 μg/kg in refrigerated cooked meat (9,10), ~12 μg/kg in soybean oil stored in the dark (4), and 13–20 μg/kg in virgin olive oils (18). These values were obtained by the isotope dilution assay (IDA) quantification method (4,10), which is based on spiking the sample with known amounts of a labeled substance prior to sample preparation and analysis by gas chromatography–mass spectrometry (GC–MS) (19). In this way, losses can be accounted for because of the almost identical chemical and physical properties of the labeled internal standard and the analyte to be quantified. Unfortunately, quantification of **1** implies laborious cleanup steps such as distillation in high vacuum, CC and high-performance liquid chromatography

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Abbreviations: CC, column chromatography; COSY, homonuclear correlation spectroscopy; EI, electron ionization; GC, gas chromatography; HETCOR, heteronuclear correlation spectra; IDA, isotope dilution assay; MS, mass spectrometry; NCI, negative chemical ionization; NMR, nuclear magnetic resonance; NOE, nuclear Overhauser effect; PC, phosphatidylcholine; PCI, positive chemical ionization; PE, phosphatidylethanolamine; RI, retention index; SIM, selected ion monitoring; THF, tetrahydrofuran.

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leading to extraction yields of about 1% (4). Thus, very sensitive detection methods are required for the quantification of **1**. Furthermore, neither **1** nor ***d*-1** is commercially available, but both are required for applying IDA. In this paper, we report a newly developed synthesis procedure of ***d*-1** and its unlabeled analog and an improved quantification method of **1** based on IDA using negative chemical ionization (NCI) as ionization technique.

EXPERIMENTAL PROCEDURES

Materials. Lithium aluminum deuteride ($LiAl^2H_4$, >99% deuterium), deuterium oxide (^2H_2O, heavy water, >99.8% ^2H), manganese oxide (MnO_2), formylmethylene triphenylphosphorane, anhydrous tetrahydrofuran (THF) (H_2O <0.005%, stored over molecular sieves), diethyl ether (Et_2O), methylenechloride (CH_2Cl_2), and Celite 545 were purchased from Fluka (Buchs, Switzerland). (*E*)-2-Octenal was obtained from Aldrich (Buchs, Switzerland) and 2-octyn-1-ol from Lancaster (Morecambe, England). Hydrogen peroxide (H_2O_2), salt (NaCl), sodium hydroxide (NaOH), sodium hydrogen carbonate ($NaHCO_3$), sodium sulfate (Na_2SO_4), hexane, heptane, methanol (MeOH), and silica gel 60 (70–230 mesh) were from Merck (Darmstadt, Germany). The egg phospholipids phosphatidylcholine (PC) and phosphatidyl-ethanolamine (PE) were obtained from Avanti Polar Lipids (Copenhagen, Denmark).

*Formation of **1** in heated phospholipids.* One gram of egg PC or egg PE was dispersed in a phosphate buffer (50 mL, 0.5 M, pH 5.6) by stirring magnetically. The dispersion was then heated for 30 min from room temperature to 145°C in a laboratory autoclave (Berghof, Eningen, Germany). After cooling, ***d*-1** (2.44 µg) was added to the reaction mixture that was then saturated with NaCl. The neutral compounds were continuously extracted from the aqueous phase for 15 h with Et_2O using a liquid-liquid extractor. The solvent extract was applied to high-vacuum transfer (20) in order to remove nonvolatile compounds. The condensate containing volatile compounds was concentrated to 1 mL before GC–MS analysis using a Vigreux column (50 × 1 cm).

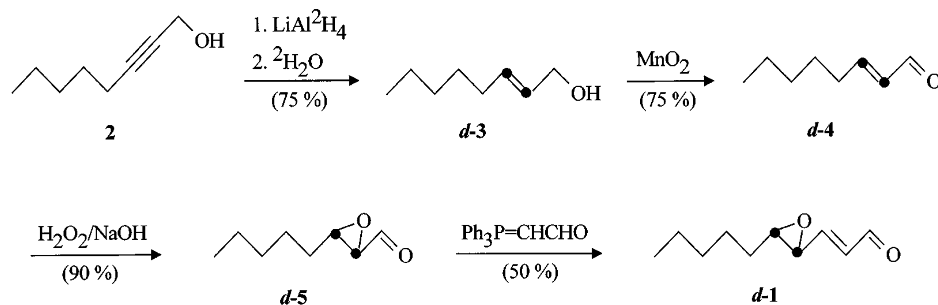
Capillary GC. This was performed on a Hewlett-Packard HP-5890 gas chromatograph (Geneva, Switzerland) equipped with a splitless injector and a flame-ionization detector. Fused-silica capillary columns were used, i.e., DB-5, DB-OV 1701, DB-FFAP, and DB-WAX (J&W Scientific, Folsom, CA; 30 m × 0.32 mm, film thickness 0.25 µm). Helium was used as carrier gas (100 kPa). The gas chromatograph was operated at an injector temperature of 250°C and a detector temperature of 250°C. The ovens were programmed as follows: 20°C, 70°C/min to 50°C, 4°C/min to 180°C, 10°C/min to 240°C (10 min) for DB-5; 20°C (2 min), 40°C/min to 50°C (2 min), 4°C/min to 150°C, 10°C/min to 240°C (15 min) for FFAP; and 20°C (1 min), 70°C/min to 60°C, 6°C/min to 180°C, 10°C/min to 240°C (15 min) for OV-1701 and DB-Wax. Linear retention indices (RI) were calculated (21).

GC–MS. Qualitative and quantitative GC–MS analysis was performed on a Finnigan SSQ 7000 mass spectrometer (Bremen, Germany) using the following conditions: electron ionization (EI) mass spectra were generated at 70 eV; positive chemical ionization (PCI) and negative chemical ionization (NCI) were carried out at 200 eV with ammonia or isobutane as reagent gas. Samples (2 µL) were introduced *via* a Hewlett-Packard HP-5890 gas chromatograph equipped with an HP-7673 autosampler using the following conditions: splitless injection at 250°C on fused-silica capillary column DB-OV 1701 described above. The carrier gas was helium (90 kPa). The temperature program was as follows: 60°C (2 min), 6°C/min to 180°C, 10°C/min to 240°C (10 min). Quantitative measurements were carried out in the selective ion monitoring (SIM) mode measuring characteristic ions of **1** and ***d*-1**. Calibration curves were obtained using mixtures of defined amounts of analyte (**1**) and labeled internal standard (***d*-1**) (4). Nine mixtures **1/*d*-1** were used, i.e., from 0.5 + 9.5, 1 + 9, 2 + 8, 3 + 7, and 5 + 5 to 7 + 3, 8 + 2, 9 + 1, and 9.5 + 0.5. The concentrations (µg/mL) of **1** and ***d*-1** used for preparing the mixtures were adapted to the varying sensitivity of the ionization modes used, i.e., 101.6/105.8 (PCI/isobutane), 10.2/10.6 (NCI/isobutane; PCI/ammonia), and 1.0/1.1 (NCI/ammonia). Samples for establishing the calibration curves and for quantification were injected twice.

Nuclear magnetic resonance (NMR) spectroscopy. The samples for NMR spectroscopy were prepared in WILMAD 528-PP 5-mm Pyrex NMR tubes, using as solvent about 0.7 mL of 99.8% C^2HCl_3 (Dr. Glaser AG, Basel, Switzerland) from a sealed vial. The NMR spectra were acquired on a Bruker AM-360 spectrometer (Karlsruhe, Germany), equipped with a quadrinuclear 5-mm probe head, at 360.13 MHz for ^1H and at 90.56 MHz for ^{13}C under standard conditions. The probe temperature was 21°C for the proton spectra and slightly higher for the carbon spectra, owing to heteronuclear composite pulse decoupling. All shifts are cited in ppm from the internal tetramethylsilane (TMS) standard. Where appropriate, proton nuclear Overhauser effect (NOE) difference spectra, distortionless enhancement by polarization transfer (DEPT) and/or fully proton-coupled spectra, and two-dimensional homonuclear and heteronuclear correlation spectra (COSY and HETCOR, respectively) were acquired as described earlier (22).

*Synthesis of [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (**d-1**).* The synthesis procedure for ***d*-1** is shown in Scheme 1. Commercially available 2-octyn-1-ol (**2**) was used as starting material to prepare [2,3-$^2H_2$]-(*E*)-2-octen-1-ol (***d*-3**) by reduction with $LiAl^2H_4$ followed by deuterolysis (22). Oxidation of ***d*-3** with MnO_2 according to (23) resulted in [2,3-$^2H_2$]-(*E*)-2-octenal (***d*-4**). [2,3-$^2H_2$]-*trans*-2,3-Epoxyoctanal (***d*-5**) was obtained by reaction of ***d*-4** with alkaline H_2O_2 (24). Chain elongation was achieved by a Wittig reaction of ***d*-5** with the ylide formylmethylene triphenylphosphorane (25) resulting in ***d*-1** as the major product.

[2,3-$^2H_2$]-(E)*-2-Octen-1-ol* (**d-3**) (Scheme 1, step ***a***). In a three-necked reactor (200 mL) fitted with a reflux condenser and a thermometer, $LiAl^2H_4$ (4.2 g, 100 mmol) was suspended in anhydrous THF (50 mL). 2-Octyn-1-ol (10.0 g, 79 mmol) dissolved in anhydrous THF (50 mL) was slowly added to the



SCHEME 1

magnetically stirred solution. The mixture was refluxed for 1 h and then stored overnight at room temperature. GC analysis indicated complete reduction of **2** to ***d*-3**. On cooling in an ice bath, heavy water (25 mL) was added drop by drop, followed by aqueous H_2SO_4 (4 N, 80 mL) to dissolve any insoluble residual materials. The organic phase was separated and the aqueous solution was then extracted with Et_2O (3 × 50 mL). The combined organic phases were washed successively with saturated solutions of $NaHCO_3$ (2 × 10 mL) and NaCl (2 × 10 mL), then dried over anhydrous Na_2SO_4. After removing the solvent by evaporation, 7.7 g (59 mmol, 75% yield) of a colorless oil of ***d*-3** with a purity of >97% (GC) was obtained. GC: RI (DB-5) = 1169, RI (OV-1701) = 1188, RI (FFAP) = 1617, RI (DB-WAX) = 1615. MS (EI) *m/z* (rel%): 130 (1, $[M]^+$), 112 (6, $[M-H_2O]^+$), 97 (7), 96 (5), 85 (8), 84 (10), 83 (37), 82 (18), 71 (11), 70 (25), 69 (32), 68 (23), 59 (100), 58 (40), 57 (32), 56 (48), 55 (33), 45 (32), 44 (16). The molecular ion was confirmed by PCI, isobutane, *m/z* (rel%): 113 (100, $[M - H_2O + H]^+$).

*[2,3-$^2H_2$]-(E)-2-Octenal (**d-4**) (Scheme 1, step b).* MnO_2 (20 g) was suspended in a solution of ***d*-3** (2.0 g, 15.4 mmol) in hexane (50 mL). Oxidation of ***d*-3** to ***d*-4** was complete after stirring the mixture at room temperature for 18 h. The mixture was filtered through a short pad of Celite to remove the remaining solid, and the Celite was washed with Et_2O. The solvent was removed by evaporation, obtaining 1.5 g (11.7 mmol, 75% yield) of a pale yellow oil of ***d*-4** without further purification. GC: RI (DB-5) = 1059, RI (OV-1701) = 1171, RI (FFAP) = 1428, RI (DB-WAX) = 1424. MS (EI) *m/z* (rel%): 128 (1, $[M]^+$), 127 (2), 113 (5), 110 (5, $[M - H_2O]^+$), 100 (12), 99 (32, $[M - CHO]^+$], 97 (5), 96 (5), 95 (7), 94 (10), 86 (18), 85 (84), 84 (34), 83 (40), 82 (15), 81 (8), 73 (8), 72 (55), 71 (58), 70 (100), 69 (48), 68 (24), 67 (22), 59 (56), 58 (35), 57 (60), 56 (48), 55 (68), 53 (7), 44 (15), 43 (36), 42 (52), 41 (93), 40 (12), 39 (32). The molecular ion was confirmed by PCI, isobutane *m/z* (rel%): 129 (100, $[M + 1]^+$), 128 (10, $[M]^+$), 111 (10, $[M + H - H_2O]^+$). ^{1}H NMR δ (ppm, C^2HCl_3): 9.51 (1:1:1, pattern typical for coupling to ^{2}H, $^3J_{1\text{-H},2\text{-}^2H}$ = 1.0 Hz, 0.9 H, intensity slightly reduced because of the long spin-lattice relaxation time T_1, 1-H); 2.33 (*t*, slightly broad, $^3J_{4\text{-H},5\text{-H}}$ = 7.5 Hz, 2 H, 4-CH_2), 1.52 ("quintet," $^3J_{avg}$ = 7.3 Hz, ≥2 H, 5-CH_2), 1.40–1.25 (*m*, complex, ≥4 H, 6-CH_2 and 7-CH_2), 0.91 ("*t*," *J* ~ 6.9 Hz, ≥3 H, 8-CH_3). Residual signals 6.85 (*t* 1:1:1, $^3J_{3\text{-H},4\text{-H}}$ = 6.7 Hz, $^3J_{3\text{-H},2\text{-}^2H}$ = 2.3 Hz, 0.07 H, 3-H) and 6.12 (*m*, $^3J_{1\text{-H},2\text{-H}}$ = 8.0 Hz and further couplings, 0.02 H, 2-H) indicated mono- or undeuterated (*E*)-2-octenal as byproducts that accounted for about 9%. No evidence for the occurrence of (*Z*) isomer was seen in the NMR spectrum. NOE-difference experiments with unlabeled (*E*)-2-octenal proved the spatial vicinity of the 1-CHO and 3-CH protons, indicating the all-*trans* arrangement of the C=C and C=O double bonds. ^{13}C NMR (proton decoupled) δ (ppm, C^2HCl_3): 194.21 (*d*, 1-CHO), 158.67 (*s* 1:1:1, $^1J_{C^2H}$ = 23.2 Hz, 3-C^2H), 132.59 (*s* 1:1:1, $^1J_{C^2H}$ = 24.5 Hz, 2-C^2H), 32.55 (*t*, 4-CH_2), 31.30 (*t*, 6-CH_2), 27.49 (*t*, 5-CH_2), 22.41 (*t*, 7-CH_2), 13.95 (*q*, 8-CH_3). These chemical shifts corresponded well to the spectrum of the unlabeled compound (26). Small signals for the residual nondeuterated 2-C and 3-C in about 1:3 ratio were observed at 132.89 (*d*) and 159.04 ppm (*d*), respectively. The NMR assignments above were based on our data of undeuterated (*E*)-2-octenal (not cited here), the assignments of which were in turn derived from those of natural abundance and 2,3-deuterated (*E*)-2-nonenal (22).

*[2,3-$^2H_2$]-trans-2,3-Epoxyoctanal (**d-5**). (Scheme 1, step **c**).* A two-necked flask (100 mL) equipped with a thermometer was charged with a solution of ***d*-3** (2.6 g, 20 mmol) in MeOH (25 mL). After cooling the solution to 1–3°C in an ice bath, 6.6 g of a 30% aqueous H_2O_2 solution (6 mL, 60 mmol) was added. The mixture was stirred vigorously and kept well cooled in the ice bath. Then, 40 µL of a 15% aqueous NaOH solution (0.15 mmol) was added in one portion. After 1 h reaction at 3–5°C, brine (30 mL) was added and the resulting suspension was extracted with CH_2Cl_2 (3 × 50 mL). The extracts were dried over Na_2SO_4, and the solvent was removed by distillation through a Vigreux column, obtaining 2.9 g (20.1 mmol, 99% yield) of *trans*-2,3-epoxyoctanal (***d*-5**) with a purity of 90% (GC). The sample, containing the corresponding *cis* isomer as main byproduct, was used as such for the Wittig reaction. GC: RI (DB-5) = 1093, RI (OV-1701) = 1218, RI (FFAP) = 1542, RI (DB-WAX) = 1533. MS (EI) *m/z* (rel%): 144 (1, $[M]^+$), 97 (3), 85 (3), 73 (100, $[M - C_5H_{11}]^+$), 72 (8), 69 (9), 59 (10), 57 (10), 56 (15), 55 (15), 45 (5), 43 (32), 42 (10), 41 (40), 40 (15), 39 (10). The molecular ions were confirmed by PCI, ammonia, *m/z* (rel%): 162 (100, $[M + NH_4]^+$), 146 (8, $[M + NH_4 - O]^+$); PCI, isobutane *m/z* (rel%): 145 (10, $[M + H]^+$), 129 (100, $[M + H - O]^+$), 128 (15), 127 (5, $[M + H - H_2O]^+$).

*[4,5-$^2H_2$]-trans-4,5-Epoxy-(E)-2-decenal (**d-1**) (Scheme 1, step **d**).* A solution of ***d*-5** (1.9 g, 13.2 mmol) in heptane (50 mL) was added to a suspension of formylmethylene triphenylphos-

phorane (4.1 g, 13.3 mmol) in heptane (50 mL). The reaction mixture was refluxed for 30 min. After cooling, the precipitated phosphorine oxide was filtered off. The filtrate was evaporated to remove the solvent. Four isomers of epoxydecenal were formed, with one predominant product: the *trans*-(*E*) isomer accounted for 87% of the total peak area. Major by-products were the *cis*-(*E*) and *trans*-(*Z*) isomers with 7 and 5%, respectively. Isomers were separated by CC using a glass column (20 × 2 cm) packed with a slurry of silica gel in hexane. Elution was carried out with hexane/Et_2O (95 + 5, vol/vol), collecting fractions of 10 mL. The target compound ***d*-1** was found by GC analysis in the fractions eluted from 200 to 400 mL. About 0.9 g (5.3 mmol, 40% yield) of the target compound was obtained as a colorless oil smelling intensely metallic with a purity of 95% [GC, 5% *cis*-(*E*) isomer]. GC: RI (DB-5) = 1380, RI (OV-1701) = 1558, RI (FFAP) = 2011, RI (DB-WAX) = 1995. MS, ^{1}H NMR, and ^{13}C NMR data are shown in Figure 1 and Tables 1–3.

Synthesis of trans-*4,5-Epoxy-(*E*)-2-decenal (**1**).* The unlabeled compound was prepared by adapting the procedure illustrated in Scheme 1 using commercially available (*E*)-2-octenal (**4**) as starting material.

trans-*2,3-Epoxyoctanal (**5**).* This was obtained in analogy to ***d*-5** by epoxidation of **4** (5.0 g, 40 mmol) with alkaline H_2O_2. About 5.1 g of **5** was obtained (36 mmol, 91% yield) by distillation under vacuum (130°C, 2 mbar) with a purity of 94% (GC). GC: RI (DB-5) = 1092, RI (OV-1701) = 1218, RI (FFAP) = 1540, RI (DB-WAX) = 1531. MS (EI) *m/z* (rel%): 142 (1, $[M]^+$), 95 (5), 83 (5), 72 (5), 71 (100, $[M - C_5H_{11}]^+$), 69 (15), 57 (25), 55 (50), 43 (35), 41 (65), 39 (20). The molecular ions were confirmed by PCI, ammonia, *m/z* (rel%): 160 (100, $(M + NH_4]^+$), 144 (5, $(M + NH_4–O]^+$); PCI, isobutane *m/z* (rel%): 143 (10, $(M + H]^+$), 127 (100, $(M + H - O]^+$), 125 (5, $[M + H - H_2O]^+$).

trans-*4,5-Epoxy*-(E)-*2-decenal* (**1**). This was obtained in analogy to ***d*-1** with 7.1 g (23 mmol) formylmethylene triphenylphosphorane and 3.3 g (23 mmol) of compound **5**. The *trans*-(*E*) isomer accounted for 92%, the *cis*-(*E*) isomer for 7% of the total peak area. After purification by CC using the same conditions as described above, about 1.5 g (8.9 mmol, 39% yield) of **1** was obtained as a colorless oil with a purity of 95% [GC, 5% *cis*-(*E*) isomer]. GC: RI (DB-5) = 1378, RI (OV-1701) = 1557, RI (FFAP) = 2010, RI (DB-WAX) = 1993. MS, ^{1}H NMR and ^{13}C NMR data are shown in Figure 1 and Tables 1–3.

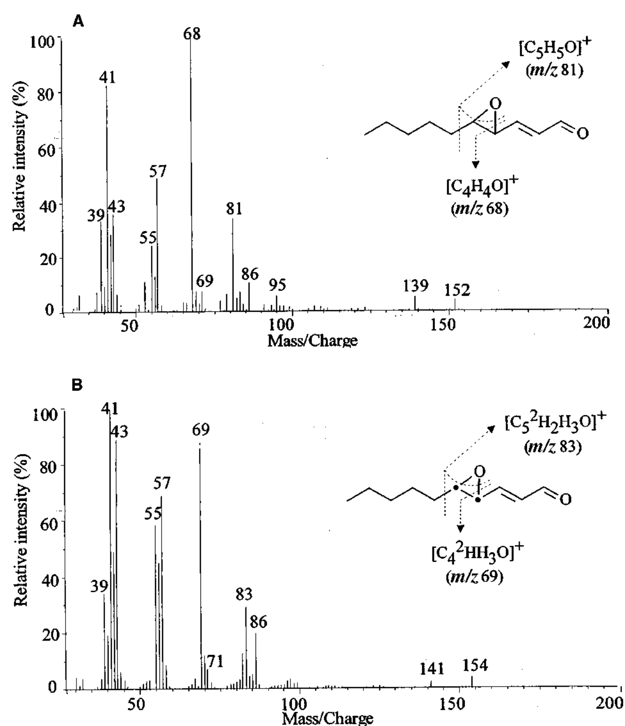


FIG. 1. Mass spectra of (A) *trans*-4,5-epoxy-(*E*)-2-decenal and (B) [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal obtained by electron impact ionization.

RESULTS AND DISCUSSION

*Preparation and characterization of [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (**d-1**) and* trans-*4,5-epoxy-(*E*)-2-decenal (**1**). (i) Synthesis procedure.* The newly developed synthesis procedure for ***d*-1** is based on the preparation of deuterated (*E*)-2-octenal (***d*-4**) followed by *trans*-epoxidation to 2,3-epoxyoctanal (***d*-5**) and subsequent chain elongation by a Wittig-type reaction (Scheme 1). In general, the preparative procedures and purification techniques used were simple and the yields satisfactory. Deuteration of commercially available 2-octyn-1-ol (**2**) resulted in [2,3-$^2H_2$]-(*E*)-2-octen-1-ol (***d*-3**) in 75% yields. As recently shown, reduction of α-acetylenic alcohols with $LiAl^2H_4$ leads preferably to *trans*-configured olefinic alcohols (22). The high stereospecificity was explained by geometrical constraints of cyclic intermediate structures. Oxidation of the primary allylic alcohol ***d*-3** with MnO_2 led to the corresponding aldehyde (***d*-4**) in 75% yield. The reaction of ***d*-4** with alkaline H_2O_2 was nearly quantitative. The resulting labeled 2,3-epoxyoctanal (***d*-5**) was obtained as a mixture of *trans* and *cis* isomers in the ratio 9:1. Wittig reaction of ***d*-5** gave rise to a mixture of epoxydecenal isomers in about 50% yield. As major isomer, the target compound (***d*-1**) was separated from isomeric by-products by CC. Similarly, the unlabeled compound (**1**) was synthesized in a two-step procedure in good overall yields using (*E*)-2-octenal (**4**) as starting material.

(ii) Structure characterization by MS. MS–PCI data indicated the incorporation of two deuterium atoms in ***d*-1**, i.e., at *m/z* 171 ($[M + H]^+$), compared to *m/z* 169 for **1**, when using isobutane as reagent gas (Table 1). Similarly, MS–PCI (ammonia) resulted in *m/z* 188 for ***d*-1** compared to *m/z* 186 for **1**. These ions are represented by the species $[M + NH_4]^+$ with abundances of about 50–90%. Fragmentation of these ions resulted in two major products that correspond to $[M + NH_4 - O]^+$ (100%) and $[M + H - O]^+$ (~60%), suggesting that the molecular ions are unstable and readily lose oxygen. This phenomenon was also observed when using isobutane as reagent gas. Apart from the molecular ions at *m/z* 171 and 169, the major signals were at *m/z* 155 and 153, representing $[M + H - O]^+$ of the labeled and unlabeled compound, respectively.

Loss of oxygen also generated characteristic fragments in the

TABLE 1
Ions Detected for *trans*-4,5-Epoxy-(*E*)-2-decenal (1) and [4,5-$^2H_2$]*trans*-4,5-Epoxy-(*E*)-2-decenal (*d*-1) Using Various Chemical Ionization Techniques[a]

Ionization mode	1	*d*-1
PCI (isobutane)	169 (25, $[M + H^+]$, 153 (100, $[M + H - O]^+$), 151 (10, $[M + H - H_2O]^+$)	171 (15, $[M + H^+]$, 155 (100, $[M + H - O]^+$), 153 (5, $[M + H - H_2O]^+$)
PCI (ammonia)	186 (90, $[M + NH_4]^+$), 170 (100, $[M + NH_4 - O]^+$), 169 (15, $[M + H]^+$), 153 (65, $[M + H - O]^+$)	188 (55, $[M + NH_4]^+$), 172 (100, $[M + NH_4 - O]^+$), 171 (20, $[M + H]^+$), 155 (55, $[M + H - O]^+$)
NCI (isobutane)	167 (40, $[M - H]^-$), 152 (30, $[M - O]^-$), 97 (100, $[M - C_5H_{11}]^-$)	168 (20, $[M - {}^2H]^-$), 154 (35, $[M - O]^-$), 99 (100, $[M - C_5H_{11}]^-$)
NCI (ammonia)	167 (20, $[M - H]^-$), 97 (100, $[M - C_5H_{11}]^-$)	168 (5, $[M - {}^2H]^-$), 99 (100, $[M - C_5H_{11}]^-$)

[a]Conditions of measurements are described in the Experimental Procedures section. Mass spectral data are given as ions (*m/z*) with intensities relative to the base peak (% relative abundance). PCI, positive chemical ionization; NCI, negative chemical ionization.

TABLE 2
^{1}H NMR Data of *trans*-4,5-Epoxy-(*E*)-2-decenal (1) and [4,5-$^2H_2$]-*trans*-4,5-Epoxy-(*E*)-2-decenal (*d*-1)[a]

Proton	1	*d*-1
1-CHO	9.57, *d*, $^3J_{1\text{-H},2\text{-H}}$ = 7.6 Hz, 1 H	9.56, *d*, $^3J_{1\text{-H},2\text{-H}}$ = 7.7 Hz, 1H
2-CH	6.39, *d d*, $^3J_{2\text{-H},3\text{-H}}$ = 15.7, $^3J_{1\text{-H},2\text{-H}}$ = 7.6 Hz, 1H	6.39, *d d*, *J* = 15.8, 7.7 Hz, 1H
3-CH	6.56, *d d*, $^3J_{2\text{-H},3\text{-H}}$ = 15.7, $^3J_{3\text{-H},4\text{-H}}$ = 6.9 Hz, 1 H	6.56, *d*, $^3J_{2\text{-H},3\text{-H}}$ = 15.7 Hz, 1H
4-CH	3.33, *d d*, $^3J_{3\text{-H},4\text{-H}}$ = 6.9, $^3J_{4\text{-H},5\text{-H}}$ = 2.1 Hz, 1 H	(residual signal 3.33, *d*, $^3J_{3\text{-H},4\text{-H}}$ = 6.7 Hz, ~0.02 H)
5-CH	2.96, *t d*, $^3J_{5\text{-H},6\text{-H}}$ = 5.5, $^3J_{4\text{-H},5\text{-H}}$ = 2.1 Hz, 1 H	(residual signal 2.96, *t*, $^3J_{5\text{-H},6\text{-H}}$ = 5.7 Hz, ~0.07 H)
6-CH_2	1.65, *m*, $^3J_{5\text{-H},6\text{-H}}$ = 5.5 Hz, 2 H	1.65, *m*, 2 H
7-CH_2	1.48, *m*, 2 H	1.48, *m*, 2 H
8,9-CH_2	~1.33, *m*, 4 H	~1.33, *m*, 4 H
10-CH_3	0.91, "*t*," 3 H	0.91, "*t*," 3 H

[a]Shifts in ppm from internal tetramethylsilane. Multiplicity abbreviations used to describe ^{1}H nuclear magnetic resonance (NMR) signals: *s* = singlet, *d* = doublet, *t* = triplet, *m* = complex multiplet. Quotation marks "…" mean approximate description of the multiplet.

MS–EI spectra, i.e., *m/z* 154 for ***d*-1** and *m/z* 152 for **1** (Fig. 1). In agreement with that, loss of oxygen has been reported for several 4,5-epoxy-2-alkenals of the chain length C_7–C_{11} (6). The fragment *m/z* 81 detected in the MS–EI spectra of this class of compounds, including **1**, is most likely a fragmentation product of $[M - O]^+$ with the composition C_5H_5O (12,27) that is formed by cleavage of the bonds at 5-C and 6-C. As ***d*-1** was labeled in the position 4-C and 5-C, the corresponding ion detected was *m/z* 83 with the composition $[C_5H_3{}^2H_2O]$. Therefore, the oxygen atom eliminated from the molecule is most likely originating from the oxirane ring.

However, the major ions did not reflect the shift of two units, i.e., *m/z* 69 and 68, which probably correspond to $[C_4{}^2HH_3O]^+$ and $[C_4H_4O]^+$, respectively. The data suggest that the said ions were generated by cleavage between 4-C and 5-C, which is in contrast to the fragmentation pattern proposed in the literature (6). Finally, the ions *m/z* 141 and 139 correspond to $[M - CHO]^+$. Loss of the aldehyde group has also been observed for 4,5-epoxy-2-heptenal (27) and 4,5-epoxy-2-nonenal (7).

(iii) Structure elucidation by NMR spectroscopy. NMR analysis was based on the non-deuterated compound **1**, for which a set of one- and two-dimensional spectra were acquired,

TABLE 3
^{13}C NMR Data of *trans*-4,5-Epoxy-(*E*)-2-decenal (1) and [4,5-$^2H_2$]-*trans*-4,5-Epoxy-(*E*)-2-decenal (*d*-1)[a]

Carbon	1	*d*-1
1-CHO	192.58, *d*	192.60, *d*
2-CH	133.51, *d*	133.51, *d*
3-CH	153.16, *d*	153.10, *d*
4-CH	56.19, *d*	(small residual signal 56.12, *d*)
4-C^2H	—	55.74, *s* (1:1:1), $^1J_{C^2H}$ = 27.2 Hz
5-CH	61.95, *d*	(small residual signal 61.88, *d*)
5-C^2H	—	61.48, *s* (1:1:1), $^1J_{C^2H}$ = 26.5 Hz
6-CH_2	31.87, *t*	31.69, *t*
7-CH_2	25.47, *t*	25.44, *t*
8-CH_2	31.50, *t*	31.50, *t*
9-CH_2	22.52, *t*	22.52, *t*
10-CH_3	13.96, *q*	13.96, *q*

[a]Shifts in ppm from internal tetramethylsilane. Multiplicity: abbreviations *s*, *d*, *t*, and *q* represent quaternary carbons, CH, CH_2, and CH_3 carbons, respectively. For abbreviation see Table 2.

including proton NOE difference spectra, COSY, and HETCOR for direct carbon-proton couplings. The structure of **1** could be confirmed, as shown in Tables 2 and 3. The coupling constant $J_{2\text{-H},3\text{-H}}$ 15.7 Hz revealed the *trans* configuration of the carbon-carbon double bond. In analogy to literature values on α-epoxy alcohols (28), the coupling constant $J_{4\text{-H},5\text{-H}} = 2.1$ Hz suggested a *trans* form of the oxirane ring. NOE-difference experiments with 10-s pre-irradiation and 4.23-s acquisition periods yielded signal enhancements, i.e., irradiation of 4-H: 7% on 2-H, 3% on 3-H, and 1.8% on 6-CH_2; irradiation of 5-H: 4.7% on 3-H, 2.2% on 2-H, and 1.4% on both 5-CH_2 and 6-CH_2. The absence of an NOE between 4-H and 5-H confirmed the *trans* arrangement of the oxirane ring. These NOE-difference results also suggested a non-coplanar arrangement of the enal group with respect to the oxirane ring. For the enal group, an all-*trans* conformation could be assumed, based on the close similarity of the $^3J_{1\text{-CHO},2\text{-H}}$ coupling constant in **1** and in (*E*)-2-octenal.

The NMR spectra also indicated the presence of about 7% of an isomer. Among the clearly distinguished, isolated ^{1}H-NMR signals of this isomer (between 10 and 2 ppm), the shifts of the putative 4- and 5-protons deviated more from the shifts of the main compound than those of any other proton. The coupling patterns of those same protons were the only ones to change, whereas the couplings of the 1-CHO and 3-H signals were practically the same for both isomers. The isomer is therefore presumably *cis*-4,5-epoxy-(*E*)-2-decenal. The low-field deviations (*ca.* 0.3 ppm) of the 4-H and 5-H signals could also indicate *cis* substitution of the oxirane ring, according to the shift rules established by Pierre *et al.* (28) for α-epoxy alcohols. Complete data for this second isomer were not available because of signal overlap with the dominating compound (especially for the 2-H and the aliphatic signals in the proton spectrum).

Both the ^{1}H NMR data of **1** in Table 2 and the ^{13}C-NMR data in Table 3 were in good agreement with those recently reported by Zamora and Hidalgo (12). The data of **1** are only given in this paper for closer comparison with the deuterated analog ***d*-1** that is reported for the first time. Other ^{1}H-NMR results on **1** (6) are not directly comparable with ours because of a different solvent used ($C^2H_2Cl_2$). Earlier ^{1}H NMR data of *trans*-4,5-epoxy-(*E*)-2-heptenal in C^2HCl_3 (27) are well compatible with ours on **1** for the unsaturated and oxirane part of the molecule, considering the different experimental temperature (35°C) and eventual concentration effects.

The NMR spectra of ***d*-1** clearly demonstrated a nearly quantitative deuteration at 4-C and 5-C (e.g., absence of couplings $^3J_{3\text{-H},4\text{-H}}$ and $^3J_{5\text{-H},6\text{-H}}$ on the 3-H and 6-H proton signals, characteristic deuterium patterns in the ^{13}C NMR spectrum). The proton spectrum indicated about 98 and 93% deuterium substitution, respectively. No undeuterated **1** could be detected in the proton spectrum. As in **1**, about 7% of the putative, equally 4,5-deuterated *cis* isomer were found in ***d*-1**, i.e., [4,5-$^2H_2$]-*cis*-4,5-epoxy-(*E*)-2-decenal.

Quantification of trans*-4,5-epoxy-(*E*)-2-decenal by IDA. (i) Mass spectra obtained by various ionization techniques.* The characteristic ions $[M - O]^+$ at *m/z* 152 (**1**) and 154 (***d*-1**) were not sufficiently abundant for quantification by IDA (Fig. 1). The $[M - CHO]^+$ fragments at *m/z* 139 and 141, showing the mass difference of two units, were also too weak. On the other hand, the more abundant ions were less characteristic (e.g., *m/z* 81/83, 55/57, 41/43) or they did not reflect the shift of two mass units (*m/z* 68/69). As no characteristic ions with sufficient abundance could be found in the MS–EI spectra of **1** and ***d*-1**, further ionization modes were tested to evaluate their suitability for IDA.

GC–MS using chemical ionization is known as a soft ionization method generating abundant molecular ions that are most characteristic of the compound to be analyzed. PCI using ammonia as a reagent gas resulted in characteristic and abundant ions for **1** and ***d*-1** (Table 1), i.e., at *m/z* 186/188 (~60–90%), 170/172 (100%), and 153/155 (~60%), respectively. The PCI spectra with isobutane generated mainly the ions at *m/z* 153/155 (100%). The NCI spectra of **1** and ***d*-1** obtained with ammonia and isobutane revealed abundant ions at *m/z* 97/99 (100%) which represent the characteristic part of the molecules. They correspond to the species $[C_5H_5O_2]^-$ and $[C_5H_3{}^2H_2O_2]^-$, respectively. Fragments formed by loss of oxygen ($[M - O]^-$) were also found, particularly when ionized with isobutane, i.e., at *m/z* 152 and 154. All these ions were estimated as suitable for IDA, and those finally selected were *m/z* 170/172 (PCI/ammonia), 153/155 (PCI/isobutane), and 97/99 (NCI with either ammonia or isobutane).

(ii) Sensitivity. Decreasing amounts of mixtures containing the analyte (**1**) and internal standard (***d*-1**) were analyzed by PCI and NCI in the SIM detection mode using isobutane or ammonia as reagent gas (Fig. 2). In general, NCI was found to result in more abundant signals than PCI. The sensitivity could further be improved when replacing isobutane by ammonia as a reagent gas. Signal-to-noise ratios (S/N) revealed NCI/ammonia as the most suitable method for the quantitative analysis of **1** (Fig. 2). The detection limit was determined to be at about 1 pg injected into the ion source with an acceptable S/N ratio of 3 (Table 4). PCI/isobutane was about 200 times less sensitive (detection limit: ~200 pg) whereas PCI/ammonia and NCI/isobutane gave comparable results, i.e., a detection limit of about 10 and 20 pg, respectively.

(iii) Selectivity. In addition to the high sensitivity, the NCI technique was also found to significantly improve selectivity in the detection of **1** and ***d*-1** (Fig. 3). When analyzing an aroma extract obtained from heated egg PE, no interferences were detected with NCI using either isobutane or ammonia. On the contrary, PCI resulted in further peaks, thus complicating data interpretation and calculation of reliable values. Therefore, NCI can be suggested as an ideal ionization technique for the quantitative analysis of **1**, particularly when using ammonia as a reagent gas.

(iv) Calibration curves. The calibration curve was established using mixtures containing defined amounts of analyte (**1**) and labeled internal standard (***d*-1**) in different ratios by measuring the characteristic ions of **1** and ***d*-1** (4). The ions indicated in Table 4 were monitored in the SIM mode. Linear curves were obtained for all of the ionization modes tested. As an example, the calibration curve using NCI/ammonia is presented in Figure 4A. Similar curves were obtained for the other ionization modes

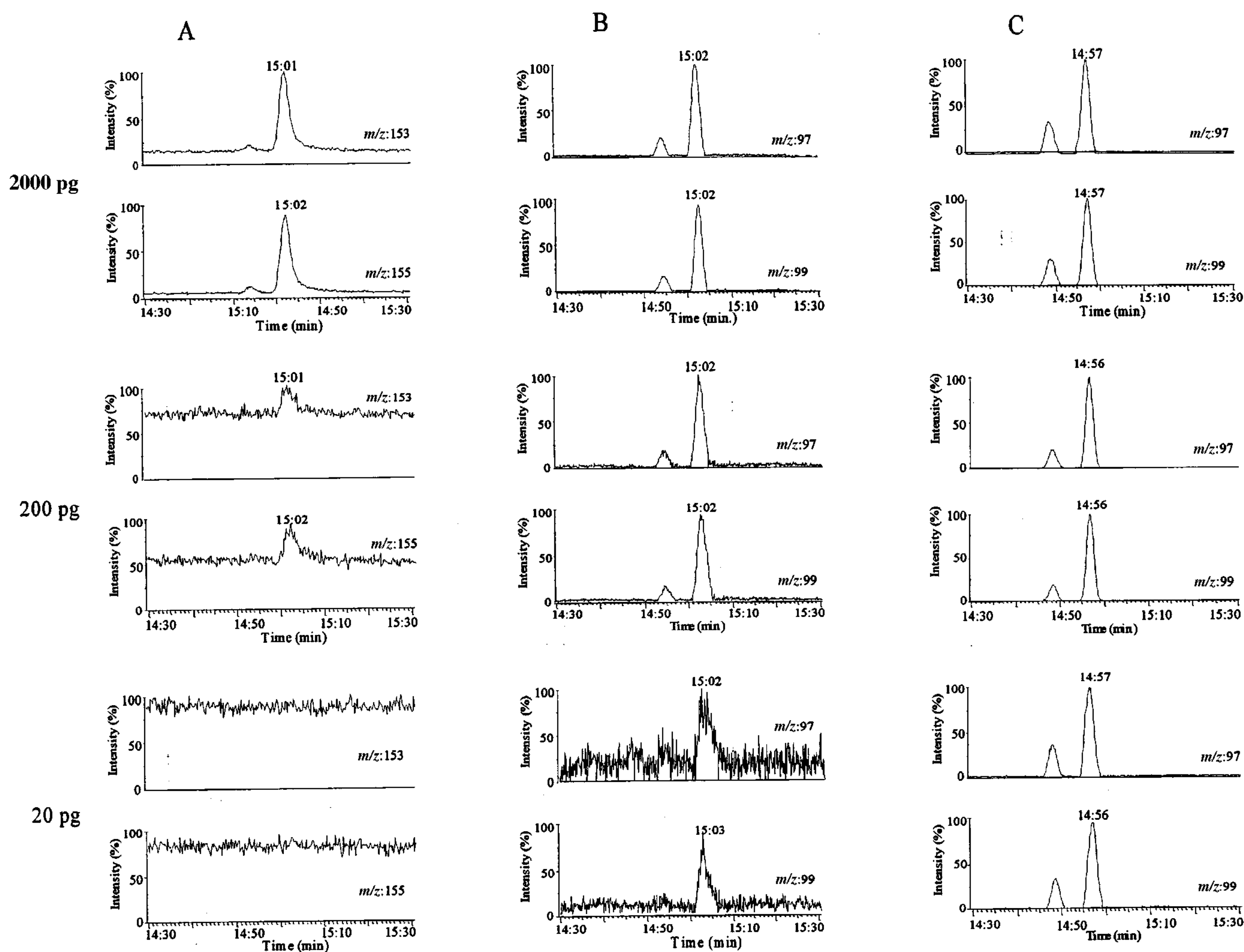


FIG. 2. Traces of decreasing amounts (2000, 200, and 20 pg) of *trans*-4,5-epoxy-(*E*)-2-decenal (**1**) and [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-decenal (***d*-1**) using various ionization techniques in the selected ion monitoring (SIM) mode. (A) Positive chemical ionization (PCI), isobutane; (B) negative chemical ionization (NCI), isobutane; (C) NCI, ammonia. The major peak represents the *trans* isomer, the minor peak the *cis* isomer of **1** and ***d*-1**.

tested, i.e., $y = 0.8404x + 0.155$, $r^2 = 0.9952$ (PCI/isobutane), $y = 0.7181x + 0.1121$, $r^2 = 0.9966$ (PCI/ammonia), and $y = 0.6365x + 0.0732$, $r^2 = 0.9985$ (NCI/isobutane). It should be pointed out, however, that if the amount ratios were extended to values higher than 10, second-order curves were obtained because of the natural deuterium isotope peaks of **1**, which coincide with the acquired ions of ***d*-1** (29).

The accuracy of the measured values with the theoretical values was checked according to the procedure described by Staempfli and coworkers (30). On the basis of knowing the amount of **1** and ***d*-1** in the mixture, the theoretical enrichment of ***d*-1** was calculated for each calibration point and expressed in mol percent excess (MPE). The measured deuterium enrichment was plotted vs. theoretical deuterium enrichment, resulting in linear curves for the four ionization modes (see example with NCI/ammonia in Fig. 4B). The slopes of the calibration curves were close to 1.000 proving the accuracy of the measurement, i.e., 0.9558 (PCI/isobutane), 0.9723 (PCI/ammonia), and 0.9029 (NCI/isobutane). The observed differences might be due to chemical impurity of **1**, chemical and isotopic impurities of ***d*-1**, and slight variation in the fragmentation pattern of **1** and ***d*-1**.

Quantification of trans-*4,5-epoxy-(*E*)-2-decenal (1) in heated phospholipids.* The linear ranges shown in Table 4 of the calibration curves were used to quantify **1** in heated aqueous dispersions of egg PC. Comparison of the data obtained from the same sample by the four ionization modes revealed good agreement in the results (Table 4), i.e., 1.61–1.67 μg **1** per g PC with an average of 1.64 ± 0.02, which correspond to a variation coefficient of less than 2%. As IDA has already been shown to be an accurate method for the quantification of **1** (4,9,10), the use of NCI/ammonia represents a significant improvement in terms of higher sensitivity and selectivity, thus allowing reduction of sample preparation time by applying simple cleanup procedures.

Conclusions. The newly developed synthesis procedure allows the preparation of both [4,5-$^2H_2$]-*trans*-4,5-epoxy-(*E*)-2-

TABLE 4
Comparison of Various Chemical Ionization Modes for the Quantification of *trans*-4,5-Epoxy-(*E*)-2-decenal (1) in Heated Egg Phosphatidylcholine (PC) by Isotope Dilution Assay[a]

Ionization mode (reagent gas)	Selected ions (*m/z*) **1**	***d*-1**	Detection limit (pg)[b]	Linearity (r^2)	Linear range (ratio **1**/***d*-1**)[c]	Concentration of 1 in egg PC (µg/g)[d]
PCI (isobutane)	153	155	200	0.999	0.05–9.0	1.67
PCI (ammonia)	170	172	10	0.999	0.05–9.0	1.61
NCI (isobutane)	97	99	20	0.999	0.05–4.0	1.64
NCI (ammonia)	97	99	1	0.998	0.05–4.0	1.62

[a][4,5-$^2H_2$]-*trans*-4,5-Epoxy-(*E*)-2-2decenal (***d*-1**) used as internal standard. Linear ranges and linearity were obtained from the calibration graphs using selected ions (see Experimental Procedures section).
[b]Detection limits correspond to a signal-to-noise ratio of 3.
[c]Linear range with $r^2 > 0.99$.
[d]Values represent the average of two injections using various chemical ionization techniques in the selected ion monitoring mode.

decenal (***d*-1**) and its unlabeled analog **1** in high purity and good yields. Optimization of mass spectrometric conditions, such as ionization mode and reagent gas, resulted in a powerful quantification method for **1** based on IDA. Comparison of various ionization modes revealed NCI as the most suitable technique with respect to both sensitivity and selectivity, particularly when

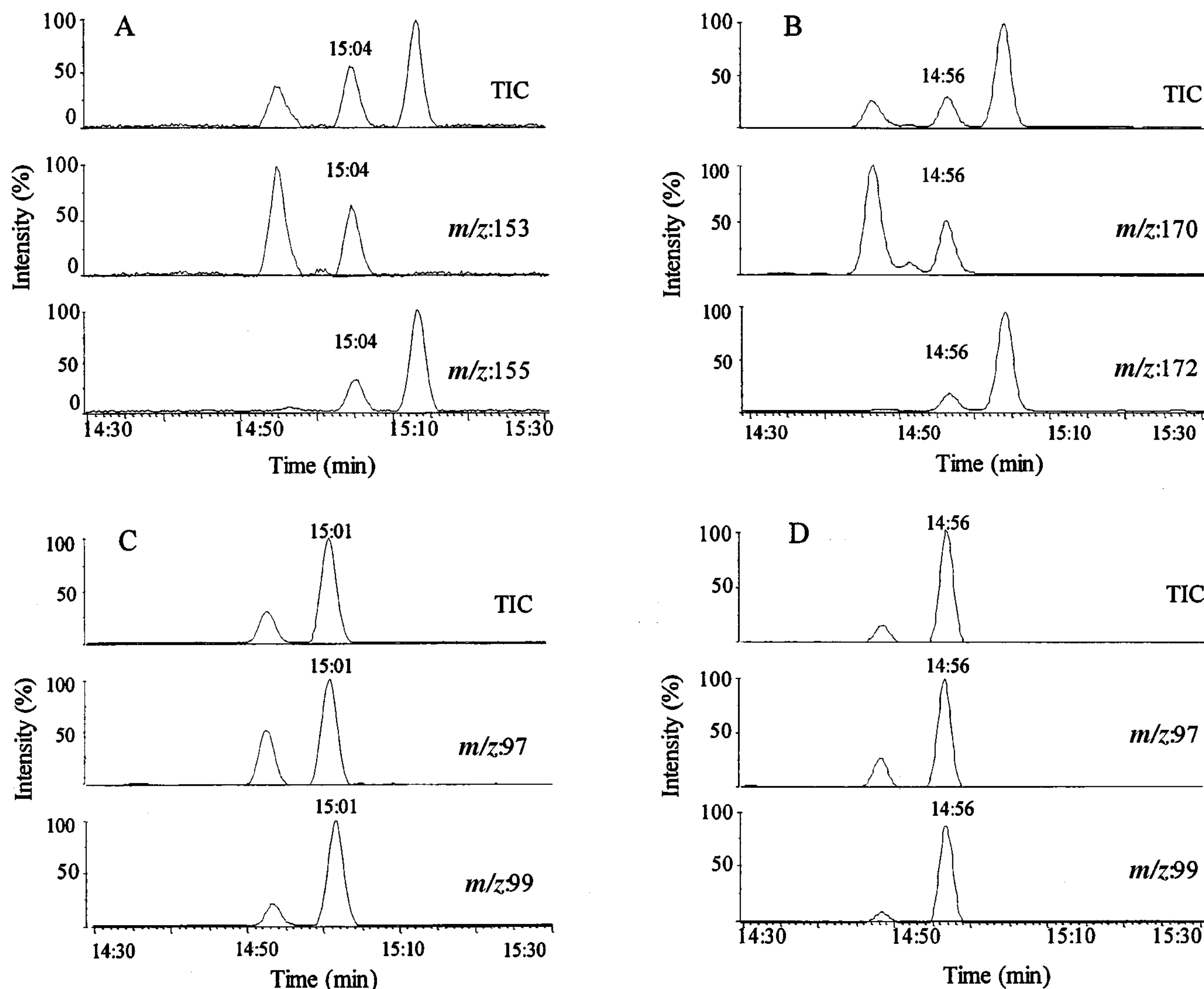


FIG. 3. Improvement of selectivity for the quantification of *trans*-4,5-epoxy-(*E*)-2-decenal (**1**) in heated egg phosphatidylethanolamine by using various chemical ionization methods in the SIM mode. (A) PCI, isobutane; (B) PCI, ammonia; (C) NCI, isobutane; (D) NCI, ammonia. TIC, total ion current; for other abbreviations see Figure 2.

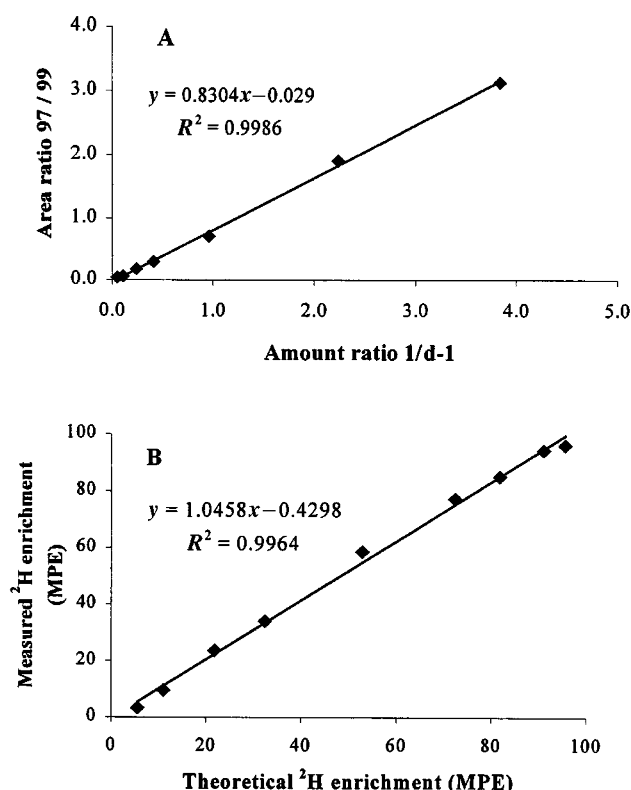


FIG. 4. (A) Calibration curve obtained for the quantification of *trans*-4,5-epoxy-(*E*)-2-decenal (**1**) using the ionization technique NCI/ammonia. The **1/*d*-1** ratios were 0.05, 0.11, 0.25, 0.43, 1.0, 2.33, 4.0, and 9.0. (B) Determination of the accuracy of the calibration curve obtained by using the ionization technique NCI/ammonia. The theoretical enrichments were calculated according to Wolfe (29) using known amounts of labeled and unlabeled *trans*-4,5-epoxy-(*E*)-2-decenal (**1**). MPE, mol percent excess; for other abbreviation see Figure 2.

using ammonia as a reagent gas. The low detection limit of 1–10 pg combined with increased selectivity by NCI are interesting features for obtaining reliable quantitative data of **1** and similar labile epoxyaldehydes at low concentration levels in complex mixtures allowing simplified clean-up procedures.

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ARTICLE

An Epoxy Alcohol Synthase Pathway in Higher Plants: Biosynthesis of Antifungal Trihydroxy Oxylipins in Leaves of Potato

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ABSTRACT: [1-^{14}C]Linoleic acid was incubated with a whole homogenate preparation of potato leaves (*Solanum tuberosum* L., var. Bintje). The methyl-esterified product was subjected to straight-phase high-performance liquid chromatography and was found to contain four major radioactive oxidation products, i.e., the epoxy alcohols methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate (14% of the recovered radioactivity) and methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate (14%), and the trihydroxy derivatives methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate (18%) and methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (30%). The structures and stereochemical configurations of these oxylipins were determined by chemical and spectral methods using the authentic compounds as references. Incubations performed in the presence of glutathione peroxidase revealed that lipoxygenase activity of potato leaves generated the 9- and 13-hydroperoxides of linoleic acid in a ratio of 95:5. Separate incubations of these hydroperoxides showed that linoleic acid 9(*S*)-hydroperoxide was metabolized into epoxy alcohols by particle-bound epoxy alcohol synthase activity, whereas the 13-hydroperoxide was metabolized into α- and γ-ketols by a particle-bound allene oxide synthase. It was concluded that the main pathway of linoleic acid metabolism in potato leaves involved 9-lipoxygenase-catalyzed oxygenation into linoleic acid 9(*S*)-hydroperoxide followed by rapid conversion of this hydroperoxide into epoxy alcohols and a slower, epoxide hydrolase-catalyzed conversion of the epoxy alcohols into trihydroxy-octadecenoates. Trihydroxy derivatives of linoleic and linolenic acids have previously been reported to be growth-inhibitory to plant-pathogenic fungi, and a role of the new pathway of linoleic acid oxidation in defense reactions against pathogens is conceivable.

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Abbreviations: FTIR, Fourier transform infrared; GC–MS, gas chromatography–mass spectrometry; GLC, gas–liquid chromatography; GSH, glutathione (reduced form); GSH-px, glutathione peroxidase; 9-H(P)OD, 9-hydro(pero)xy-10(*E*),12(*Z*)-octadecadienoic acid; 13-H(P)OD, 13-hydro(pero)xy-9(*Z*),11(*E*)-octadecadienoic acid; MC, (–)-menthoxycarbonyl; RP-HPLC, reversed-phase high-performance liquid chromatography; SP-HPLC, straight-phase high-performance liquid chromatography; TLC, thin-layer chromatography; UV, ultraviolet.

Oxylipins constitute a family of oxygenated derivatives of fatty acids. Hydroperoxides, formed from fatty acids by action of lipoxygenases (1,2) or α-dioxygenase (3), serve as important primary products in oxylipin formation. Further conversion of the hydroperoxides into various compounds belonging to the plant oxylipin family is catalyzed by the enzymes allene oxide synthase, divinyl ether synthase, hydroperoxide lyase, peroxygense, vicinal diol synthase, and epoxy alcohol synthase (4–7). One specific lipoxygenase product, i.e., the 13(*S*)-hydroperoxide derivative of linolenic acid, is of special importance because of its precursor role in the biosynthesis of 12-oxo-10,15(*Z*)-phytodienoic acid and the plant hormone jasmonic acid (2,5,8).

Certain oxylipins are involved as mediators in plants' defensive reactions against pathogens and also exert a direct inhibitory effect on the growth of pathogenic fungi. Leaves are the most important site of attack by plant pathogens, and knowledge about the pathways of oxylipin biosynthesis and metabolism in leaf tissue is therefore a prerequisite for understanding the roles of oxylipins in plant–pathogen interactions. This paper is concerned with a new oxylipin pathway in green leaves consisting of sequential oxidation of linoleic and linolenic acids into fatty acid epoxy alcohols and trihydroxy fatty acids.

EXPERIMENTAL PROCEDURES

Plant materials. Potato (*Solanum tuberosum* L., var. Bintje) was grown in soil under a 16-h light/8-h dark photoperiod. Leaves of 2–5-wk-old plants were used.

Fatty acids. Linoleic, linolenic, and oleic acids were purchased from Nu-Chek-Prep (Elysian, MN). [1-^{14}C]Linoleic, [1-^{14}C]linolenic, and [9,10-$^{3}H_2$]oleic acids (Du Pont NEN, Boston, MA) were mixed with the corresponding unlabeled acids and purified by SiO_2 chromatography to afford specimens with specific radioactivities of 4.6, 6.7, and 184 kBq/μmol, respectively. [1-^{14}C]9(*S*)-Hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HPOD] and [1-^{14}C]13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HPOD] were prepared by incubation of linoleic acid with tomato lipoxygenase (9) and soybean lipoxygenase (10), respec-

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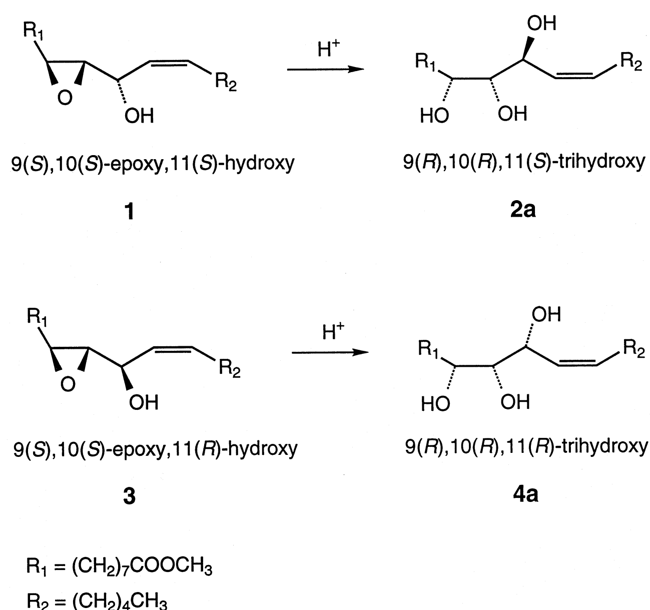
tively. The ketols methyl 13-hydroxy-12-oxo-9(*Z*)-octadecenoate and methyl 9-hydroxy-12-oxo-10(*E*)-octadecenoate were obtained by incubation of 13(*S*)-HPOD with allene oxide synthase from corn followed by isolation of the methyl esters by thin-layer chromatography (TLC) (11).

*Methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate (**13**) and methyl 10(*R*),11(*R*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate*. The title compounds were prepared from the methyl ester of 9(*S*)-HPOD by vanadium-catalyzed epoxidation as described in detail elsewhere (12,13).

*Methyl 9(*S*),10(*S*)-epoxy-11(*S*)-hydroxy-12(*Z*)-octadecenoate (**1**) and methyl 9(*S*),10(*S*)-epoxy-11(*R*)-hydroxy-12(*Z*)-octadecenoate (**3**)*. 9(*S*)-HPOD (23 mg) in ethanol (0.5 mL) was treated with a solution of bovine hemoglobin (2.5 g) in 0.1 M potassium phosphate buffer pH 7.4 (50 mL) at 37°C for 10 min (*cf.* Ref. 14). The solution was acidifed to pH 3 and kept at this pH for 3 min in order to hydrolyze the allylic epoxy alcohol 9,10-epoxy-13-hydroxy-11-octadecenoic acid into trihydroxy acids and in this way facilitate isolation of the nonallylic epoxy alcohols. Material extracted with diethyl ether was methyl-esterified by treatment with diazomethane and subjected to TLC using solvent system B, described below in the paragraph on "Chromatographical methods." Bands appeared due to methyl 9-oxooctadecadienoate, methyl 9-hydroxyoctadecadienoate, and methyl trihydroxyoctadecenoates as well as **1** (nonallylic *erythro* epoxy alcohol; R_f 0.53; approx. 9% of the product) and **3** (nonallylic *threo* epoxy alcohol; R_f 0.48; approx. 14% of the product) (Scheme 1) (bold Arabic numbers represent methyl esters of epoxy alcohol and trihydroxy fatty acids. Enantiomers are designated by **a** or **b**). The two latter compounds were recovered from the silica gel and further purified by reversed-phase high-performance liquid chromatography (RP-HPLC) using a solvent system of acetonitrile/water (60:40, vol/vol) (Table 1). The mass spectra of the Me_3Si derivatives of **1** and **3** were very similar. Prominent ions in the mass spectrum of the derivative of **1** were observed at *m/z* 383 (2%; $M^+ - 15$; loss of $\cdot CH_3$), 277 [1; $M^+ - (90 + 31)$; loss of Me_3SiOH plus $\cdot OCH_3$], 241 [3; $M^+ - 157$; loss of $\cdot(CH_2)_7$-$COOCH_3$], 212 (10),



SCHEME 1

199 [91; Me_3SiO^+=CH–CH=CH–$(CH_2)_4$–CH_3], 155 [40; tentatively HO^+=CH–$(CH_2)_6$–CH=C=O formed by cleavage of the C-9/C-10 bond and loss of CH_3OH], 129 (38; Me_3SiO^+=CH–CH=CH_2), and 73 (100; Me_3Si^+). Fourier transform infrared (FTIR) spectrometry of **1** and **3** showed bands at 3460 cm^{-1} (alcohol), 1741 cm^{-1} (ester carbonyl), and 903 cm^{-1} (*trans* epoxide). It has been shown earlier for the two *trans*-12,13-epoxy-11-hydroxyoctadecenoates produced from 13(*S*)-HPOD upon treatment with hemoglobin that the less polar diastereomer has the *erythro* relative configuration at C-11/C-12, whereas the more polar one is *threo* (14,15). In order to confirm the relative configurations of C-10/C-11 of **1** and **3** obtained from 9(*S*)-HPOD, aliquots of the hydrogenated derivatives (**14** and **17**, respectively) were deoxygenated by treatment with triphenylphosphine selenide and trifluoroacetic acid (15). The (–)-menthoxycarbonyl (MC) derivatives of the resulting methyl 11-hydroxy-9(*E*)-octadec-

TABLE 1
Chromatographic Properties of Epoxy Alcohols

Compound[a]	GLC[b]	SP-HPLC[c]	RP-HPLC[d]
Methyl 9(*S*),10(*S*)-epoxy-11(*S*)-hydroxy-12(*Z*)-octadecenoate (**1**)	20.76	12.0	22.8
Methyl 9(*S*),10(*S*)-epoxy-11(*R*)-hydroxy-12(*Z*)-octadecenoate (**3**)	20.75	15.7	20.4
Methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate (**13**)	20.85	8.9	26.1
Methyl 10(*R*),11(*R*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate	20.76	12.9	22.1
Methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate	21.28	34.7	18.0
Compound **I**	20.85	8.9	26.1
Compound **II**	21.28	34.7	18.0

[a]Bold numbers designate methyl esters of epoxy alcohol and trihydroxy fatty acids. Compound **I**, methyl 10(*S*),11(*S*)-epoxy-9(*S*)hydroxy-12(*Z*)-octadecenoate; compound **II**, methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate.
[b]The retention times found on gas–liquid chromatography (GLC; column temp., 230°C) are expressed as C-values (*cf.* Ref. 20).
[c]Retention volumes (mL) using a solvent system of 2-propanol/hexane (1:99, vol/vol) are given. SP-HPLC, straight-phase high-performance liquid chromatography.
[d]Retention volumes (mL) using a solvent system of acetonitrile/water (60:40, vol/vol) are given. RP-HPLC, reversed-phase HPLC.

enoates were subjected to oxidative ozonolysis, and the configurations of the resulting 2-hydroxynonanoates (MC derivatives) were established by gas–liquid chromatography (GLC) using the authentic derivatives as references (16). It was found that **1** produced 2(*S*)-hydroxynonanoate, thus proving that this epoxy alcohol had the *S* configuration at C-11. This result combined with the configuration of C-9 (*S*) and the geometry of the epoxide group (*trans*) showed that the relative configuration of C-10/C-11 of **1** was *erythro*. In the same way, **3** produced 2(*R*)-hydroxynonanoate, and thus had the *R* configuration at C-11 and the *threo* relative configuration at C-10/C-11 (Scheme 1).

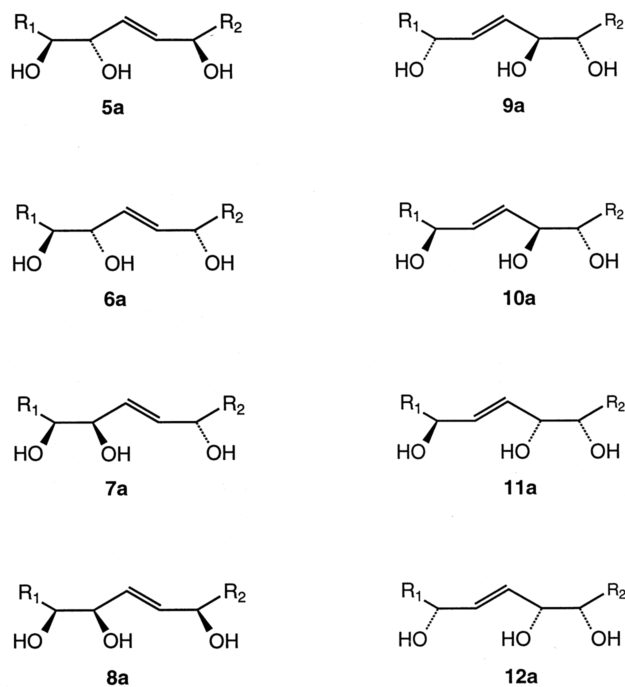
*Methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate*. Either of three enzymes could be used to prepare 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid and its methyl ester from 9(*S*)-HPOD, i.e., epoxy alcohol synthase from the fungus *Saprolegnia parasitica* (13), peroxygenase from seeds of broad bean (17), or peroxygenase from oat seeds (18). The biosynthesized epoxy alcohol was isolated as its methyl ester by TLC (solvent system B, R_f 0.39) followed by straight-phase HPLC (SP-HPLC) [solvent system, 2-propanol/hexane (1:99, vol/vol); effluent volume, 34.7 mL].

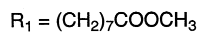
*Methyl 9(*R*),10(*R*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (***2a***)*. *erythro*-Epoxy alcohol **1** (1 mg) was dissolved in 50% aqueous tetrahydrofuran (2 mL), and 70% perchloric acid (0.2 mL) was added. The solution was kept at 23°C for 1 h and subsequently extracted with diethyl ether. Analysis by TLC using solvent system A, described below in the paragraph on "Chromatographic methods." showed the presence of a major trihydroxyester (**2a**; R_f 0.72). The Me_3Si derivative of **2a** had a C-value of 22.30 and a mass spectrum which showed prominent ions at *m/z* 560 (0.5%; M^+), 361 (26; M^+ – 199), 313 [5; M^+ – (157 + 90); loss of $\cdot(CH_2)_7$-$COOCH_3$ plus Me_3SiOH], 301 (1; M^+ – 259); 271 (100; 361 – 90), 259 [12; Me_3SiO^+=CH–$(CH_2)_7$–$COOCH_3$], 199 [15; Me_3SiO^+=CH–CH=CH–$(CH_2)_4$–CH_3], 147 (15; Me_3Si–O^+=$SiMe_2$), and 73 (80; Me_3Si^+). In another experiment, **2a** was prepared by treatment of **1** with perchloric acid in tetrahydrofuran/$H_2{}^{18}O$. Mass spectrometric analysis of the Me_3Si derivative of **2a** obtained in this way demonstrated a shift of the ions *m/z* 259 and 361 to 261 and 363, respectively, whereas the ions *m/z* 199 and 301 remained unchanged. This result showed that formation of **2a** from **1** occurred by solvent attack and epoxide opening at C-9 (with inversion of the configuration of that carbon). Consequently, **2a** could be assigned the structure methyl 9(*R*),10(*R*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (Scheme 1).

*Methyl 9(*R*),10(*R*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate (***4a***)*. *threo*-Epoxy alcohol **3** (1 mg) was hydrolyzed by treatment with perchloric acid, and the major trihydroxyester (**4a**; R_f 0.59) was isolated by TLC as described for **2a**. The C-value of the Me_3Si derivative was 22.47, and the mass spectrum was closely similar to that of the corresponding derivative of **2a**. Hydrolysis carried out with $H_2{}^{18}O$ resulted in incorporation of ^{18}O in the C-9 position, demonstrating that the configuration of this carbon was inverted in the formation of **4a** from **3** (Scheme 1).

*Methyl 9,10,13-trihydroxy-11(*E*)-octadecenoates and methyl 9,12,13-trihydroxy-10(*E*)-octadecenoates (***5a–12a***)*. Methyl 9,10,13- and 9,12,13-trihydroxyoctadecenoates with defined configurations at C-9 to C-13 (eight diastereomers; Scheme 2) were prepared by chemical hydrolysis of diastereomeric methyl 10,11-epoxy-9-hydroxyoctadecenoates and methyl 11,12-epoxy-13-hydroxyoctadecenoates as decribed in detail (12). Methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (**10a**) was also prepared by enzymatic hydrolysis of 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid (18).

*Methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate (***2b***) and methyl 9(*S*),10(*S*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (***4b***)*. Allylic *erythro* epoxy alcohol **13** (1 mg) was dissolved in 67% aqueous tetrahydrofuran (3 mL) and treated with 2 M hydrochloric acid (10 µL). After 3 min at 23°C, the solution was extracted with diethyl ether and subjected to TLC using solvent system A. In analogy with previous results obtained for epoxy alcohols generated from 13(*S*)-HPOD (19), this led to the appearance of two bands due to methyl 9,10,13-trihydroxy-11(*E*)-octadecenoates epimeric at C-13 [**7a** (R_f 0.38) and **8a** (R_f 0.47)] as well as two weaker bands due to methyl 9,10,11-trihydroxy-12(*Z*)-octadecenoates epimeric at C-11 [**2b** (R_f 0.72) and **4b** (R_f 0.59)] (Scheme 3). Hydrolysis of epoxy alcohol **13** carried out in tetrahydrofuran/$H_2{}^{18}O$ resulted in the formation of 9,10,11-trihydroxyoctadecenoates, which had incorporated ^{18}O at C-11 but not at C-9 or C-10. This was apparent from the mass spectra of the Me_3Si derivatives, in which the ion *m/z* 199 was shifted to





$R_1 = (CH_2)_7COOCH_3$
$R_2 = (CH_2)_4CH_3$

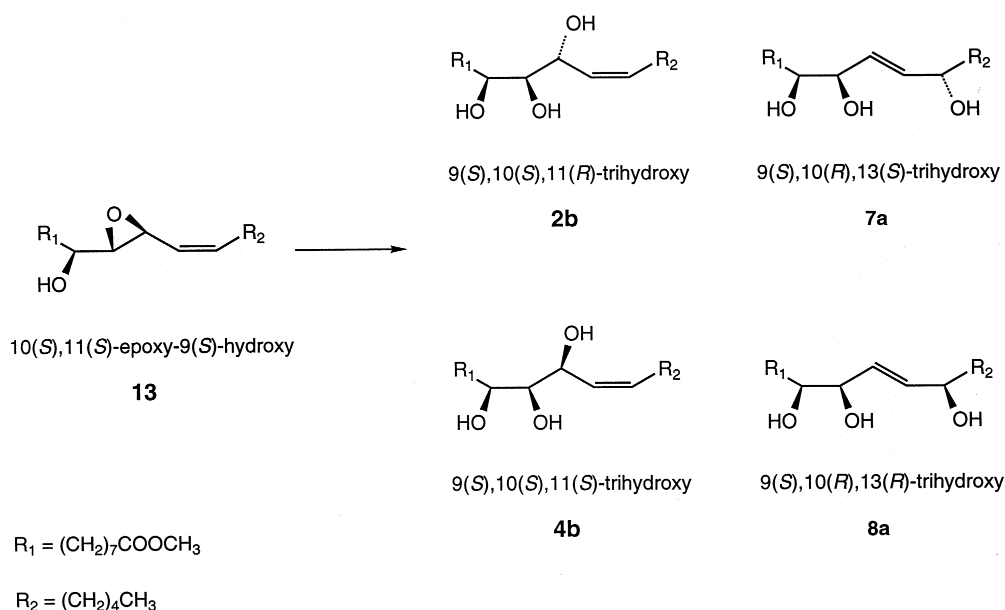
SCHEME 2

201, whereas the ions *m/z* 259 and 361 were not affected. Thus, in the formation of **2b** and **4b** from **13**, epoxide opening took place at C-11 (with or without inversion of absolute configuration), and the C–O bonds at C-9 and C-10 remained intact. Consequently, the two 9,10,11-trihydroxyoctadecenoates had the *S* configuration at C-9 and C-10, and were epimeric at C-11. Assignment of the absolute configuration at this carbon was made possible by chromatographic matching of **2b** and **4b** obtained from **13** with the 9(*R*),10(*R*),11(*S*)-trihydroxyoctadecenoate (**2a**) and the 9(*R*),10(*R*),11(*R*)-trihydroxyoctadecenoate (**4a**) prepared from **1** (Scheme 1). **2b** obtained from **13** was found to cochromatograph with **2a** on TLC (R_f 0.72) and GLC (C-value, 22.30); consequently **2b** could be identified as 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate. In the same way, **4b** formed from **13** had chromatographic properties identical to **4a** (R_f 0.59, C-22.47) and was therefore identical to 9(*S*),10(*S*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (Scheme 3).

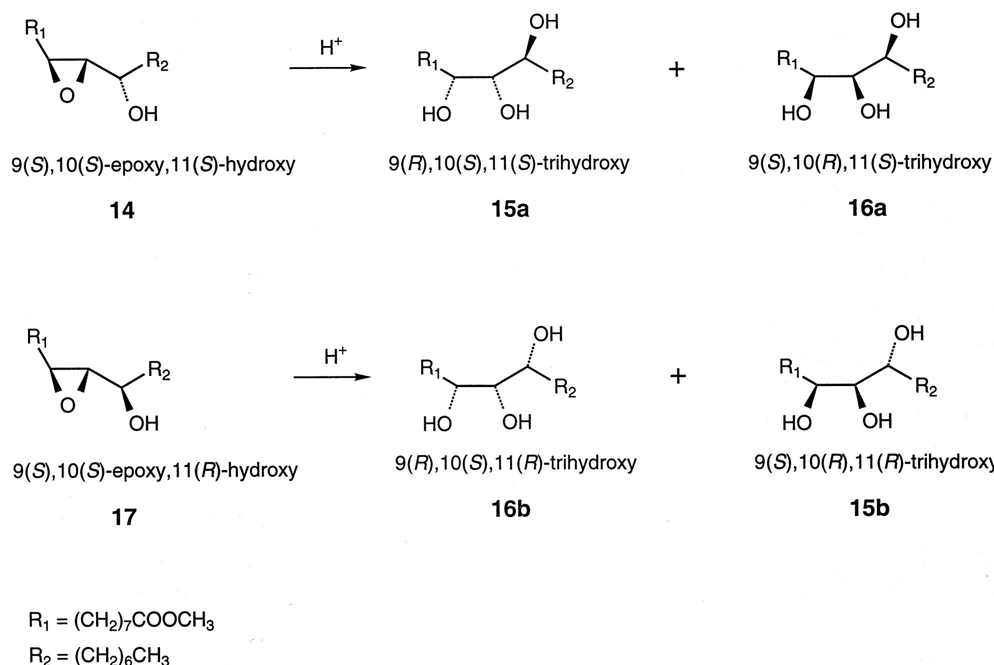
*Methyl 9(*R*),10(*S*),11(*S*)-trihydroxyoctadecanoate (***15a***) and methyl 9(*S*),10(*R*),11(*S*)-trihydroxyoctadecanoate (***16a***).* Epoxy alcohol **1** (2 mg) was hydrogenated and the resulting dihydro derivative (**14**) was dissolved in 50% aqueous tetrahydrofuran (2 mL) and treated with 70% perchloric acid (0.2 mL) at 23°C for 1 h. Analysis of the trimethylsilylated product by GLC showed the presence of two diastereomeric trihydroxyesters, i.e., **15a** (95%; C-22.77) and **16a** (5%; C-22.69) (Scheme 4). The mass spectra recorded on these compounds were closely similar and showed ions at *m/z* 531 (0.1%; $M^+ - 31$; loss of $\cdot OCH_3$), 434 [8; $M^+ - 128$; rearrangement with loss of OHC–$(CH_2)_6$–CH_3], 373 [7; $M^+ - (99+90)$; loss of $\cdot(CH_2)_6$-CH_3 plus Me_3SiOH], 303 (12; $M^+ - 259$), 259 [88; Me_3SiO^+=CH–$(CH_2)_7$–$COOCH_3$], 201 [63; Me_3SiO^+=CH–$(CH_2)_6$–CH_3], 155 [23; OHC–$(CH_2)_7$–C≡O^+], and 73 (100; Me_3Si^+). In a separate experiment, **14** was treated with perchloric acid in the presence of $H_2{}^{18}O$. Mass spectrometric analysis revealed that this led to the formation of trihydroxyesters **15a** and **16a,** which had incorporated one atom of ^{18}O at C-9 and C-10, respectively. Accordingly, **15a**, the major trihydroxyester, was formed from **14** by solvent attack at C-9 (with inversion of the configuration of that carbon) and had the structure methyl 9(*R*),10(*S*),11(*S*)-trihydroxyoctadecanoate. In the same way, the minor trihydroxyester (**16a**) was formed by epoxide opening at C-10 and was due to methyl 9(*S*),10(*R*),11(*S*)-trihydroxyoctadecanoate (Scheme 4).

*Methyl 9(*R*),10(*S*),11(*R*)-trihydroxyoctadecanoate (**16b**) and methyl 9(*S*),10(*R*),11(*R*)-trihydroxyoctadecanoate (**15b**).* Catalytic hydrogenation of epoxy alcohol **3** into the dihydro derivative **17** followed by perchloric acid-catalyzed hydrolysis afforded two diastereomeric 9,10,11-trihydroxyoctadecanoates, i.e., **16b** (90%; C-22.69) and **15b** (10%; C-22.77). Hydrolysis carried out in the presence of $H_2{}^{18}O$ afforded **16b** and **15b** which were labeled with ^{18}O at C-9 and C-10, respectively. Consequently, the configurations of these trihydroxyoctadecanoates were 9(*R*),10(*S*),11(*R*) (**16b**) and 9(*S*),10(*R*),11(*R*) (**15b**) (Scheme 4).

Enzyme preparations. Potato leaves were minced and homogenized at 0°C in 0.1 M potassium phosphate buffer pH 7.4 (1:10, wt/vol) with an Ultra-Turrax. The homogenate was filtered through gauze, and the filtrate (protein, 1.5 mg/mL) was directly used for the incubations. In some experiments, the filtrate was centrifuged at 1,100 × *g* for 15 min to afford a low-speed sediment and supernatant. Further centrifugation of the latter at 105,000 × *g* for 60 min provided a high-speed particulate fraction and a particle-free supernatant (protein, 0.9 mg/mL). The high-speed particulate fraction was resuspended in a volume of buffer equal to that of the high-speed supernatant (protein, 0.4 mg/mL). Reduced glutathione



SCHEME 3



SCHEME 4

(GSH) and glutathione peroxidase (GSH-px) were purchased from Sigma Chemical Co. (St. Louis, MO). $H_2{}^{18}O$ (95.0 atom% ^{18}O) was obtained from IsoTec (Miamisburg, OH).

Incubations and treatments. [1-^{14}C]Linoleic acid, [1-^{14}C]linolenic acid, [1-^{14}C]9(*S*)-HPOD, or [1-^{14}C]13(*S*)-HPOD (200 μM) was stirred with enzyme preparation (2–50 mL) at 23°C for 20 or 60 min. The mixtures were acidified to pH 5 and rapidly extracted with 2 vol of diethyl ether. Material obtained after evaporation of the solvent was methyl-esterified by treatment with diazomethane and analyzed by SP-HPLC. For assay of peroxygenase activity (18), a suspension of the 105,000 × *g* particulate fraction from homogenate of potato leaves was stirred at 23°C for 10 min with [9,10-$^3H_2$]oleic acid (200 μM) and hydrogen peroxide (5 mM). The methyl-esterified product was subjected to radio-TLC using solvent system C, described in the paragraph below on "Chromatographic methods." in order to separate radioactive methyl 9,10-epoxystearate from methyl oleate.

Chemical methods. Catalytic hydrogenation of hydroxyesters (3) and epoxy alcohols (15), oxidative ozonolysis (16), and vanadium-catalyzed epoxidation (19) were performed as indicated. Configurational determinations of monohydroxy acids (16), nonallylic fatty acid epoxy alcohols (15), and trihydroxyesters (12) were carried out as described in detail.

Chromatographical and instrumental methods. SP-HPLC was carried out with a column of Nucleosil 50 (200 × 4.6 mm) purchased from Macherey-Nagel (Düren, Germany) and a mixture of 2-propanol/hexane (1:99 or 5:95, vol/vol) was used as the solvent. RP-HPLC was performed with a column of Nucleosil 100-5 C_{18} using a solvent system of acetonitrile/water (60:40 or 40:60, vol/vol). The absorbance (210 nm) and radioactivity of HPLC effluents were determined online, using a Spectromonitor III ultraviolet (UV) detector (Laboratory Data Control, Riviera Beach, FL) and a liquid scintillation counter (IN/US Systems, Tampa, FL), respectively. TLC was carried out with precoated plates (Kieselgel 60, 0.25 mm) from E. Merck (Darmstadt, Germany) and solvent systems consisting of either ethyl acetate (system A), ethyl acetate/hexane (30:70, vol/vol; system B), or ethyl acetate/hexane (10:90, vol/vol; system C). Radioactivity on TLC plates was determined using a Berthold Dünnschichtscanner II (Wildbad, Germany) interfaced with a Macintosh IIsi computer. GLC was performed with a Hewlett-Packard (Avondale, PA) model 5890 gas chromatograph equipped with a methylsilicone capillary column (length, 25 m; film thickness, 0.33 mm). Helium at a flow rate of 25 cm/s was used as the carrier gas. Retention times found on GLC were converted into C-values as described (20). 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. FTIR 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, Downers Grove, IL).

RESULTS

Isolation of oxidation products of linoleic acid. [1-^{14}C]-Linoleic acid (200 μM) was stirred at 23°C for 20 min with a whole homogenate of leaves of potato and the methyl-esterified product was subjected to SP-radio-HPLC. As seen in Figure 1A, four peaks of radioactive compounds appeared (compounds **I–IV**), in addition to the peak for methyl linoleate due

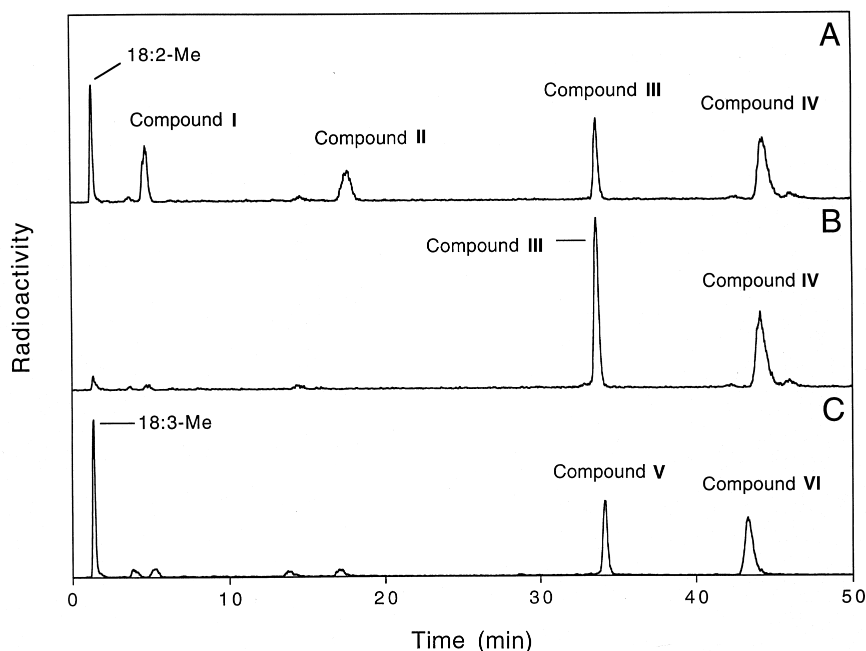


FIG. 1. Analysis by straight-phase radio high-performance liquid chromatography (SP-radio-HPLC) of methyl-esterified reaction products obtained following incubations of fatty acids with whole homogenate of potato leaves (10 mL; 15 mg of protein) at 23°C. (A) Incubation of 200 μM [1-^{14}C]linoleic acid for 20 min; (B) incubation of 200 μM [1-^{14}C]linoleic acid for 60 min; (C) incubation of 200 μM [1-^{14}C]linolenic acid for 20 min. The column was eluted with 2-propanol/hexane (1:99, vol/vol) (0–25 min) followed by 2-propanol/hexane (5:95, vol/vol) (25–50 min) at a flow rate of 2 mL/min. Compound **I**, methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate; compound **II**, methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate; compound **III**, methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate; compound **IV**, methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate; compound **V**, tentatively identified as methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*),15(*Z*)-octadecadienoate; compound **VI**, tentatively identified as methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*),15(*Z*)-octadecadienoate.

to remaining unconverted substrate. Prolongation of incubation to 60 min led to the disappearance of compounds **I** and **II** and an increased yield of compounds **III** and **IV** (Fig. 1B). [1-^{14}C]Linolenic acid incubated with the enzyme preparation at 23°C for 20 min afforded two main products, i.e., compounds **V** and **VI**, which had polarities similar to those of compounds **III** and **IV**, respectively (Fig. 1C). The labeled oxidation products obtained by SP-HPLC were contaminated by chlorophyll and other pigments. To allow structural work, compounds **I** and **II** were further purified by RP-HPLC (Table 1) or by TLC using solvent system B. Purification of compounds **III** and **IV** was accomplished by RP-HPLC using acetonitrile/water (40:60, vol/vol) (effluent volumes of compounds **III** and **IV**, 34.5 and 22.2 mL, respectively) or by TLC (Table 2).

Structure of compound **I**. The UV spectrum of compound **I** was featureless, demonstrating the absence of conjugated diene or similar chromophores. The mass spectrum of the Me_3Si derivative showed prominent ions at *m/z* 398 (0.5%; M^+), 383 (7; $M^+ - 15$; loss of $\cdot CH_3$), 259 [100; $Me_3SiO^+=CH-(CH_2)_7-COOCH_3$], 225 (10), 155 [42; $OHC-(CH_2)_7-C\equiv O^+$], 143 (23), 130 (26), 109 (20), and 73 (72; Me_3Si^+), thus being in agreement with an epoxyhydroxyoctadecenoate having its hydroxyl group at C-9 and its epoxide group at C-10/C-11. Comparison of the mass spectrum and the chromatographic properties of compound **I** with those of reference epoxy alcohols oxygenated in the C-9, -10, and -11 positions showed a complete agreement between compound **I** and methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate **13** (Table 1). Catalytic hydrogenation of compound **I** was accompanied by hydrogenolysis of the allylic C–O bond at C-11 and resulted in the formation of methyl 9,10-dihydroxystearate. Analysis of the Me_3Si derivative of this product by GLC showed a C-value identical to that of the Me_3Si derivative of methyl *erythro*-9,10-dihydroxystearate (C-21.45) but different from that of the corresponding derivative of *threo*-9,10-dihydroxystearate (C-21.29). This finding thus provided independent proof for the *erythro* configuration of C-9/C-10 of compound **I**. Allylic epoxyhydroxyoctadecenoates undergo rapid hydrolysis into specific trihydroxyoctadecenoates at pH 3 and lower (12). As would be expected, compound **I** behaved identically to **13** in this respect and afforded 9,10,13-trihydroxyoctadecenoates **7a** and **8a** in a 1:1 ratio together with smaller amounts of 9,10,11-trihydroxyoctadecenoates **2b** and **4b** (Scheme 3). On the basis of these results, compound **I** was identified as methyl 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate.

TABLE 2
Chromatographic Properties of Trihydroxyesters

Compound[a]	C-value[b]	R_f value[c]
Methyl 9(*R*),10(*R*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (**2a**)	22.30	0.72
Methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate (**2b**)	22.30	0.72
Methyl 9(*R*),10(*R*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate (**4a**)	22.47	0.59
Methyl 9(*S*),10(*S*),11(*S*)-trihydroxy-12(*Z*)-octadecenoate (**4b**)	22.47	0.59
Methyl 9(*S*),10(*R*),11-trihydroxy-12(*Z*)-octadecenoate[d]	22.33	0.66
Methyl 9(*S*),10(*R*),11-trihydroxy-12(*Z*)-octadecenoate[d]	22.50	0.69
Methyl 9(*S*),10(*R*),11(*R*)-trihydroxyoctadecanoate (**15b**)	22.77	—
Methyl 9(*S*),10(*R*),11(*S*)-trihydroxyoctadecanoate (**16a**)	22.69	—
Methyl 9(*S*),10(*S*),13(*R*)-trihydroxy-11(*E*)-octadecenoate (**5a**)	22.24	0.39
Methyl 9(*S*),10(*S*),13(*S*)-trihydroxy-11(*E*)-octadecenoate (**6a**)	22.31	0.50
Methyl 9(*S*),10(*R*),13(*S*)-trihydroxy-11(*E*)-octadecenoate (**7a**)	22.52	0.37
Methyl 9(*S*),10(*R*),13(*R*)-trihydroxy-11(*E*)-octadecenoate (**8a**)	22.53	0.47
Methyl 9(*R*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (**9a**)	22.32	0.39
Methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (**10a**)	22.40	0.50
Methyl 9(*S*),12(*R*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (**11a**)	22.57	0.37
Methyl 9(*R*),12(*R*),13(*S*)-trihydroxy-10(*E*)-octadecenoate (**12a**)	22.60	0.47
Compound **III**	22.30	0.72
Compound **IV**	22.40	0.50
Compound **III**-H_2	22.77	—
Compound **V**-H_2	22.77	—

[a]Enantiomers are designated with letters **a** and **b** following the compound number. Compound **III**, methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate; compound **IV**, methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate; and compound **V**, tentatively identified as methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*),15(*Z*)-octadecadienoate.
[b] Gas–liquid chromatography was carried out with a methylsilicone column operated at 230°C.
[c]The solvent system for thin-layer chromatography was ethyl acetate.
[d]Methyl 9(*S*),10(*R*),11-trihydroxy-12(*Z*)-octadecenoates epimeric at C-11 were prepared by hydrolysis of 10(*R*),11(*R*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoate. The configurations at C-11 of these compounds have not been established.

*Structure of compound **II***. No absorption bands in the range 210–300 nm were noticeable in the UV spectrum of compound **II**. The mass spectrum of the Me_3Si derivative showed ions at *m/z* 398 (2%; M^+), 383 (3; $M^+ - 15$; loss of $\cdot CH_3$), 259 [18; $Me_3SiO^+{=}CH{-}(CH_2)_7{-}COOCH_3$], 241 [87; $M^+ - 157$; loss of $\cdot(CH_2)_7{-}COOCH_3$], 225 (21), 99 [100; $^+O{\equiv}C{-}(CH_2)_4{-}CH_3$], and 73 (80; Me_3Si^+), thus indicating the presence of an epoxide group at C-12/C-13 and a hydroxyl group at C-9. The mass spectrum and the chromatographic data found for compound **II** (Table 1) were identical to those of a reference sample of the allylic epoxy alcohol methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate. Oxidative ozonolysis performed on the MC derivative of compound **II** followed by methyl-esterification afforded the MC derivative of dimethyl 2(*S*)-hydroxy-1,10-decanedioate, thus establishing the presence of a double bond in the Δ^{10} position and the *S* configuration of C-9. In agreement with the allylic epoxide structure of compound **II**, rapid hydrolysis took place at pH 3 with the formation of trihydroxyoctadecenoate **10a** as the major product (67%). In addition, smaller amounts of trihydroxyoctadecenoates **11a**, **7a**, and **6a** appeared. As shown in previous work (12,18), such a pattern of hydrolysis products is diagnostic for one specific epoxy alcohol, i.e., methyl 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoate.

Structure of compound **III**. The mass spectrum of the Me_3Si derivative of compound **III** (Fig. 2A) was in agreement with a 9,10,11-trihydroxy-12(*Z*)-octadecenoate. This structure possesses three chiral carbons, and eight stereoisomers (four separable diastereomers) are possible. For reference purposes, the *erythro*-9,10-*erythro*-10,11 diastereomer (enantiomers **2a** and **2b**), as well as the *erythro*-9,10-*threo*-10,11 diastereomer (enantiomers **4a** and **4b**), was prepared by acid-catalyzed hydrolysis of the appropriate epoxy alcohol precursors (Schemes 1 and 3). The *threo*-9,10-*erythro*-10,11 and *threo*-9,10-*threo*-10,11 diastereomers also were prepared (Table 2), although the absolute configurations of these compounds could not be established due to lack of the necessary *cis*-9,10-epoxy-11-hydroxy-12(*Z*)-octadecenoate precursor. By comparing the chromatographic data of compound **III** with those of the four chemically prepared diastereomers (Table 2), it was apparent that compound **III** had the *erythro*-9,10-*erythro*-10,11 configuration and was identical to **2a** or **2b**. As shown below, 9(*S*)-HPOD served as the precursor of compound **III**, and an incubation of [9-$^{18}O_2$]9(*S*)-HPOD led to the formation of compound **III** in which the ^{18}O-label at C-9 was retained. The fact that the carbon–oxygen bond at C-9 remained intact and that the stereochemistry of this carbon was thus preserved during the conversion of 9(*S*)-HPOD into compound **III** showed that compound **III** had the *S* configuration at C-9 and was identical to the enantiomer **2b**, i.e., methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate. In a separate experiment, compound **III** was hydrogenated and the Me_3Si derivative was analyzed by GC–MS. As expected,

the mass spectrum and C-value (22.77) were identical to those of the Me_3Si derivative of **15b** prepared by perchloric acid-catalyzed hydrolysis of the dihydro epoxy alcohol **17** (Scheme 4).

Structure of compound **IV**. The mass spectrum of the Me_3Si derivative of compound **IV** (Fig. 2B) showed ions at *m/z* 545 (0.4%; $M^+ - 15$; loss of $\cdot CH_3$), 460 [15; $M^+ - 100$; rearrangement with loss of $OHC–(CH_2)_4–CH_3$], 439 [2; $M^+ - (31 + 90)$; loss of $\cdot OCH_3$ plus Me_3SiOH], 387 (4; $M^+ - 173$), 259 [16; $Me_3SiO^+=CH–(CH_2)_7–COOCH_3$], 230 [5; rearrangement with loss of $OHC–CH=CH–CH(OSiMe_3)–CH(OSiMe_3)–(CH_2)_4–CH_3$], 173 [100; $Me_3SiO^+=CH–(CH_2)_4–CH_3$], 147 (11; $Me_3Si–O^+=SiMe_2$), 103 (11; $Me_3SiO^+=CH_2$), and 73 (69; Me_3Si^+), thus indicating a 9,12,13-trihydroxyoctadecenoate (*cf.* Ref. 12). Comparison of the chromatographic properties of compound **IV** with those of the eight possible diastereomers of methyl 9,12,13-trihydroxy-10(*E*)-octadecenoate (**9a**–**12a** and **9b**–**12b**) established that compound **IV** was identical to **10a** or its enantiomer (Table 2). Oxidative ozonolysis performed on the tris-MC derivative of compound **IV** followed by methyl-esterification afforded the MC derivative of dimethyl 2(*S*)-hydroxy-1,10-decanedioate, thus locating the double bond to the Δ^{10} position and assigning the *S* configuration to C-9. The latter finding demonstrated that compound **IV** was identical to the enantiomer **10a**, i.e., methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate.

Incubations in the presence of GSH-px. [1-^{14}C]Linoleic acid (200 μM) was stirred at 23°C for 20 min with a whole homogenate of potato leaves in the presence of GSH (2 mM) and GSH-px (0.5 U/mL). This led to a virtually complete block of the formation of compounds **I**–**IV** and also to approximately 50% inhibition of the rate of oxygenation of linoleic acid. The product was mainly due to two monohydroxy esters, i.e., the methyl ester of 9(*S*)-hydroxy-10(*E*),12(*Z*)-octadecadienoic acid [9(*S*)-HOD] (91%) and the methyl ester of 13(*S*)-hydroxy-9(*Z*),11(*E*)-octadecadienoic acid [13(*S*)-HOD] (9%). The structures and stereochemistry of these compounds were established by GC–MS and by steric analysis of chiral fragments formed upon oxidative ozonolysis. By performing a series of incubations with increasing activity of GSH-px (0–2 U/mL) and plotting the yields of 9- and 13-HOD vs. GSH-px activity, it was obvious that the 9-lipoxygenase activity was partially inhibited by GSH-px. Extrapolating the proportions of 9- and 13-HOD to zero activity of GSH-px gave a result of 95:5.

*Incubation of 9(*S*)-HPOD*. Incubation of [1-^{14}C]9(*S*)-HPOD (200 μM) with whole homogenate of potato leaves at 23°C for 20 min gave a product consisting of compounds **I**–**IV** in amounts closely similar to those observed after incubation of linoleic acid. When a suspension of the 105,000 × *g* sediment fraction in buffer was used as the enzyme source, formation of compounds **III** and **IV** was almost completely abolished and compounds **I** and **II** were obtained as the major products (Fig. 3A).

*Incubation of 13(*S*)-HPOD*. Incubation of [1-^{14}C]13(*S*)-HPOD (200 μM) with either whole homogenate of potato leaves or the 105,000 × *g* sediment fraction of leaf homogenate and analysis of the methyl-esterified product by SP-radio-HPLC showed the presence of one main radioactive product (6.2 mL effluent) (Fig. 3B). This material was identified as methyl 13-hydroxy-12-oxo-9(*Z*)-octadecenoate (α-

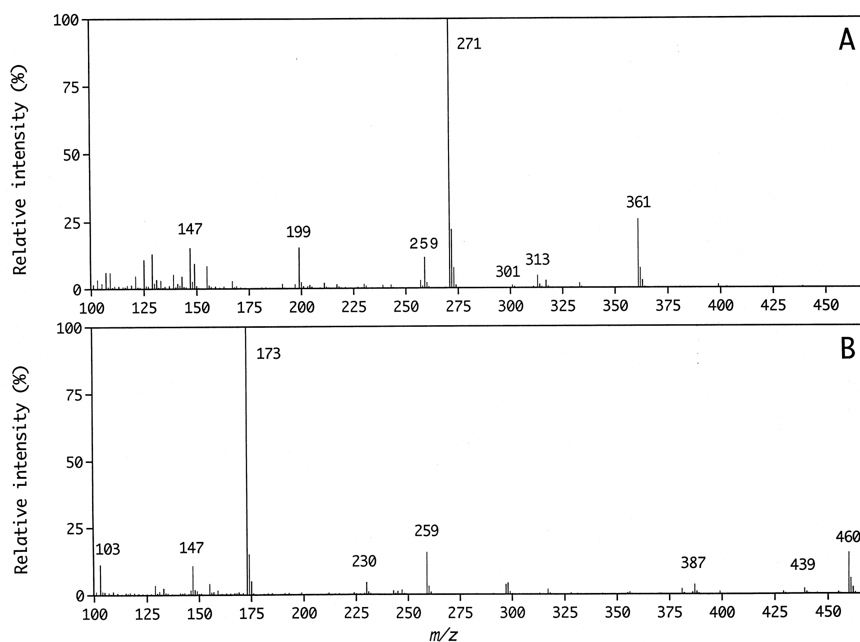


FIG. 2. Mass spectra of the Me_3Si derivatives of (A) compound **III** [methyl 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*)-octadecenoate] and (B) compound **IV** [methyl 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoate].

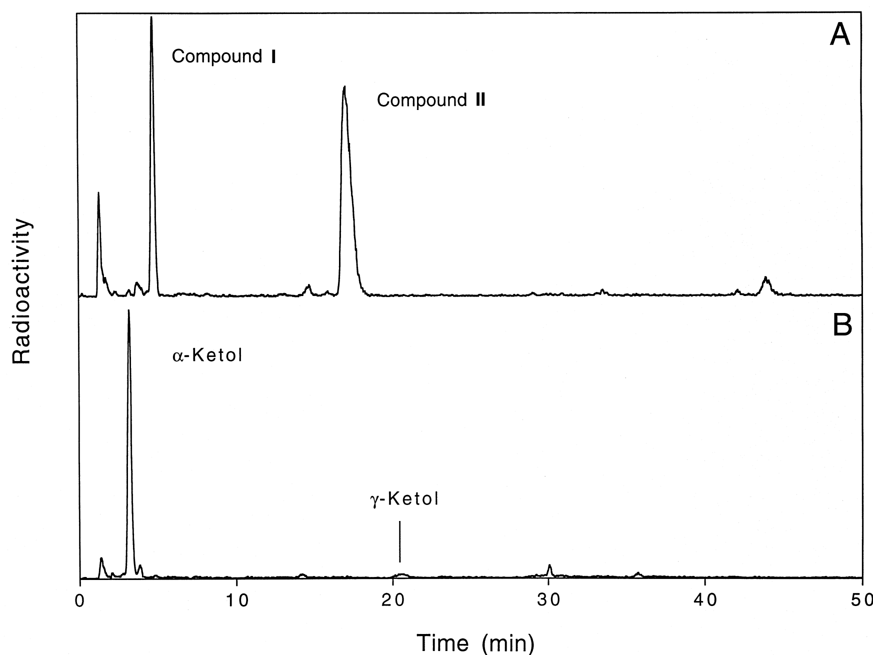


FIG. 3. SP-HPLC radiochromatograms of methyl-esterified products obtained following incubations of (A) 200 μM [1-^{14}C]9(*S*)-hydroperoxy-10(*E*),12(*Z*)-octadecadienoic acid or (B) 200 μM [1-^{14}C]13(*S*)-hydroperoxy-9(*Z*),11(*E*)-octadecadienoic acid with suspensions of the 105,000 × *g* particulate fraction of homogenate of potato leaves (5 mL; 2 mg of protein) at 23°C for 20 min. α-Ketol, methyl 13-hydroxy-12-oxo-9(*Z*)-octadecenoate; γ-ketol, methyl 9-hydroxy-12-oxo-10(*E*)-octadecenoate. For HPLC conditions and structures of compounds **I** and **II,** and abbreviation, see Figure 1.

ketol) by GC–MS using a sample of the authentic compound as the reference. A minor peak of radioactivity appeared at 40.9 mL effluent. This material was identified as methyl 9-hydroxy-12-oxo-10(*E*)-octadecenoate (γ-ketol) by comparison with an authentic sample. Formation of these ketols demonstrated the presence of allene oxide synthase activity in the 105,000 × *g* particle fraction of potato leaf homogenate (*cf.* Ref. 11). 13(*S*)-HPOD incubated in concentrations up to 1 mM was completely metabolized into ketols, whereas incubations of 9(*S*)-HPOD at concentrations >300 μM led to incomplete conversion into epoxy alcohols and accumulation of residual hydroperoxide. These results showed that 13(*S*)-HPOD, but not 9(*S*)-HPOD, served as a substrate for allene oxide synthase in potato leaves. No conversion of 13(*S*)-HPOD into epoxy alcohols was detectable in these experiments, however, it remains to be determined whether this was due to the specificity of the epoxy alcohol synthase or whether its activity on 13(*S*)-HPOD was masked by the strong allene oxide synthase activity.

Characterization of oxidation products of linolenic acid. Analysis the methyl-esterified product obtained after incubation of [1-^{14}C]linolenic acid (200 μM) with potato leaf homogenate showed two main products, i.e., compounds **V** and **VI** (Fig. 1C). The mass spectrum of the Me_3Si derivative of the first-mentioned compound (C-value, 22.30) showed ions at *m/z* 558 (0.4%; M^+), 361 (24; $M^+ - 197$), 271 (100; 361 – 90), 259 [17; Me_3SiO^+=CH–$(CH_2)_7$–$COOCH_3$], 197 [6; Me_3SiO^+=CH–(CH=CH–$CH_2)_2$–CH_3], and 73 (88; Me_3Si^+), thus suggesting a 9,10,11-trihydroxyoctadecadienoate. Catalytic hydrogenation afforded a stereoisomer of methyl 9,10,11-trihydroxyoctadecanoate which had the same C-value (22.77) and mass spectrum as methyl 9(*S*),10(*R*),11(*R*)-trihydroxyoctadecanoate (**15b**) prepared by hydrolysis of dihydro epoxy alcohol **17** or by hydrogenation of compound **III**. These data suggested that compound **V** was identical to the methyl ester of 9(*S*),10(*S*),11(*R*)-trihydroxy-12(*Z*),15(*Z*)-octadecadienoic acid.

The mass spectrum of the Me_3Si derivative of compound **VI** (C-value, 22.46) showed prominent ions at *m/z* 543 (0.5%; $M^+ - 15$; loss of $\cdot CH_3$), 460 [16; $M^+ - 98$; rearrangement with loss of OHC–CH_2–CH=CH–CH_2–CH_3], 387 (5; $M^+ - 171$), 259 [28; Me_3SiO^+=CH–$(CH_2)_7$–$COOCH_3$], 171 (100; Me_3SiO^+=CH–CH_2–CH=CH–CH_2–CH_3), and 73 (95; Me_3Si^+), suggesting the identity of compound **VI** with a di-unsaturated 9,12,13-trihydroxyoctadecanoate. That this compound was a more unsaturated analog of compound **IV** was indicated by the fact that catalytic hydrogenation of compound **VI** resulted in the formation of a trihydroxyoctadecanoate which had a C-value (22.74) and mass spectrum identical to those of methyl 9(*S*),12(*S*),13(*S*)-trihydroxyoctadecanoate prepared by hydrogenation of compound **IV**. These data suggested that compound **VI** was identical to the methyl ester of 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*),15(*Z*)-octadecadienoic acid; however, unambigous identification of com-

pounds **V** and **VI** produced from linolenic acid must await synthesis of the appropriate, stereochemically defined reference compounds.

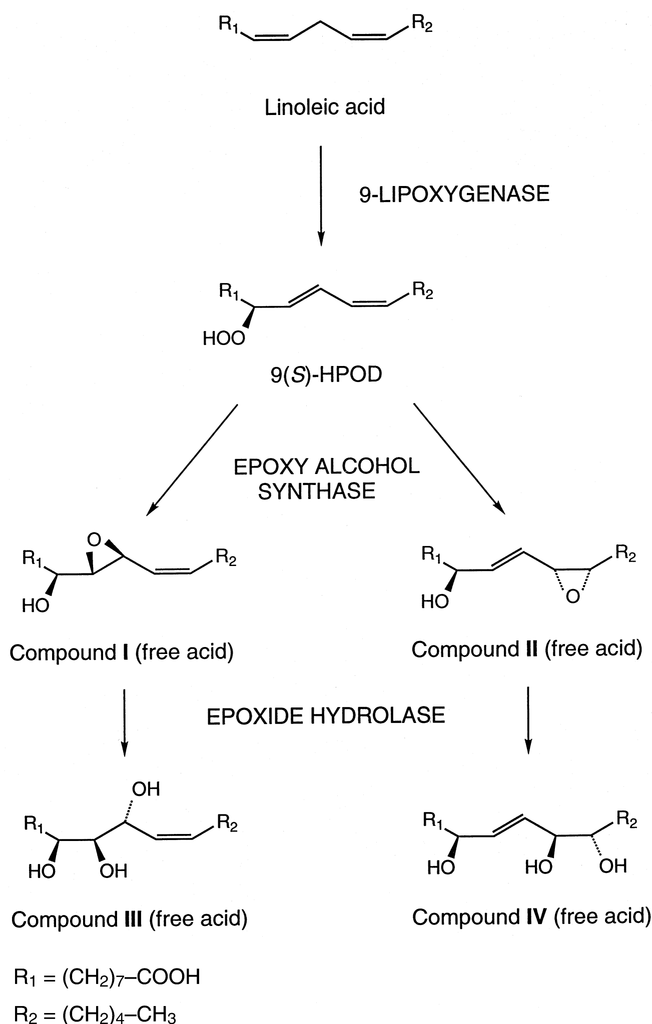
Assay of peroxygenase activity. Suspensions of the 105,000 × *g* particle fraction of potato leaf homogenate were incubated with [1-^{14}C]oleic acid and 5 mM hydrogen peroxide. Labeled 9,10-epoxystearate was undetectable by radio-TLC, indicating the absence of peroxygenase activity (<0.5 nmol/min · mg protein).

DISCUSSION

The present study is concerned with a new pathway of fatty acid metabolism in a higher plant, i.e. biosynthesis of epoxyhydroxy and trihydroxy fatty acids in leaves of potato. The first step of the transformation was catalyzed by a 9-lipoxygenase as indicated by the structures of the products formed, and by the fact that incubation of linoleic acid performed in the presence of GSH-px led to the formation of 9(*S*)-HOD by diversion of 9(*S*)-HPOD. That the major lipoxygenase activity in leaves of potato was of the 9 type was in agreement with the recent isolation from infected potato leaves of colneleic and colnelenic acids, compounds which are formed from the 9(*S*)-hydroperoxides of linoleic and linolenic acids, respectively (21). In contrast to these results, it has been reported that the two mainly expressed lipoxygenase genes in leaves of potato code for 13-lipoxygenases, and that wounding of potato leaves leads to induction of 13- and not 9-lipoxygenase (22). No data concerning lipoxygenase activity or lipoxygenase products in potato leaves were given in this study.

The second step of the transformation studied in the present work consisted of enzymatic isomerization of 9(*S*)-HPOD into two acid-labile fatty acid epoxy alcohols, i.e., the α,β-epoxy alcohol 10(*S*),11(*S*)-epoxy-9(*S*)-hydroxy-12(*Z*)-octadecenoic acid (free acid form of compound **I**) and the γ,δ-epoxy alcohol 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid (free acid form of compound **II**) (Scheme 5). This step was conveniently studied using the 105,000 × *g* particle fraction of potato leaf homogenate, which lacked the enzyme activity catalyzing breakdown of the epoxy alcohols (Fig. 3). A related conversion of hydroperoxides has been observed in preparations of the primitive fungus *S. parasitica*, in which case linoleic acid 9- and 13-hydroperoxides, as well as arachidonic acid 15-hydroperoxide, were each converted into an α,β- and a γ,δ-epoxy alcohol by intramolecular epoxidation of the α,β- and γ,δ-double bonds, respectively (13,23). Interestingly, the γ,δ-epoxy alcohol formed in the potato leaf homogenate was identical to the γ,δ-epoxy alcohol formed from 9(*S*)-HPOD in the fungal preparation, whereas the α,β-epoxy alcohols biosynthesized in the two systems differed with respect to the stereochemistry of the epoxide group, i.e., 10(*S*),11(*S*) in the potato leaf epoxy alcohol and 10(*R*),11(*R*) in the *S. parasitica* epoxy alcohol. The enzyme peroxygenase also catalyzes formation of epoxy alcohols from fatty acid hydroperoxides (17), and this enzyme is responsible for the biosynthesis of 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid in preparations of oat seeds (18,24). Peroxygenase activity was undetectable in the preparations of potato leaves used in the present study, and formation of epoxy alcohols is proposed to take place by action of a specific epoxy alcohol synthase activity (Scheme 5). It should be mentioned in this context that the epoxy alcohols *trans*-9,10-epoxy-*threo*-11-hydroxy-12(*Z*)-octadecenoic and *trans*-12,13-epoxy-*threo*-11-hydroxy-9(*Z*)-octadecenoic acids have previously been generated from 9- and 13-HPOD, respectively, in a 9-lipoxygenase-promoted process (25) or by "pseudo-enzymic" reactions taking place in preparations of potato tuber (26). These epoxy alcohols differ chemically from those biosynthesized in *S. parasitica* or potato leaves and are formed from hydroperoxides by a mechanism involving alkoxyl radical cyclization rather than by a double bond epoxidation mechanism (see Ref. 27 for a discussion of mechanisms of formation of fatty acid epoxy alcohols).

The third step of the transformation studied in the present work consisted of conversion of fatty acid epoxy alcohols into trihydroxyoctadecenoic acids. It seemed likely that the en-



SCHEME 5

zyme responsible for this step was an epoxide hydrolase similar to or identical to the epoxide hydrolase in oat seeds (18). This enzyme catalyzed regio- and stereospecific hydrolysis of 12(*R*),13(*S*)-epoxy-9(*S*)-hydroxy-10(*E*)-octadecenoic acid into 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid and was characterized as a soluble 47 kD protein (18). The epoxide hydrolase activity of potato leaves was relatively weak, thus allowing epoxy alcohol intermediates to be isolated when the time of incubation was kept short (*cf.* Fig. 1A and 1B). From the stereochemistry of the epoxy alcohols and their trihydroxy acid products, it could be deduced that the epoxide hydrolase reaction consisted of stereospecific attack by water at the allylic epoxide carbons C-11 and C-12 of the α,β- and γ,δ-epoxy alcohols, respectively, followed by opening of the epoxide ring by an S_N2-type reaction.

The existence of two partially different pathways for biosynthesis of 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid (free acid form of compound **IV**), i.e., one involving peroxygenase in oat seeds (18) and one involving epoxy alcohol synthase activity in potato leaves, is noteworthy and may suggest a specific function for this particular trihydroxy acid. Studies in the rice plant have shown that 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid is produced in plants suffering from rice blast disease and that it exerts a growth-inhibitory effect against the rice blast fungus (28,29). Furthermore, a 9,12-13-trihydroxy-10(*E*)-octadecenoate (stereochemistry not determined) has been isolated as an antifungal principle from tubers of taro infected with the black root fungus (*Ceratocystis fimbriata*) (30). Further studies are needed to establish whether 9(*S*),12(*S*),13(*S*)-trihydroxy-10(*E*)-octadecenoic acid and the other oxylipins encountered in the present study play a role as endogenous defensive substances in potato.

ACKNOWLEDGMENTS

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Factors Enhancing Diacylglycerol Acyltransferase Activity in Microsomes from Cell-Suspension Cultures of Oilseed Rape

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ABSTRACT: Several factors, including an unidentified endogenous component, were found to stimulate microsomal diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) from a microspore-derived cell-suspension culture of oilseed rape (*Brassica napus* L. cv. Jet Neuf). At a concentration of 25 mM, $MgSO_4$ and $MgCl_2$ stimulated microsomal DGAT 25- and 10-fold, respectively. ATP and CoA at concentrations of 2 and 1 mM stimulated the enzyme 2.4- and 12-fold, respectively, although the effects were lessened in the presence of higher Mg^{2+} concentrations. Although microsomal DGAT activity was increased only slightly by the addition of exogenous *sn*-1,2-diacylglycerol to the reaction mixture, it was increased substantially by the addition of exogenous phosphatidate. *sn*-Glycerol-3-phosphate and other phospholipids tested did not have this stimulatory effect. DGAT activity did not decrease when microsomes were incubated with ATP in the presence of the cytosolic fraction. This fraction, however, contained a small organic compound(s) that stimulated microsomal DGAT activity.

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In developing oilseeds, triacylglycerol (TAG) is formed through a series of enzymatically catalyzed reactions known as the Kennedy pathway (1,2). Diacylglycerol acyltransferase (DGAT, EC 2.3.1.20) catalyzes the final step in this pathway *via* the acyl-CoA dependent acylation of *sn*-1,2-diacylglycerol (DAG) and has been implicated as a rate-limiting step in the formation of TAG in both plants (3–6) and animals (7). An examination of the factors that may affect the activity of this enzyme in plants may enable the development of strategies to increase seed oil content and/or alter the fatty acid composition of the oil.

DGAT activity is associated with the endoplasmic reticulum (2), and in mammalian systems, the activity of the enzyme may be altered through allosteric interactions and covalent modification (8,9). While the characteristics of microsomal DGAT have been studied extensively in mammalian systems, studies with the plant enzyme have been more limited. Factors affecting DGAT activity have been investigated in several systems. For example, phospholipids have been found to stimulate fungal DGAT, with phosphatidate (PA) having the greatest effect (10). Organic molecules known as chalcones (11) and amidepsines (12) also have been shown to inhibit mammalian DGAT activity. The chalcones were extracted from hops (*Humulus lupulus*) and inhibited DGAT activity in rat liver microsomes and inhibited TAG production in intact Raji cells (11). The amidepsines were isolated from the fungal culture, *Humicola* sp. FO-2942 (12).

Cultures derived from oil-forming plants have proven useful for studying the biochemistry of TAG accumulation (13,14). The synthesis of storage oils in seeds and fruits is a dynamic and stage-dependent process. The use of these cultures, however, permits the study of oil-forming species year-round without the need to obtain seeds (zygotic embryos) at specific stages of development. Recently, a microspore-derived (MD) cell-suspension culture of winter oilseed rape (*Brassica napus* L. cv. Jet Neuf) has been utilized in biochemical and molecular genetic studies of TAG accumulation (15–18). The cell-suspension culture was generated in 1983 (19) and initially was used for studies of freezing tolerance (20–22). The cell-suspension culture has been shown to accumulate about 3–6% TAG on a dry weight basis when maintained in growth medium containing 6% (wt/vol) sucrose (15,18). Increasing the sucrose concentration to 22%, however, has been shown to increase TAG content to about 9% (18). In contrast, mature seed and late-cotyledonary-stage MD embryos (35 d in culture) of oilseed rape have been shown to accumulate TAG in excess of 45% of the dry weight of the tissue (23). MD cell-suspension cultures, however, are easier to maintain than MD embryos and offer the advantage of allowing study of TAG biosynthesis in the absence of the cellular differentiation associated with zygotic and MD embryos.

The current study examined the effects of various compounds on microsomal DGAT activity in the MD cell-suspension culture in an effort to further characterize this enzyme. The effects of various salts, ATP, CoA, exogenous substrates, phospholipids, and *sn*-glycerol-3-phosphate (G-3-P) on DGAT activity were determined. During investigation of the possible alteration of microsomal DGAT activity by phosphorylation, the cytosolic fraction was found to stimulate TAG biosynthesis.

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Abbreviations: BSA, bovine serum albumin; CL, cardiolipin; DAG, *sn*-1,2-diacylglycerol; DGAT, diacylglycerol acyltransferase; G-3-P, *sn*-glycerol-3-phosphate; MD, microspore-derived; PA, phosphatidate; SDS, sodium dodecyl sulfate; TAG, triacylglycerol; TLC, thin-layer chromatography.

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MATERIALS AND METHODS

Chemicals. [1-^{14}C]Oleic acid (51 Ci mol^{-1}) was obtained from Amersham Canada, Ltd. (Oakville, Ontario, Canada). Merck silica gel 60 H, used for preparing thin-layer chromatography (TLC) plates, was from VWR Canlab (Mississauga, Ontario, Canada). Ecolite^{TM} (+) biodegradable scintillant was from ICN Biomedicals, Inc. (Irvine, CA). Dye reagent concentrate for protein assays was from Bio-Rad (Richmond, CA). High-performance liquid chromatography-grade solvents were from BDH, Inc. (Toronto, Ontario, Canada). *sn*-1,2-Dioleoylglycerol was from Avanti Polar Lipids, Inc. (Alabaster, AL). Ultrafiltration membranes were from Amicon, Ltd. (Oakville, Ontario, Canada). Acyl-CoA synthetase from *Pseudomonas fragi* was from Boehringer Mannheim Canada (Laval, Québec, Canada). Acyl-CoA was synthesized from radiolabeled fatty acids (24). Disodium ATP (treated to remove divalent cations) and all other biochemicals used were of the highest purity available and were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO).

Plant material. The MD cell-suspension culture of *B. napus* L. cv. Jet Neuf was provided by Dr. J. Singh of the Eastern Cereal and Oilseed Research Centre, Agriculture and Agri-Food Canada, Ottawa, and essentially maintained according to Orr *et al.* (20). The culture was grown on a rotary shaker (set at 150 rpm) at 25°C under constant light with an intensity of 36 μmol m^{-2} s^{-1}. One-third of the mass of cells grown in 125-mL Erlenmeyer flasks was routinely transferred to fresh medium at 2-wk intervals. The remainder of the cells, obtained after 2 wk of culture, were washed with water over a nylon sieve, blotted with filter paper to remove excess water, and the fresh weight was determined. Cells were either immediately used or frozen in liquid N_2 and stored at –80°C until use.

Preparation of microsomes. Cells were ground in four volumes of grinding buffer (0.5 M sucrose, 0.2 M Hepes-NaOH, pH 7.4) using a chilled mortar and pestle. Differential centrifugation of the homogenate was performed at 4°C. The homogenate was centrifuged at 10,000 × *g* for 20 min and the supernatant was filtered through glass wool. The 10,000 × *g* supernatant was then centrifuged at 100,000 × *g* for 1 h. The resulting microsomal pellet was washed with 10 mM Hepes-NaOH buffer (pH 7.4) and resedimented at 100,000 × *g* for 1 h. The washed pellet was resuspended in a volume equivalent to one-tenth of the original mass of cells using 10 mM Hepes-NaOH buffer (pH 7.4) (i.e., microsomes were resuspended in 100 μL of buffer if 1 g of cells was used). Both the first 100,000 × *g* supernatant (cytosolic fraction) and the resuspended microsomes were divided into small aliquots, flash-frozen with liquid N_2, and stored at –80°C.

Enzyme assays and protein determination. Microsomal DGAT was assayed essentially as described by Little *et al.* (16) except that ATP and CoA were not included in the standard reaction mixture. Microsomes used in the enzyme assays were thawed once. Different batches of microsomes were used, but each series of experiments (e.g., effect of salt concentration) was conducted with the same batch of microsomes. Each experiment was repeated three times in triplicate, resulting in a total of nine enzyme assays for each condition. The 60-μL reaction mixture contained 0.2 M Hepes-NaOH (pH 7.4), 330 μM *sn*-1,2-dioleoylglycerol, 15 μM [1-^{14}C]oleoyl-CoA (51 Ci mol^{-1}), 0.1% (wt/vol) Tween-20, 6 mg bovine serum albumin (BSA) mL^{-1} and 5–10 μL resuspended microsomes. Microsomal protein introduced into the reaction mixture ranged from 20 to 90 μg. Enzyme reactions were allowed to proceed at 30°C for 10 min. The enzyme reaction was initiated with microsomes and terminated by adding 10 μL of 5% (wt/vol) sodium dodecyl sulfate (SDS). A reaction mixture consisting of 120 μL (final reaction volume) was used to test the effect of various volumes of cytosolic fraction on DGAT activity in order to allow for a greater range of concentrations. Studies of other reaction components and additives were carried out under the standard conditions except that the concentration of the component of interest was varied. A 50-μL aliquot of each reaction mixture was spotted onto a silica gel 60 H preparative TLC plate and developed in hexane/diethyl ether (80:20, vol/vol) with a trioleoylglycerol standard. Sections of silica containing TAG from each lane were scraped into scintillation vials, combined with 5 mL Ecolite^{TM} (+), and assayed for radioactivity.

Thioesterase activity was assayed under the same reaction conditions described above for the DGAT assay except that the preparative TLC plates were developed in hexane/diethyl ether/acetic acid (70:30:1, by vol) with oleic acid as a standard.

Acyl-CoA synthetase from *P. fragi* was used to test cytosolic fractions for CoA content. The enzyme was assayed at 35°C for 3 h using a 90-μL reaction mixture containing 0.1 M Mops [3-(*N*-morpholino)propanesulfonic acid]-NaOH (pH 7.5), 10 mM dithiothreitol, 10 mM ATP, 0.05 μCi [1-^{14}C]oleic acid, 0.1% (wt/vol) Triton X-100, 2 μL of acyl-CoA synthetase (0.5 mg in 50 μL 0.1 M Mops-NaOH, pH 7.4), and varying concentrations of CoA or quantities of cytosolic fraction. Reactions were quenched with 10 μL 5 % (wt/vol) SDS, and 75 μL of each mixture was run on a silica gel H plate in *n*-butanol/water/acetic acid (5:3:2, by vol) as described by Taylor *et al.* (25) with oleoyl-CoA as a standard. Silica sections containing acyl-CoA were assayed for radioactivity as described for the DGAT assay.

The protein content of microsomes was determined using the Bio-Rad protein microassay based on the Bradford procedure (25), using BSA as a standard.

Ashing. Two milliliters of the 100,000 × *g* supernatant were ashed by placing the sample into a crucible in a steam bath and slowly adding 20 drops of concentrated aqueous HCl. When the sample approached dryness and was beginning to char, the crucible was placed in a cold muffle furnace and ignited by gradually heating to 600°C overnight. The sample was then cooled to room temperature and resuspended in 2 mL of 10 mM Hepes-NaOH buffer (pH 7.4).

Ultrafiltration. Ultrafiltration experiments were carried out on the 100,000 × *g* supernatant using Amicon ultrafiltration cells with YM 30, YM 10, and YM 1 membranes (nominal molecular mass cutoff of 30, 10, and 1 kDa, respectively).

RESULTS AND DISCUSSION

Effect of salts on DGAT activity. A systematic study of potential factors affecting the activity of DGAT was undertaken. In vari-

ous systems, divalent cations have been shown to alter acyltransferase activity either negatively or positively (26–28). Mg^{2+} is sometimes included in reaction mixtures for assaying acyltransferase activity (26–30). The effects of various salts on microsomal DGAT activity from the cell-suspension culture are shown in Figure 1. DGAT activity of the microsomes prepared from MD cell-suspension cultures was not affected by one cycle of freezing and thawing. Mg^{2+} salts had the greatest effect on DGAT activity with $MgSO_4$ and $MgCl_2$ at 25 mM concentrations stimulating microsomal DGAT by about 25- and 10-fold, respectively. Mg^{2+} also has been shown to activate DGAT from *Mortierella ramanniana* var. *angulispora* (10). High concentrations of Mg^{2+} previously have been shown to decrease the solubility of fatty acyl-CoA (31). Consequently, it may be favorable to use a moderate concentration of Mg^{2+} in order to balance high activity levels with substrate solubility. $CaCl_2$ and NaCl were found to have lesser effects on DGAT in this system (results not shown). Ichihara and Noda (32) reported that DGAT activity in microsomes from maturing safflower seeds is not stimulated by Ca^{2+}.

Effect of ATP and CoA on DGAT activity. ATP and CoA are sometimes added to reaction mixtures for assaying TAG biosynthetic enzymes (16,33). The concentration dependence of microsomal DGAT on ATP and CoA was systematically examined using various concentrations of each compound. Addition of 2 mM ATP increased DGAT activity up to 2.4-fold in the absence of added Mg^{2+} (Fig. 2A) and up to 1.5-fold in the presence of 3 mM Mg^{2+} (Fig. 2B). ATP did not have a significant effect, however, in the presence of 25 mM Mg^{2+} (Fig. 2C). Bacterial diacylglycerol kinase activity has been shown to be dependent on the concentration of a Mg-ATP complex as well as being activated by free Mg^{2+} (34).

CoA, at a concentration of 1 mM, stimulated DGAT up to 14- and 13-fold in the absence of added Mg^{2+} and in the presence of 3 mM Mg^{2+}, respectively (Figs. 3A and 3B). In the presence of 25 mM Mg^{2+}, however, 1 mM CoA only increased DGAT activity 1.5-fold (Fig. 3C).

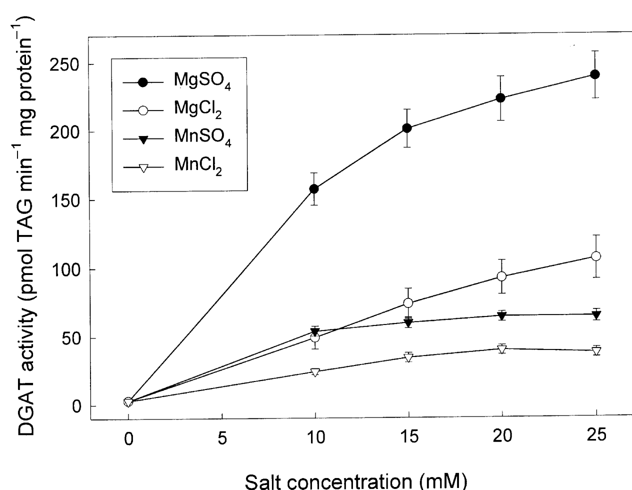

FIG. 1. Effect of various concentrations of $MgSO_4$, $MgCl_2$, $MnSO_4$, and $MnCl_2$ on microsomal diacylglycerol acyltransferase (DGAT) activity from microspore-derived (MD) cell-suspension cultures of *Brassica napus* L. cv. Jet Neuf. Data points represent the mean ± SE (*n* = 9).

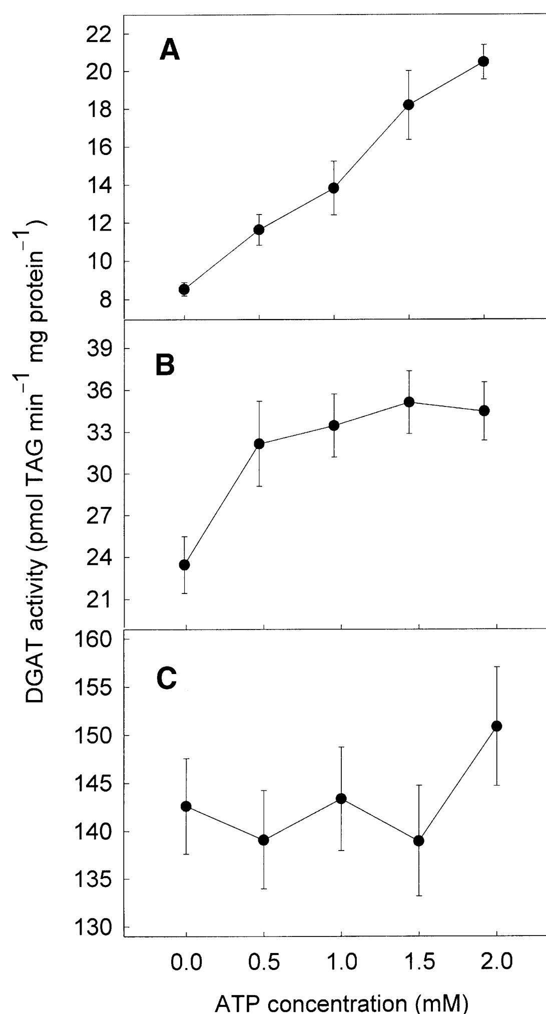

FIG. 2. Effect of ATP concentration on microsomal DGAT activity from MD cell-suspension cultures of *B. napus* L. cv Jet Neuf (A) without Mg^{2+}; (B) with 3 mM Mg^{2+}; (C) with 25 mM Mg^{2+}. Note the differences in the scales for DGAT activity among the three panels. Data points represent the mean ± SE (*n* = 9). See Figure 1 for abbreviations.

It was previously reported that thioesterase activity was high enough in MD embryos of *B. napus* L. to interfere with the determination of DGAT activity and that ATP and CoA were required to restore the acyl-CoA pool through the action of an endogenous acyl-CoA synthetase (23). Microsomes from the MD cell-suspension culture degraded between 1 and 6.5% of the radiolabeled acyl-CoA under the same conditions that the DGAT assay was conducted. This suggested that although ATP and CoA caused an increase in DGAT activity, it did not occur through restoration of the acyl-CoA pool. Instead, these compounds may be acting as allosteric modulators of DGAT in this system.

Effect of exogenous substrates on DGAT activity. The dependence of DGAT activity on oleoyl-CoA and *sn*-1,2-dioleoylglycerol concentration in the presence of 3 and 25 mM $MgCl_2$

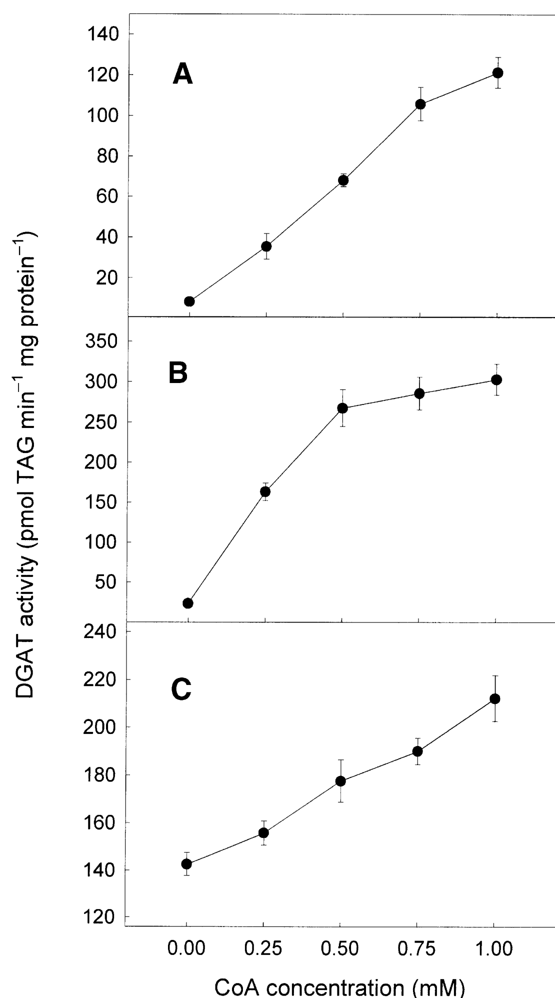

FIG. 3. Effect of CoA concentration on microsomal DGAT activity from MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. (A) Without Mg^{2+}; (B) 3 mM Mg^{2+}; (C) 25 mM Mg^{2+}. Note the differences in the scales for DGAT activity among the three panels. Values represent the mean ± SE (n = 9). See Figure 1 for abbreviations.

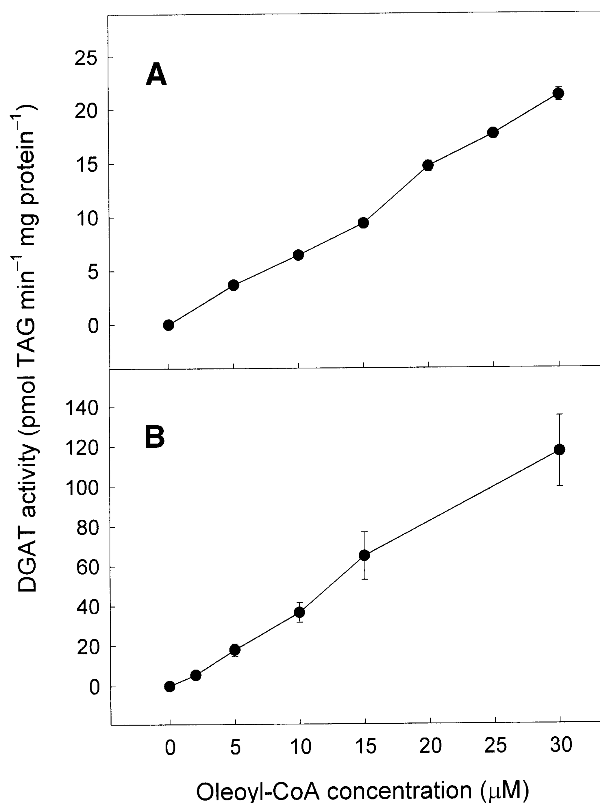

FIG. 4. Effect of oleoyl-CoA concentration on microsomal DGAT activity from MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. (A) 3 mM Mg^{2+}; (B) 25 mM Mg^{2+}. Note the differences in the scales for DGAT activity between the two panels. Data points represent the mean ± SE (n = 9). See Figure 1 for abbreviations.

was examined. The effect of oleoyl-CoA concentration seemed to be similar in the presence of both low (Fig. 4A) and high Mg^{2+} concentrations (Fig. 4B), even though the solubility of fatty acyl-CoA has been shown to be greatly reduced in the presence of high Mg^{2+} concentrations (31).

DGAT activity was not dependent on exogenous *sn*-1,2-dioleoylglycerol when assayed in the presence of 3 mM Mg^{2+} (Fig. 5A), but 100 μM (bulk concentration) *sn*-1,2-dioleoylglycerol caused a 1.3-fold increase in activity when 25 mM Mg^{2+} was present (Fig. 5B). This may have been a result of the increased activity at high Mg^{2+} concentration that led to rapid utilization of the endogenous substrate during the reaction. Little *et al.* (16) previously reported that DGAT activity in fractions solubilized from microsomes of cell-suspension cultures was not dependent on exogenous DAG. Cao and Huang (29) also reported that endogenous DAG was present in microsomes from maturing oil seeds of maize.

Effect of phospholipids and G-3-P on DGAT activity. Phospholipids have been shown to affect DGAT activity in mammalian (35) and fungal (36) systems, and may represent allosteric modulators of the enzyme. In the current study, the addition of 500 μM (bulk concentration) PA to the reaction mixture caused a twofold increase in DGAT activity (Fig. 6). Other phospholipids and G-3-P, tested at 500 μM, had no significant effect on DGAT activity (Fig. 6). Although PA is a precursor for DAG production, the addition of DAG to our system (in the presence of 3 mM Mg^{2+}) did not stimulate DGAT activity. This suggested that PA may be acting as an allosteric modulator. Alternatively, the PA may have been used by microsomal PA phosphatase to produce DAG that was presented to DGAT in a manner that enabled it to be used more efficiently than exogenous DAG. Stimulation by PA was concentration dependent with almost a 2.5-fold stimulation at 1 mM PA (Fig. 7). Although we found that G-3-P had no significant effect on DGAT activity in the cell-suspension system, Taylor *et al.* (33) have reported that G-3-P results in increased TAG production in MD embryo microsomes and suggested that the compound could be a positive effector of TAG biosynthetic enzymes. Kamisaka and Nakahara (36) examined the effects of various phospholipids on solublized DGAT from *M. ramanniana* and also found that

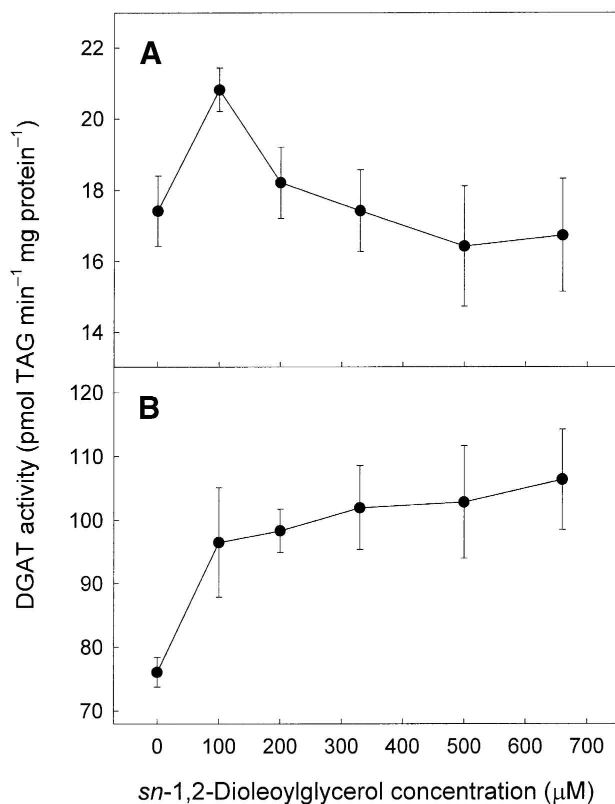

FIG. 5. Effect of bulk concentration of *sn*-1,2-dioleoylglycerol on microsomal DGAT activity in MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. (A) 3 mM Mg^{2+}; (B) 25 mM Mg^{2+}. Note the differences in the scales for DGAT activity between the two panels. Data points represent the mean ± SE (n = 9). See Figure 1 for abbreviations.

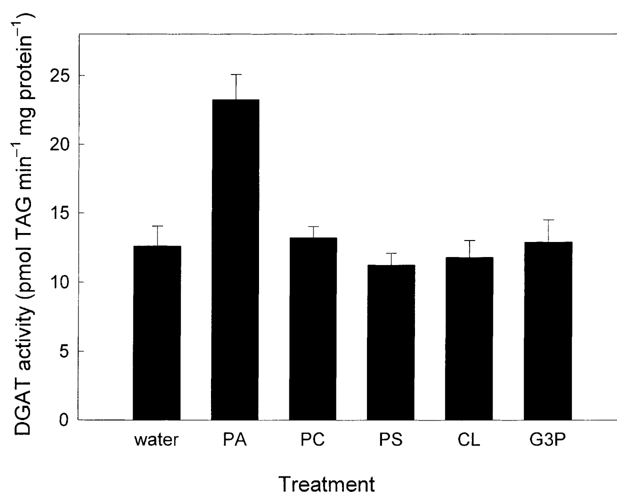

FIG. 6. Effect of 500-μM bulk concentrations of phosphatidate (PA), phosphatidylcholine (PC), phosphatidylserine (PS), cardiolipin (CL), and *sn*-glycerol-3-phosphate (G-3-P) on microsomal DGAT activity from MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. Stock mixtures (0.5 mL) of PA (from egg yolk lecithin), PC (from egg yolk lecithin), PS (from bovine brain), and CL (from bovine heart) were prepared using 10 mM Hepes-NaOH buffer, pH 7.4. The stock mixtures were sonicated in small glass vials on ice for 10 min using a Cole-Palmer Ultrasonic Cleaner, model 8845-44 (Cole-Palmer Instrument Co., Chicago, IL). The dispersed phospholipids were diluted into the reaction mixtures at the appropriate final bulk concentration immediately prior to assaying for enzyme activity. Bar heights represent the mean ± SE (n = 9). See Figure 1 for other abbreviations.

PA had the greatest effect and that G-3-P had little effect on the activity of the enzyme.

Stimulation of DGAT activity by a cytosolic factor(s). In mammalian systems, DGAT activity may be altered by a phosphorylation–dephosphorylation mechanism (37). The reversible inactivation by phosphorylation was detected by incubating rat liver microsomes with an aliquot of the cytosolic fraction, plus ATP and Mg^{2+}. The resulting inactivation was reversed upon incubation of the microsomes with the cytosolic fraction in the absence of ATP. The decrease in DGAT activity implicated the action of a soluble DGAT-kinase (9,38). In an attempt to determine if a similar mechanism was operative in the MD cell-suspension culture, an aliquot of the cytosolic fraction (100,000 × g supernatant) was added to the DGAT assay, either in the presence or absence of ATP. The cytosolic fraction stimulated DGAT activity when added back to the microsomal fraction in the presence of 3 mM Mg^{2+}, with 5 μL of the cytosolic fraction resulting in about twofold stimulation of DGAT activity (Table 1). In the presence of 25 mM Mg^{2+}, however, a much lower level of stimulation by the cytosolic fraction was observed (Table 1). Unlike results from mammalian studies, no inhibition was detected under any of the conditions tested. The stimulation by the cytosolic fraction was not dependent on exogenous ATP (Table 1) or affected by preincubation with microsomes (data not shown), but was dependent on the quantity of cytosolic fraction added. The degree of stimulation showed an essentially linear dependence on the quantity of cytosolic fraction added to the reaction mixture (Fig. 8). Thirty microliters of the cytosolic fraction resulted in a sixfold stimulation of DGAT activity.

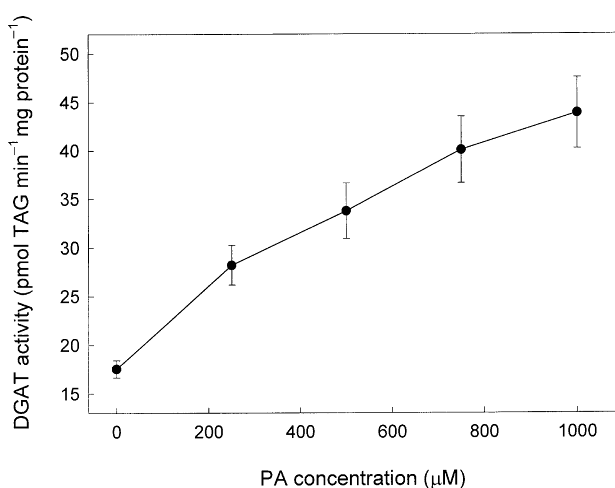

FIG. 7. Effect of bulk concentration of PA on microsomal DGAT activity in MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. A stock dispersion of PA was prepared as described in the legend to Figure 6. Data points represent the mean ± SE (n = 9). See Figures 1 and 6 for abbreviations.

TABLE 1
Effect of 5 μL Cytosolic Fraction on Microsomal DGAT Activity in MD Cell-Suspension Cultures of *Brassica napus* L. cv. Jet Neuf in the Presence of 3 or 25 mM Mg^{2+} and 0, 1, or 2 mM ATP

	Specific activity of DGAT[a] (pmol TAG min^{-1} mg $protein^{-1}$)	
	Control	Cytosolic fraction
3 mM Mg^{2+}		
0 mM ATP	18.52 ± 1.33	40.54 ± 3.40
1 mM ATP	50.12 ± 4.45	99.97 ± 8.41
2 mM ATP	52.25 ± 4.43	103.52 ± 7.42
25 mM Mg^{2+}		
0 mM ATP	98.38 ± 7.26	112.13 ± 6.64
1 mM ATP	190.11 ± 14.39	222.61 ± 16.12
2 mM ATP	189.38 ± 12.38	223.10 ± 14.01

[a]Values represent the mean ± SE (n = 9). DGAT, diacylglycerol acyltransferase; MD, microspore-derived; TAG, triacylglycerol.

Ashing of the cytosolic fraction resulted in a loss of the stimulatory effect, which indicated that the factor was organic. Furthermore, ultrafiltration indicated that the stimulatory factor(s) had a nominal molecular weight of less than 1,000 with 80% of the stimulatory activity recovered after passage through a YM1 membrane. There have not been any previous reports of a small organic stimulator of DGAT in plant systems, although xanthohumols (which are chalcones) isolated and characterized from hops of *H. lupulus* have been shown to inhibit DGAT activity in microsomes from rat liver cells (11).

CoA was the most potent of the exogenous organic stimulators of microsomal DGAT activity identified in this study (Fig. 3). In order to determine if CoA present in the cytosolic fraction might be responsible for the stimulation, the effects of CoA and the cytosolic fraction on *P. fragi* acyl-CoA synthetase were compared. Bacterial acyl-CoA synthetase activity was found to be highly dependent on exogenous CoA concentration (data not shown). Some enzyme activity (1.7 nmol h^{-1} mg $protein^{-1}$) was detectable without exogenous CoA, suggesting the enzyme preparation contained some CoA. Incorporation of 2 μM exogenous CoA into the reaction mixture increased the enzyme activity by fourfold. Addition of increasing volumes of the cytosolic fraction, in the absence of exogenous CoA, however, did not increase bacterial acyl-CoA synthetase activity. Therefore, under these experimental conditions, CoA was not detectable in the cytosolic fraction.

ATP was found to stimulate DGAT activity only up to 1.5-fold (Fig. 2B) under the conditions used to test the stimulation of DGAT by the cytosolic fraction. Thus, ATP alone could not be responsible for all of the stimulatory activity present in the cytosolic fraction.

Collectively, the results indicated that DGAT in microsomes of MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf was affected by a number of molecules, including inorganic divalent ions, PA, CoA, ATP, and an unidentified organic factor(s). Endogenous DAG was the preferred substrate of the enzyme because microsomal DGAT activity was independent of exogenous DAG. Also, the stimulatory effect of PA may have involved the generation of DAG that was more readily utilized by DGAT than exogenous DAG. Thus, it is possible that substrate

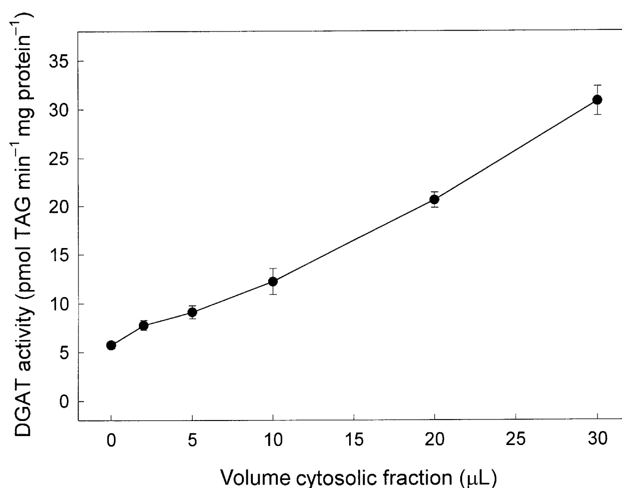

FIG. 8. Effect of the volume of cytosolic fraction on microsomal DGAT activity in MD cell-suspension cultures of *B. napus* L. cv. Jet Neuf. Enzyme assays were conducted using standard reaction conditions (the reaction mixture contained 3 mM Mg^{2+} in the absence of ATP and CoA). Data points represent the mean ± SE (n = 9). See Figure 1 for abbreviations.

channeling (39) is involved in TAG biosynthesis in the endoplasmic reticulum. Although addition of exogenous CoA and ATP resulted in enzyme stimulation, the cytosolic fraction, prepared from cell homogenate, may have been too dilute to reflect the endogenous effects of these compounds. Attempts to demonstrate a decrease in DGAT activity by phosphorylation revealed the presence of a cytosolic factor(s) that stimulated the enzyme. Future identification of the stimulator(s) may provide additional insight into the characteristics of plant DGAT.

ACKNOWLEDGMENTS

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Acyclic and Incompletely Cyclized Triterpene Alcohols in the Seed Oils of Theaceae and Gramineae[1]

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ABSTRACT: The triterpene alcohol constituents of the nonsaponifiable lipids of two Theaceae seed oils, sasanqua and camellia oils, and two Gramineae seed oils, wheat germ and rice bran oils, were investigated. This led to the isolation and characterization of one acyclic and eight incompletely cyclized triterpene alcohols. They are camelliol A, camelliol B, camelliol C, achilleol A, helianol, isohelianol, sasanquol, graminol A [(13*R*,14*R*)-3,4-*seco*-25(10→9)*abeo*-8α,9β,10α-podioda-4,17,21-trien-3-ol], and (2*Z*,6*Z*,10*Z*,14*E*,18*E*)-farnesylfarnesol. Two other compounds isolated were characterized as (2*Z*,6*Z*,10*E*,14*E*)-geranylfarnesol, a sesterterpene alcohol, and phytol, a diterpene alcohol. Graminol A and (2*Z*,6*Z*,10*E*,14*E*)-geranylfarnesol are considered to be new natural products.

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In the course of our study on the triterpene alcohol constituents of higher plants, we have isolated seven incompletely cyclized triterpene alcohols from the nonsaponifiable lipids (NSL) of sasanqua oil and characterized them as camelliol A (**I**), camelliol B (**II**), camelliol C (**III**), and achilleol A (**IV**) (1); helianol (**V**) and isohelianol (**VI**) (2); and sasanquol (**VII**) (3) (see Scheme 1 for the structures), in addition to several tetracyclic and pentacyclic triterpene alcohols (4). In continuing our work on camellia oil, wheat germ oil, and rice bran oil, we have isolated four compounds, in addition to **I–VII**, from their NSL fractions and characterized them as graminol A [**VIII**: (13*R*,14*R*)-3,4-*seco*-25(10→9)*abeo*-8α,9β,10α-podioda-4,17,21-trien-3-ol], (2*Z*,6*Z*,10*Z*,14*E*,18*E*)-farnesylfarnesol (**IX**), (2*Z*,6*Z*,10*E*,14*E*)-geranylfarnesol (**X**), and phytol (**XI**; phyt-2-en-1-ol). This paper describes further characterization of the incompletely cyclized triterpene alcohols (**I–VIII**), and acyclic triterpene (**IX**), sesterterpene (**X**), and diterpene alcohols (**XI**) in the NSL fractions of the four seed oils investigated in our recent (1–3) and present studies.

[1]Based on a paper presented at the symposium, "Chemistry, Biochemistry, and Function of Sterols," AOCS Annual Meeting & Expo, Orlando, FL, May 1999.

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Abbreviations: GC–MS, gas chromatography–mass spectroscopy; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography; HREIMS, high-resolution electron impact mass spectra; IR, infrared; NOE: nuclear Overhauser effect; NMR, nuclear magnetic resonance; NSL, nonsaponifiable lipids; RRT, relative retention time.

MATERIALS AND METHODS

General. Crystallizations were performed from MeOH. Infrared (IR) spectra were recorded on a JASCO IR-300 IR spectrometer as liquid films. Optical rotations were measured on a JASCO DIP-370 polarimeter in $CHCl_3$ at 25°C. High-resolution electron impact mass spectra (HREIMS) were recorded on a Hitachi M-80B double-focusing gas chromatography–mass spectroscopy (GC–MS) instrument (70 eV) using a direct inlet system. Nuclear magnetic resonance (NMR) spectra were recorded with a JEOL LA-500 spectrometer at 500 MHz (^{1}H NMR) and 125 MHz (^{13}C NMR) in $CDCl_3$ with Me_4Si (^{1}H NMR) and $CDCl_3$ at δ 77.0 (^{13}C NMR) as internal standard. Chemical shifts were recorded in δ values. Reversed-phase high-performance liquid chromatography (HPLC) was carried out on an ODS column [Superiorex ODS S-5 µm column, 25 cm × 10 mm i.d. (Shiseido Co., Ltd., Tokyo, Japan), temperature 25°C] with MeOH as mobile phase (flow rate 4 mL/min) using an SSC Flow System 3100K (Senshu Scientific Co., Ltd., Tokyo, Japan) and an ERC-7520 refractive index detector (ERC Co., Ltd., Tokyo, Japan). Gas–liquid chromatography (GLC) was run on a Shimadzu GC-14B apparatus using a DB-17 fused-silica capillary column (30 m × 0.3 mm i.d., column temperature 275°C). For both HPLC and GLC, cholesterol (cholest-5-en-3β-ol) was the standard for the determination of the relative retention times (RRT) (i) of hydroxy terpenes; cholesterol acetate was the standard for the determination of RRT (ii) for the acetoxy terpenes. Acetylation (Ac_2O/pyridine) and hydrolysis of acetates (5% KOH in MeOH) were performed at room temperature overnight.

Materials. Sources of commercially prepared crude oils—sasanqua oil from the seeds of *Camellia sasanqua* Thunb. (4), camellia oil from the seeds of *C. japonica* L. (4), and wheat germ oil from the seeds of *Triticum aestivum* L. (5)—were described in our previous articles. Crude rice bran oil from the seeds of *Oryza sativa* L. was donated by Boso Oil and Fat

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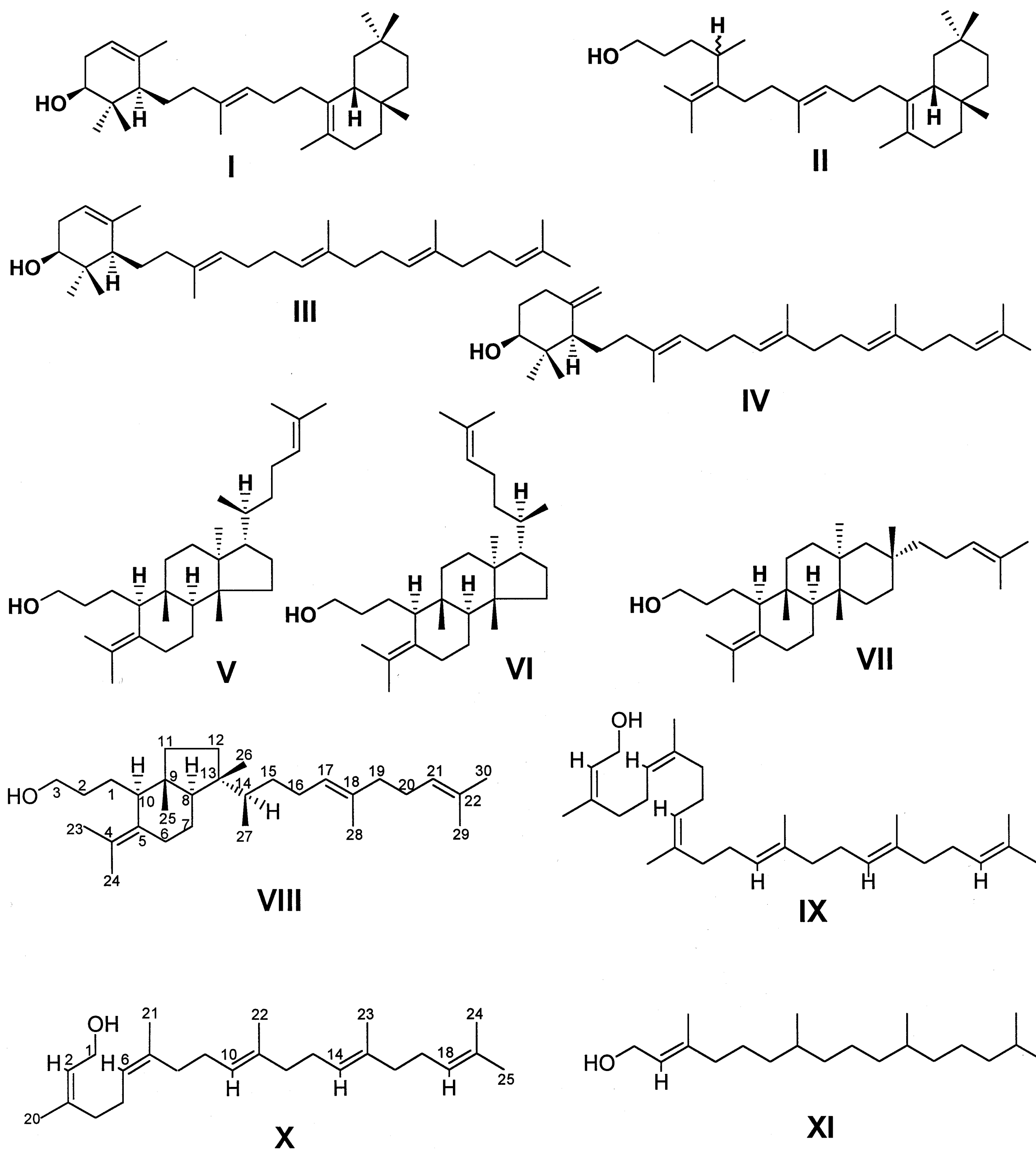


SCHEME 1

Co. (Tokyo, Japan). **I–IV** (1), **V** and **VI** (2) and **VII** (3) and their acetyl derivatives were used as the reference specimens.

Extraction and isolation. Alkaline hydrolysis (5% KOH in MeOH, reflux, 3 h) of oil materials followed by diisopropyl ether extraction yielded a neutral NSL fraction. The NSL were chromatographed on a silica gel (silica gel 60, 230–400 mesh; Merck KGaA, Darmstadt, Germany) column with hexane, hexane/EtOAc (9:1 → 4:1, vol/vol) as eluants. Hexane/EtOAc (9:1) eluted a fraction that, after rechromatography over silica gel, yielded a triterpene alcohol fraction (a fraction containing predominantly triterpene alcohols) which, upon acetylation, gave an acetylated fraction. Chromatography of this fraction over an ODS (Chromatorex ODS, 100 mesh; Fuji Silysia Chemical Ltd., Kasugai-shi, Aichi, Japan) column, eluted with MeOH, followed by HPLC afforded individual compounds as the acetyl derivatives. Alkaline hy-

TABLE 1
Chromatographic Data of the Compounds Described in This Paper, and Yields of Triterpene Alcohol Fractions and Isolated Compounds from the Oil Materials

		Chromatographic data				Yield from 1 kg of oil material			
		RRT(i)[a]	Free alcohol	RRT(ii)[b]	Acetate	Sasanqua	Camellia	Wheat germ	Rice bran
Code	Compound	HPLC	GLC	HPLC	GLC	2.20 g[c]	1.42 g	2.49 g	9.34 g
I	Camelliol A	0.65	0.78	0.51	0.68	1 mg[d]	0.6 mg		
II	Camelliol B	0.60	0.60	0.51	0.57	1 mg	1 mg		
III	Camelliol C	0.39	0.85	0.41	0.74	2 mg	0.7 mg		
IV	Achilleol A	0.46	0.79	0.35	0.66	2 mg	0.7 mg	56 mg	12 mg
V	Helianol	0.68	1.44	0.63	1.32	1 mg	0.7 mg		
VI	Isohelianol	0.70	1.27	0.62	1.14	22 mg	0.7 mg		
VII	Sasanquol	0.72	1.44	0.67	1.31	15 mg	2 mg		
VIII	Graminol A	0.57	0.90	0.46	0.81			3 mg	1 mg
IX	Farnesylfarnesol	0.39	0.67	0.31	0.64			3 mg	
X	Geranylfarnesol	0.23	0.23	0.19	0.23			144 mg	
XI	Phytol	0.25	0.04	0.21	0.05		1 mg	111 mg	

[a]RRT(i), retention time relative to cholesterol.
[b]RRT(ii), retention time relative to cholesterol acetate.
[c]Yield of triterpene alcohol fractions.
[d]Yield of isolated individual compounds as the acetyl derivatives. HPLC, high-performance liquid chromatography; GLC, gas–liquid chromatography.

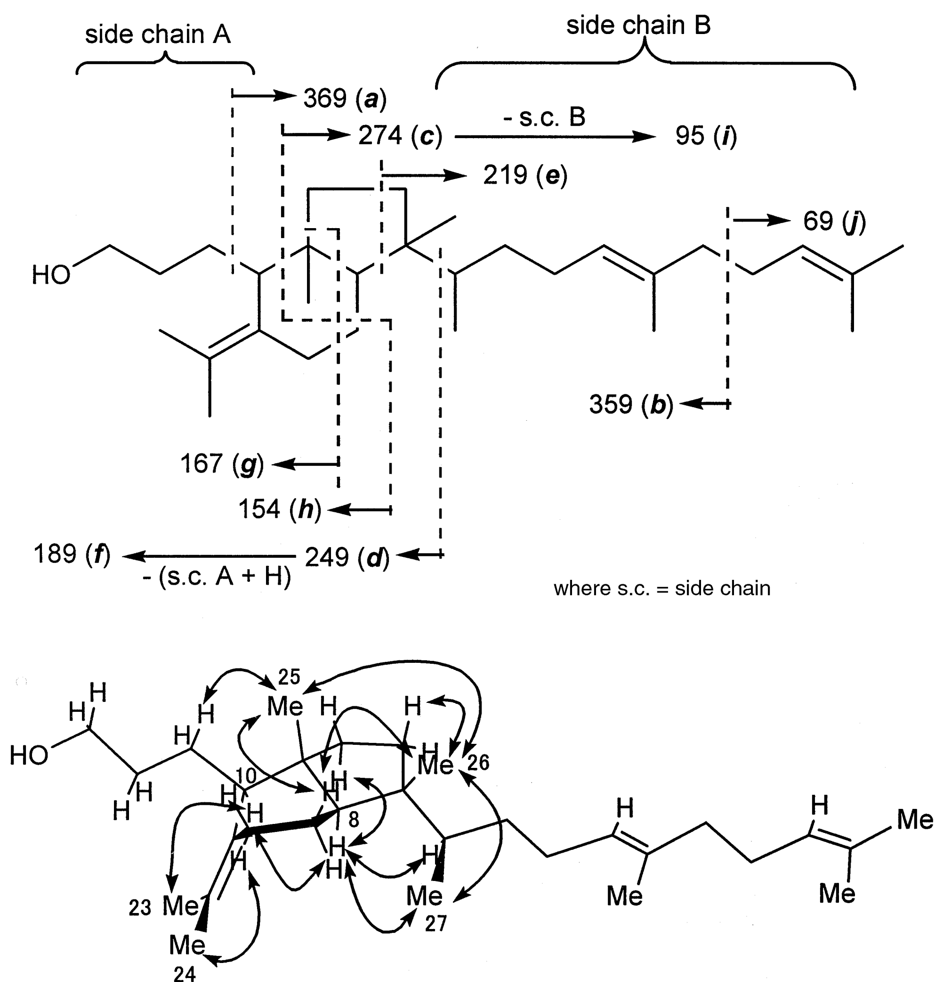
drolysis of the acetates yielded free alcohols. Yields of triterpene alcohol fractions and individual compounds from the oil materials are shown in Table 1 accompanied by their chromatographic data.

Conformational analysis for **VIII**. One thousand-step systematic Monte Carlo conformation searches were carried out for **VIII** with the extended molecular mechanics 3 (MM3*) force field as implemented in MacroModel Version 6.0 to predict the fully optimized lowest-energy structure (6).

RESULTS

As shown in Table 1, 11 acyclic and incompletely cyclized terpene alcohols, **I**–**XI**, were isolated in our recent and present studies, i.e., seven compounds, **I**–**VII**, from sasanqua oil (1–3); eight compounds, **I**–**VII** and **XI**, from camellia oil; five compounds, **IV**, **VIII**, **IX**, **X**, and **XI**, from wheat germ oil; and two compounds, **IV** and **VIII**, from rice bran oil. The seven compounds, **I**–**VII**, were identified as camelliol A (**I**), camelliol B (**II**), camelliol C (**III**), achilleol A (**IV**), helianol (**V**), isohelianol (**VI**), and sasanquol (**VII**) by chromatographic (HPLC and GLC) and spectral (MS and ^{1}H NMR) comparison with authentic compounds. Identification of two compounds, **IX** and **XI**, as (2*Z*,6*Z*,10*Z*,14*E*,18*E*)-farnesylfarnesol (7) and phytol (phyt-2-en-1-ol) (8), respectively, was performed by comparison of MS and NMR spectral data with the literature. Characterization of two other compounds, **VIII** and **X**, was performed as described below.

Characterization of compound **VIII**. Compound **VIII** was obtained as an amorphous gum after crystallization. It showed $[\alpha]_D$ −48.0° (*c* = 0.10) and IR absorptions at 3377 and 837 cm^{-1} HREIMS of **VIII** exhibited ions at *m/z* 428.4018 ($[M]^+$, relative intensity 32%; calc. $C_{30}H_{52}O$, 428.3978), 413 (38), 385 (9), 369 (35; fragment ***a*** in Scheme 2), 359 (2; ***b***), 287 (6), 274 (18; ***c***), 249 (100; ***d***), 231 (15), 219 (7; ***e***), 205 (13), 189 (24; ***f***), 167 (61; ***g***), 154 (17; ***h***), 149 (37), 135 (63), 109 (75), 95 (94; ***i***), and 69 (95; ***j***). The ^{1}H and ^{13}C NMR data for **VIII** are shown in Table 2. Compound **VIII** ($C_{30}H_{52}O$) has a primary hydroxyl group [ν_{max} 3377 cm^{-1}; δ_C 63.7 (CH_2); δ_H 3.61 (2H, *dd*, *J* = 5.8, 5.8 Hz)], two trisubstituted double bonds [ν_{max} 837 cm^{-1}; δ_C 124.4 (CH) and 125.0 (CH); δ_H 5.10 (*tt*, *J* = 1.2, 6.6 Hz) and 5.11 (*dt*, *J* = 1.2, 5.8 Hz)], a tetrasubstituted double bond (four quaternary sp^2 carbon signals with δ_C 124.2, 130.9, 131.3, and 134.8, two of which are associated with the trisubstituted double bonds), and two tertiary [δ_H 0.71 and 1.01 (each *s*)], one secondary [δ_H 0.85 (*d*, *J* = 6.7 Hz)], and five olefinic [δ_H 1.60 (6H), 1.62, 1.66, and 1.68 (each *s*)] methyl groups. These data, in combination with prominent MS fragment ions at *m/z* 369 [fragment ***a*** in Scheme 2; loss of side chain A (C_3H_5O)], 249 [***d***; loss of side chain B ($C_{13}H_{23}$)], and 189 (***e***; loss of both side chains A and B), suggested that **VIII** possesses a 3,4-*seco*-triterpen-3-ol structure (1–3) with a bicyclic skeleton, constituted with one five- and one six-membered ring (9), and two side chains A and B. The other fragment ions at *m/z* 274 (***c***), 219 (***e***), 167 (***g***), 154 (***h***), and 95 (***i***) suggested that the two tertiary methyls to be located at C-9 and C-13. Analysis of the ^{13}C distortionless enhancement by polarization transfer (DEPT), ^{1}H–^{1}H correlation spectroscopy (COSY), ^{1}H detected multiple quantum coherence (HMQC), and heteronuclear multiple-bond correlation (HMBC) spectra (Table 2) revealed the structure of **VIII** to be 3,4-*seco*-25(10→9)*abeo*-podioda-4,17,21-trien-3-ol. The stereochemistry of **VIII** was determined by phase-sensitive nuclear Overhauser effect (NOE) difference spectroscopy experiment. Compound **VIII** showed significant NOE correlations between [H-1—H-25—H-26—H-27] and [H-27—H-8α—H-10α—H-23] (Scheme 2). This allowed the assignment of H-25 and H-26 to be oriented at the β-face and



SCHEME 2

H-8 and H-10 at the α-face of the ring system, and it also allowed 14*R* stereochemistry. We conclude that structure **VIII** is (13*R*,14*R*)-3,4-seco-25(10→9)*abeo*-8α,9β,10α-podioda-4,17,21-trien-3-ol, which we named graminol A. The most stable conformation of **VIII** with minimum steric energy was simulated using MacroModel (6) and the corresponded drawings were shown in Scheme 2. The conformation of **VIII** was fairly consistent with the results from the NOE experiment carried out in solution. Graminol A acetate (**VIIIa**) was obtained as an amorphous gum, which showed $[\alpha]_D$ – 10.7° (*c* = 0.15) and IR absorptions at 1730, 1243, and 840 cm^{-1}. Its electron impact-MS exhibited ions at *m/z* 470.4101 ($[M]^+$, relative intensity 3%; calc. $C_{32}H_{54}O_2$, 470.4120), 455.3879 (4; $C_{31}H_{51}O_2$), 427.3542 (1; $C_{29}H_{47}O_2$), 401.3372 (1; $C_{27}H_{45}O_2$, corresponded to fragment ***b*** in Scheme 2), 369.3519 (3; $C_{27}H_{45}$, ***a***), 291.2296 (26; $C_{19}H_{31}O_2$, ***d***), 274.2640 (3; $C_{20}H_{34}$, ***c***), 231.2135 (4; $C_{17}H_{27}$), 219.2126 (1; $C_{16}H_{27}$, ***e***), 209.1551 (8; $C_{13}H_{21}O_2$, ***g***), 196.1461 (1; $C_{12}H_{20}O_2$, ***h***), 189.1632 (6; $C_{14}H_{21}$, ***f***), 177.1641 (3; $C_{13}H_{21}$), 175.1466 (4; $C_{13}H_{19}$), 163.1484 (5; $C_{12}H_{19}$), 95.0852 (54; C_7H_{11}, ***i***), and 69.0704 (100; C_5H_9, ***j***). The ^1H NMR spectrum of **VIIIa** showed signals of H-3 [4.03 (2H, *dd*, *J* = 6.7, 6.7 Hz)], H-17 [5.13 (*dt*, *J* = 1.2, 6.7 Hz)], H-21 [5.10 (*tt*, *J* = 1.5, 7.0)], H-23 [1.65 (*br s*)], H-24 [1.63 (*s*)], H-25 [1.00 (*s*)], H-26 [0.71 (*s*)], H-27 [0.85 (*d*, *J* = 7.0 Hz)], H-28 [16.0; 1.60 (br *s*)], H-29 [1.60 (br *s*)], H-30 [1.68 (*d*, *J* = 0.9 Hz)], and OCO*Me* [2.04 (*s*)].

Characterization of compound **X**. Compound **X** was a colorless viscous mass which showed $[\alpha]_D$ + 46.7° (*c* = 0.10) and IR absorptions at 3334, 2921, 2858, 1668, 1444, 1379, 999, and 835 cm^{-1}. The HREIMS exhibited ions at *m/z* 358.3246 ($[M]^+$, relative intensity 0.4%; calc. $C_{25}H_{42}O$, 358.3413), 340.3152 (1; $C_{25}H_{40}$), 325.2862 (0.1; $C_{24}H_{37}$), 289.2519 (0.2; $C_{20}H_{33}O$, $[M - C_5H_9]^+$), 273.2572 (0.4; $C_{20}H_{33}$), 271.2390 (1; $C_{20}H_{31}$), 257.2309 (0.2; $C_{19}H_{29}$), 229.1999 (0.5; $C_{17}H_{25}$), 221.1948 (0.5; $C_{15}H_{25}O$, $[M - C_{10}H_{17}]^+$), 215.1834 (0.4; $C_{16}H_{23}$), 205.1935 (1; $C_{15}H_{25}$), 203.1756 (2; $C_{15}H_{23}$), 189.1650 (2; $C_{14}H_{21}$), 177.1595 (1; $C_{13}H_{21}$), 137.1296 (6; $C_{10}H_{17}$), 135.1153 (5; $C_{10}H_{15}$, $[M - C_{15}H_{25} - H_2O]^+$), 85.0645 (2; C_5H_9O), 81.0689 (43; C_6H_9), and 69.0695 (100; C_5H_9). The ^1H NMR of **X** showed signals of H-1 [δ_H 4.09 (2H, *dd*, *J* = 1.0, 7.3 Hz)], H-2 [5.45 (*dq*, *J* = 1.2, 7.0 Hz)], H-6 [5.12 (*tt*, *J* = 1.5, 7.0 Hz)], H-10 [5.12 (*tt*, *J* = 1.5, 7.0 Hz)], H-14 [5.13 (*tt*, *J* = 1.5, 7.0 Hz)], H-18 [5.10 (*tt*, *J* = 1.5,

TABLE 2
^{13}C (125 MHz) and ^{1}H (500 MHz) Nuclear Magnetic Resonance Spectral Data (δ values; $CDCl_3$) and ^{1}H–^{13}C Long-range Correlations[a] of Graminol A (VIII)

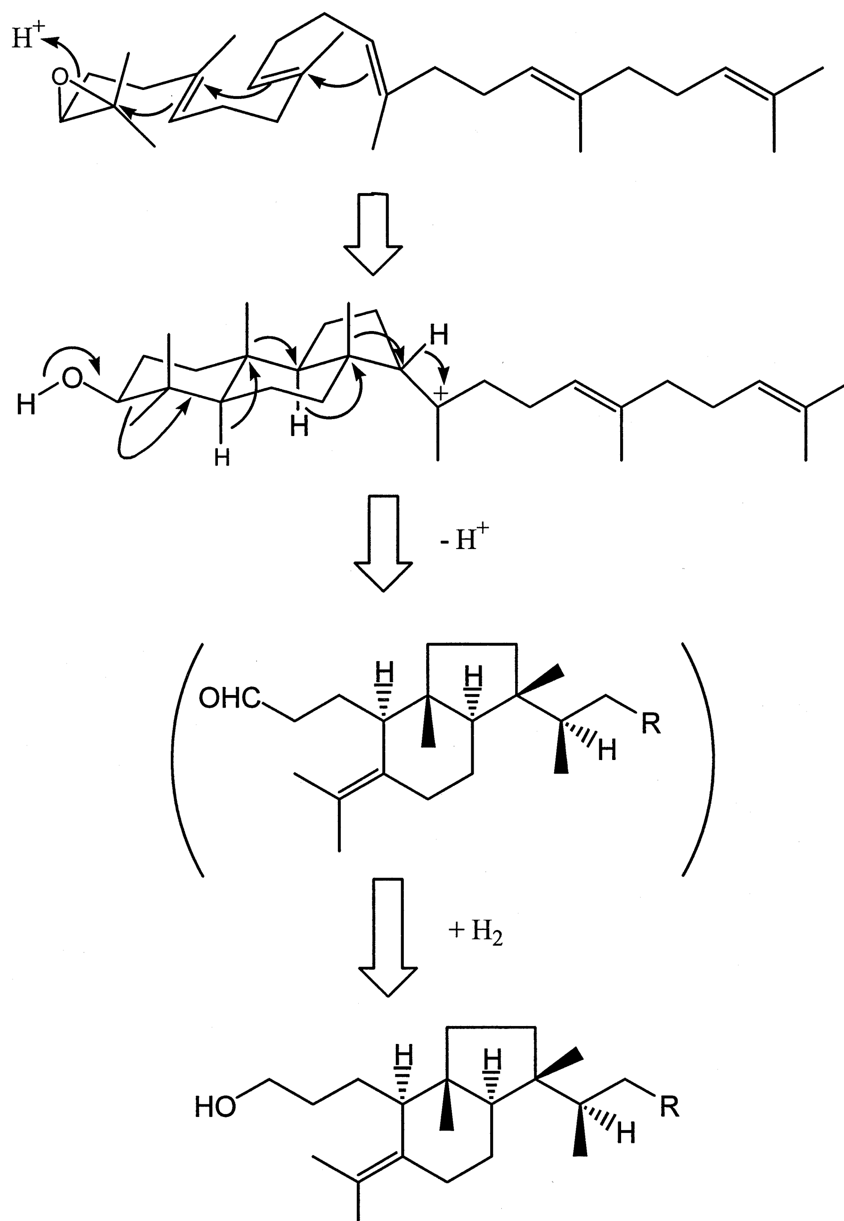
Carbon number[b]		δ_C	δ_H[c]	Cross peaks in HMBC spectrum
1	CH_2	26.4	1.36, 1.65	
2	CH_2	32.3	1.37, 1.49	
3	CH_2	63.7	3.61 (2H, *dd,* 5.8, 5.8)	C-1, C-2
4	C	124.2		
5	C	130.9		
6	CH_2	24.2	1.97 (α), 2.28 (β; *br dd,* 6.4, 6.4)	
7	CH_2	22.8	1.91 (α; *ddd,* 3.6, 7.3, 15.0), 1.62 (β)	
8	CH	50.6	1.42 (*br d,* 7.3)	C-7, C-9, C-13, C-14, C-26
9	C	46.1		
10	CH	46.3	2.34 (*dt,* 4.0, 10.1)	C-1, C-4, C-5, C-8
11	CH_2	38.4	1.05 (α), 1.59 (β)	
12	CH_2	38.7	1.48 (α), 1.31 (β; *dd,* 6.4, 12.5)	
13	C	50.4		
14	CH	46.8	1.17 (*dddd,* 1.5, 6.4, 6.4, 17.1)	
15	CH_2	32.9	0.99, 1.43	
16	CH_2	26.7	1.85 (*ddd,* 7.9, 15.3, 15.3), 2.05	
17	CH	125.0	5.11 (*dt,* 1.2, 5.8)	C-19, C-20, C-28
18	C	134.8		
19	CH_2	39.8	1.98 (2H, *br dd,* 7.0, 8.2)	C-17, C-18, C-20, C-28
20	CH_2	26.8	2.07 (2H, *br dd,* 7.0, 14.7)	C-19, C-21, C-22
21	CH	124.4	5.10 (*tt,* 1.2, 6.0)	C-19, C-29, C-30
22	C	131.3		
23	Me	20.6	1.66 (*br s*)	C-4, C-5, C-24
24	Me	20.2	1.62 (*s*)	C-4, C-5, C-23
25	Me	28.7	1.01 (*s*)	C-8, C-9, C-11
26	Me	16.3	0.71 (*s*)	C-12, C-13, C-14
27	Me	15.1	0.85 (*d,* 6.7)	C-13, C-14, C-15
28	Me	16.0	1.60 (*br s*)	C-17, C-18, C-19
29	Me	17.7	1.60 (*br s*)	C-21, C-22, C-30
30	Me	25.7	1.68 (*s*)	C-21, C-22, C-29

[a]Obtained by DEPT (distortionless enhancement by polarization transfer); ^{1}H-^{1}H COSY (correlation spectroscopy); HMQC (^{1}H detected multiple quantum coherence); and HMBC (heteronuclear multiple-bond correlation).
[b]For numbering see **VIII** in Scheme 1.
[c]Figures in parentheses in the ^{1}H chemical shift column denote *J* values (Hz).

7.0 Hz)], H-20 [1.75 (*d*, J = 0.9 Hz)], H-21 [1.70 (*d*, J = 1.2 Hz)], H-22 [1.60 (*s*)], H-23 [1.61 (*s*)], H-24 [1.60 (*s*)], and H-25 [1.68 (*d*, J = 0.9 Hz)]. Compound **X** ($C_{25}H_{42}O$) has a hydroxymethylene group [ν_{max} 3334 cm^{-1}; δ_H 4.09 (2H, *dd*, J = 1.0, 7.3 Hz)], five trisubstituted double bonds [ν_{max} 835 cm^{-1}; δ_H 5.10, 5.12 (2H) and 5.13 (each *tt*, J = 1.5, 7.0 Hz), and 5.45 (*tq*, J = 7.0, 1.2 Hz)], three *cis*-olefinic [δ_H 1.60 (6H, *s*) and 1.61 (*s*)], two *trans*-olefinic [δ_H 1.68 (*d*, J = 0.9 Hz) and 1.70 (*d*, J = 1.2 Hz)] methyl groups, and a *cis*-olefinic methyl group adjacent to a terminal hydroxymethylene group [δ_H 1.75 (*d*, J = 0.9 Hz)]. These data, in combination with a series of MS fragment ions at *m/z* 289, 273, 221, 205, 137, 85, and 69 due to allylic cleavages, suggested an acyclic pentaprenyl alcohol structure (7,10). Careful comparison of the ^{1}H NMR spectral data of **X** with those of **IX** (7) allowed us to assign the structure of **X** as (2*Z*,6*Z*,10*E*,14*E*)-geranylfarnesol. (2*Z*,6*Z*,10*E*,14*E*)-Geranylfarnesol acetate (**Xa**) was obtained as a colorless viscous mass. It showed $[\alpha]_D$ + 20.0° (*c* = 0.10) and IR absorptions at 2921, 2858, 1741, 1446, 1375, 1232, 1022, and 835 cm^{-1}. The HREIMS exhibited ions at *m/z* 400.3332 ($[M]^+$, relative intensity 1%; calc. $C_{27}H_{44}O_2$, 400.3332), 340 (2), 331 (0.5), 297 (0.5), 271 (2), 257 (0.5), 229 (1), 215 (1), 203 (3), 189 (3), 175 (1), 81 (45), and 69 (100). The ^{1}H NMR of **Xa** showed signals of H-1 [δ_H 4.55 (2H, *dd*, J = 1.5, 7.0 Hz)], H-2 [5.36 (*dq*, J = 1.5, 7.3 Hz)], H-6 [5.11 (*tt*, J = 1.5, 7.0 Hz)], H-10 [5.11 (*tt*, J = 1.5, 7.0 Hz)], H-14 [5.11 (*tt*, J = 1.5, 7.0 Hz)], H-18 [5.10 (*tt*, J = 1.5, 7.0 Hz)], H-20 [1.76 (*d*, J = 1.3 Hz)], H-21 [1.69 (*d*, J = 1.2 Hz)], H-22 [1.60 (*s*)], H-23 [1.61 (*s*)], H-24 [1.60 (*s*)], H-25 [1.68 (*d*, J = 1.2 Hz)], and OCO*Me* [2.04 (*s*)].

DISCUSSION

Among the 11 compounds **I**–**XI** detected in this study, two compounds, **VIII** and **X**, are new natural products to the best of our knowledge although the all-*trans* form of **X**, i.e., (2*E*,6*E*,10*E*,14*E*)-geranylfarnesol, has previously been isolated from the insect wax of *Ceroplastes albolineatus* (10). These acyclic and incompletely cyclized compounds occur only as minor components (less than 1%) of the triterpene alcohol fractions of the oil materials investigated, except for **VI** (*ca*. 1% of the triterpene alcohol fraction) in sasanqua oil, and



SCHEME 3

IV (*ca*. 2.7%), **X** (*ca*. 5.8%), and **XI** (*ca*. 4.5%) in wheat germ oil (Table 1). Major triterpene alcohol constituents of sasanqua and camellia oils (4) and wheat germ and rice bran oils (11) have previously been reported.

Only a few acyclic and incompletely cyclized triterpenoids possessing a monohydroxyl group at C-3 had been isolated from natural sources prior to our isolation of compounds **I–VII** from sasanqua oil (1–3) and **V** from several Compositae flowers (12). They are **IV** (13) and achilleol B (14) from *Achillea odorata* (Compositae), **IX** from *Phyllanthus niruri* (Euphorbiaceae) (7), and malabarica-14(18),17(20),24-trien-3β-ol from *Pyrethrum santalinoides* (Compositae) (15). Our present study suggests wider occurrence of these acyclic and incompletely cyclized triterpene alcohols in plants. On the other hand, various incompletely cyclized triterpene hydrocarbons, including podioda-7,17,21-triene and its Δ^8-isomer (8), and incompletely cyclized triterpenes with a hydroxyl group other than at C-3 have been isolated from fern plants (16, and references cited therein).

A possible biosynthetic pathway for the formation of **VIII** is shown in Scheme 3. Cyclization of squalene oxide would afford a tricyclic triterpene alcohol with a podiodane skeleton that, on stepwise hydride and methyl shifts, would yield an aldehyde. Reduction of the aldehyde would give compound **VIII**. This type of biosynthetic pathway for the formation of 3,4-*seco*-triterpene alcohol was proposed recently for iridals, the 3,4-*seco*-triterpenoids found in sword lilies (Iridaceae) (17). The other 3,4-*seco*-triterpene alcohols, **V–VII**, may

have this kind of biosynthetic pathway for their formation. Several other biosynthetic pathways for the ring A cleavage which lead to 3,4-*seco*-triterpene 3-oic acids have been proposed (18).

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Wax Ester-Synthesizing Activity of Lipases

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ABSTECT: The synthesis/hydrolysis of wax esters was studied in an aqueous solution using purified rat pancreatic lipase, porcine pancreatic carboxylester lipase, and *Pseudomonas fluorescens* lipase. The equilibrium between wax ester synthesis and hydrolysis favored ester formation at neutral pH. The synthesizing activities were measured using free fatty acid or triacylglycerol as the acyl donor and an equimolar amount of long-chain alcohol as the acyl acceptor. When oleic acid and hexadecanol emulsified with gum arabic were incubated with these lipases, wax ester was synthesized, in a dose- and time-dependent manner, and the apparent equilibrium ratio of palmityl oleate/free oleic acid was about 0.9/0.1. These lipases catalyzed the hydrolysis of palmityl oleate emulsified with gum arabic, and the apparent equilibrium ratio of palmityl oleate/free oleic acid was also about 0.9/0.1. The apparent equilibrium ratio of wax ester/free fatty acid catalyzed by lipase depended on incubation pH and fatty alcohol chain length. When equimolar amounts of trioleoylglycerol and fatty acyl alcohol were incubated with pancreatic lipase, carboxylester lipase, or *P. fluorescens* lipase, wax esters were synthesized dose-dependently. These results suggest that lipases can catalyze the synthesis of wax esters from free fatty acids or through degradation of triacylglycerol in an aqueous medium.

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Wax esters (long-chain fatty alcohols esterified to long-chain fatty acids) are widely distributed in living organisms. They are especially abundant in the cuticles of leaves and insects, in marine algae, and in certain bacteria. In mammals, wax esters are synthesized in most tissues, especially the liver (1). Sebaceous glands secrete a lipid mixture containing wax esters onto the skin surface (2). The microsomal membranes of meibomian glands catalyze wax ester synthesis (3,4). Rat brain and murine ascites cells also synthesize wax esters (5,6). Cofactors such as ATP, Mg^{2+}, and CoA reportedly stimulate wax ester formation (7,8). However, the wax ester-synthesizing enzymes have not been purified and characterized.

In specific organic solvents lipases have been reported to synthesize certain esters when alcohol (acyl acceptor) is present at high concentration. For example, pancreatic lipase catalyzes the acyl transfer reaction in a 99% organic medium (9), and microbial lipase synthesizes acylglycerols from fatty acids and glycerol in a glycerol medium (10). These lipases are used industrially for ester and peptide synthesis in organic media.

Riley *et al.* (11) found that pancreatic lipase catalyzes the synthesis of alcohol ester from fatty acid and ethanol in aqueous medium. Other lipases and carboxylesterase synthesize ethyl esters (12,13). However, high concentrations of ethanol (about 1 to 1.5 M) were used for the assay of ethyl ester synthesis; the ratio of ethanol (acyl acceptor)/free fatty acid (acyl donor) was over 2500.

In this study, we focused on the acyl transfer reaction catalyzed by lipases at the surface of the substrate emulsion, and demonstrated that these enzymes catalyze the formation of wax esters from free fatty acids or triacylglycerols and an equimolar amount of long-chain alcohol in an aqueous medium. Our results suggest that lipases generally catalyze wax ester formation on the surface of substrate emulsions at a low concentration of alcohol. Therefore, we postulate that wax esters can be formed in the various organs by various lipases, independently of the presence of ATP, Mg^{2+}, and CoA.

MATERIALS AND METHODS

Materials. The following enzyme substrates and reagents were used: [1-^{14}C]trioleoylglycerol (3.95 Gbq/mol) and [1-^{14}C]oleic acid (2.1 Gbq/mmol) were from Dupont NEN (Boston, MA). Trioleoylglycerol, bile salts, and colipase were from Sigma (St. Louis, MO). Oleic acid, dioleoylglycerol, monooleoylglycerol, palmityl oleate, lauryl oleate, and fatty alcohols were from Funakoshi (Tokyo, Japan). Bovine serum albumin (BSA) was from Wako Pure Chemical Industries (Osaka, Japan) and was extracted by the method of Chen to remove free fatty acid (14).

Enzyme sources. Rats (Crj: Wistar) were cared for under guidelines for animal experimentation of the Laboratory Animal Center at Ehime University School of Medicine. Pancreatic lipase from rat pancreas (3200 U/mg protein, at pH 6.8) (15) and carboxylester lipase from porcine pancreas (159 U/mg protein, at pH 6.8) (16) were purified as described previously. Crystalline lipase from *Pseudomonas fluorescens* was from Amano Pharmaceutical Co. (Nagoya, Japan), and was purified further as described previously (4200 U/mg protein, at pH 6.8) (17). A lingual lipase fraction was prepared from rat tongues. The entire lingual serous glandular region

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was homogenized in cold 25 mM potassium phosphate buffer, pH 6.3, containing 0.9% NaCl. The homogenate was centrifuged at 100,000 × *g* for 60 min, and the supernatant, which was used as the enzyme solution, was stored at −80°C (0.28 U/mg protein, at pH 5.0) (18). Crude microbial lipases from *Mucor lipolyticus* (480 U/mg protein, at pH 8.4), *Candida cylindracea* (526 U/mg protein, at pH 6.8), and *Rhizopus* sp. (99 U/mg protein, at pH 6.8) were obtained from Amano Pharmaceutical Co. One U (unit) will produce 1.0 µmol of oleic acid from trioleoylglycerol emulsified with gum arabic per minute at 37°C.

Enzyme assays. All of the enzyme assays were done in aqueous medium. Wax ester synthesis from oleic acid was determined using [^{14}C]oleic acid. A suspension of 30 µmol [^{14}C]oleic acid (5,000,000 dpm) and 30 µmol fatty alcohol in 2 mL 5% (wt/vol) gum arabic solution was placed in an ice bath and sonicated for 5 min with a Tomy model UD-200 (Tomy, Tokyo, Japan) equipped with a microtip at setting 2–3. The assay mixture consisted of 0.2 mL of 200 mM potassium phosphate buffer (pH 6.8) containing 0.75 µmol [^{14}C]oleic acid (125,000 dpm), 0.75 µmol fatty alcohol, and 2.5 mg gum arabic. Colipase (0.75 µg/mL) and cholic acid (6.25 mM) for pancreatic lipase, and cholic acid (6.25 mM) for carboxylester lipase were added in the assay mixture. The incubation was carried out for 1 h at 37°C, and the reaction was stopped by adding 1.5 mL chloroform/methanol (1:2, vol/vol) containing standard lipids (10 nmol hexadecanol, palmityl oleate, and oleic acid). The mixture was shaken for 15 s, then 0.5 mL chloroform and 0.75 mL H_2O were added, and the mixture was shaken again for 5 s, followed by centrifugation at 1,000 × *g* for 10 min. The lower phase was dried with a stream of nitrogen, solubilized in 50 µL chloroform, and separated by thin-layer chromatography (Whatman silica gel K-5). The plates were developed with hexane/diethyl ether/acetic acid (85:15:1, by vol). The radiolabeled lipid spots (substrate-product distribution) were directly determined using Bio-Imaging Analyzer, BAS 1000 (Fuji Film, Tokyo, Japan). The radioactivity of lipids was also determined using a liquid scintillation counter. Lipids were located with iodine vapor, and spots of wax ester were cut out for radioactivity measurement.

Wax ester hydrolysis was determined using palmityl [^{14}C]oleate or lauryl [^{14}C]oleate as substrate. A suspension of 30 µmol [^{14}C]wax ester (5,000,000 dpm) in 2 mL 5% (wt/vol) gum arabic solution was sonicated for 5 min as described above. The assay mixture consisted of 0.2 mL of 200 mM potassium phosphate buffer (pH 6.8) containing 0.75 µmol [^{14}C]wax ester (125,000 dpm) and 2.5 mg gum arabic. Colipase (0.75 µg/mL) and cholic acid (6.25 mM) for pancreatic lipase, and cholic acid (6.25 mM) for carboxylester lipase were added in the assay mixture. The incubation was carried out for 1 h at 37°C. The lipids were extracted as described above and separated by thin-layer chromatography (Whatman silica gel K-5) with hexane/diethyl ether/acetic acid (85:15:1, by vol). The radiolabeled lipid spots were directly determined using the BAS 1000. The radioactivity of lipids was also determined using a liquid scintillation counter as described above.

Wax ester synthesis from trioleoylglycerol was determined using [^{14}C] trioleoylglycerol. A suspension of 30 µmol [^{14}C]trioleoylglycerol (5,000,000 dpm) and 30 µmol fatty alcohol in 2 mL 5% (wt/vol) gum arabic solution was sonicated for 5 min as described above. The assay mixture consisted of 0.2 mL of 200 mM potassium phosphate buffer (pH 6.8) containing 0.75 µmol [^{14}C]trioleoylglycerol (125,000 dpm), 0.75 µmol fatty alcohol, 2.5 mg gum arabic, and 5 mg BSA. Colipase (0.75 µg/mL) and cholic acid (6.25 mM) for pancreatic lipase, and cholic acid (6.25 mM) for carboxylester lipase were added in the assay mixture. The incubation was carried out for 1 h at 37°C. The lipids were extracted as described above and separated by thin-layer chromatography (Whatman silica gel K-5). The plates were developed with hexane/diethyl ether/acetic acid first at 60:40:2, by vol, and then at 95:5:1, by vol. The separated radioactive lipid spots were determined using the BAS 1000.

Preparation of [^{14}C] wax esters. Palmityl [^{14}C]oleate and lauryl [^{14}C]oleate were synthesized by pancreatic lipase-catalyzed esterification of [^{14}C]oleic acid and isolated by preparative thin-layer chromatography. A suspension of 10 µmol [^{14}C]oleic acid (640 kBq) and 20 µmol fatty alcohol in 1 mL 5% (wt/vol) gum arabic solution was sonicated for 5 min. The oleic acid emulsion was incubated with 500 µg of pancreatic lipase at pH 6.5. After incubation for 1 h at 37°C, lipids were extracted as described above and separated by thin-layer chromatography (Whatman silica gel K-5). The plates were developed with hexane/diethyl ether/acetic acid (85:15:1, by vol), and then the lipid spots were scraped off and extracted with chloroform/methanol (2:1, vol/vol).

RESULTS

Effect of enzyme concentration. When emulsified oleic acid and hexadecanol (molar ratio 1:1) were incubated with pancreatic lipase, wax ester (palmityl oleate) was synthesized in a dose- and time-dependent manner (Fig. 1). Similar results were obtained using carboxylester lipase and *P. fluorescens* lipase (data not shown). By using high concentrations of lipases, the ratios of palmityl oleate/free oleic acid reached constant values, and the apparent equilibrium ratios were 0.90:0.10 for pancreatic lipase and 0.94:0.06 for carboxylester lipase and *P. fluorescens* lipase (Fig. 2). Similar results were obtained using oleic acid and dodecanol emulsion as substrates (data not shown).

Wax ester-hydrolyzing activities of these lipases were determined using palmityl [^{14}C]oleate as a substrate. Pancreatic lipase was able to hydrolyze palmityl oleate in a concentration-dependent manner, and the ratio of palmityl oleate/free oleic acid also reached a constant value (0.89:0.11) (Fig. 3). This ratio was essentially the same as the ratio obtained for wax ester synthesis (0.90:0.10) and was not changed during long-term incubation (at 84.5 µg/mL lipase for 48 h incubation). Similar results were observed using lauryl [^{14}C]oleate as a substrate (data not shown). Carboxylester lipase and *P. fluorescens* lipase were also able to hydrolyze palmityl

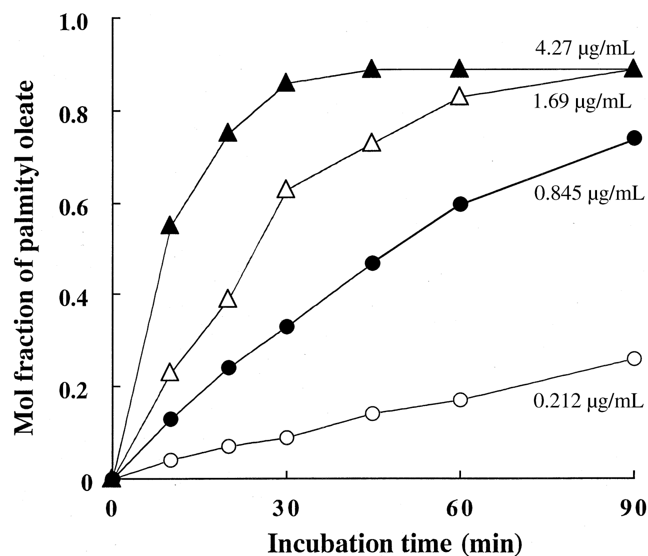


FIG. 1. Effect of incubation time on the synthesis of palmityl oleate by pancreatic lipase. The synthesis of palmityl oleate was determined using [^{14}C]oleic acid (3.75 mM) and hexadecanol (3.75 mM) as substrates. After incubation at 37°C, lipids were extracted and the molar fraction of palmityl oleate was determined.

oleate in a concentration-dependent fashion, and the apparent equilibrium ratios of palmityl oleate/free oleic acid were 0.93:0.07, essentially the same as the ratio (0.94:0.06) for the

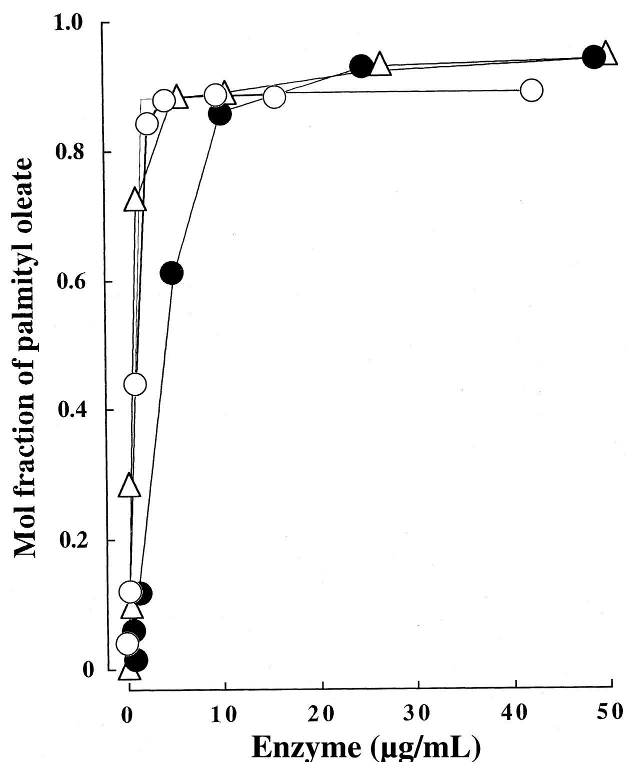


FIG. 2. Effect of lipase concentration on the synthesis of palmityl oleate. The synthesis was determined using [^{14}C]oleic acid (3.75 mM) and hexadecanol (3.75 mM) as substrates. After incubation at pH 6.8 and 37°C for 1 h, lipids were extracted and the molar fraction of palmityl oleate was determined. ○, Pancreatic lipase from rats; ●, carboxylester lipase from porcine pancreas; △, *Pseudomonas fluorescens* lipase.

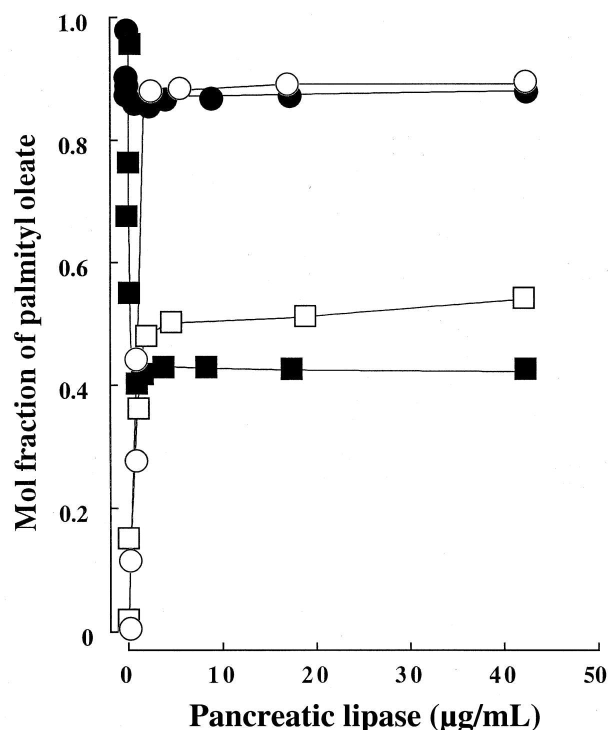


FIG. 3. Effect of pancreatic lipase concentration on the synthesis/hydrolysis of palmityl oleate with or without bovine serum albumin (BSA). Palmityl [^{14}C]oleate (3.75 mM) hydrolysis and palmityl oleate synthesis from [^{14}C]oleic acid (3.75 mM) and hexadecanol (3.75 mM) were determined with or without BSA (25 mg/mL). After incubation at 37°C for 1 h, lipids were extracted and the molar fraction of palmityl oleate was determined. ○, Synthesis without BSA; □, synthesis with BSA; ●, hydrolysis without BSA; ■, hydrolysis with BSA.

synthesis (data not shown). In these condition, these lipases completely degraded ethyl oleate: the apparent equilibrium ratios of ethyl oleate/free oleic acid were about 0.005:0.995 (data not shown). Upon addition of 2.5% BSA, the apparent equilibrium ratio changed, the ratio of palmityl oleate/free oleic acid being 0.47:0.53 for hydrolysis and 0.51:0.49 for synthesis (Fig. 3).

Effect of alcohol chain length and pH. Figure 4 shows the effect of saturated alcohol fatty acyl chain length on wax ester synthesis. The patterns of three enzymes were superimposable. The apparent equilibrium ratio of wax ester/free oleic acid was around 0.9:0.1 when fatty alcohols with acyl chains longer than decanol were used as substrates. The ratio decreased when fatty alcohols with acyl chains shorter than octanol were used, and no wax ester-synthesizing activity was detected when ethanol was used as a substrate. Figure 5 shows the pH activity curves for palmityl oleate synthesis by three lipases. The three pH activity curves were superimposable. Below pH 7.0, the apparent equilibrium ratios of palmityl oleate/free oleic acid were about 0.9:0.1. The ratio of palmityl oleate/free oleic acid decreased sharply at alkaline pH. Above pH 8.0, wax ester-synthesizing activity decreased and wax ester-hydrolyzing activity increased. When the activity of hydrolysis/synthesis was measured at pH 9.0 for 48 h using a high concentration of pancreatic lipase (84.5 μg/mL), the ratio

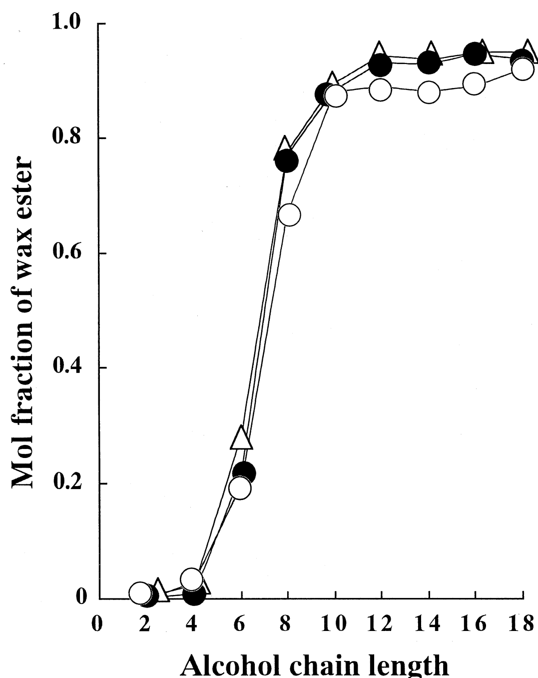


FIG. 4. Effect of fatty alcohol acyl chain length on synthesis of wax ester, as determined using [^{14}C]oleic acid (3.75 mM) and saturated alcohols of various chain lengths (3.75 mM) as substrates. After incubation at pH 6.8 and 37°C for 1 h, lipids were extracted and the molar fraction of wax ester was determined. ○, Pancreatic lipase (84.5 µg/mL); ●, carboxylester lipase (100 µg/mL); △, *Pseudomonas fluorescens* lipase (100 µg/mL).

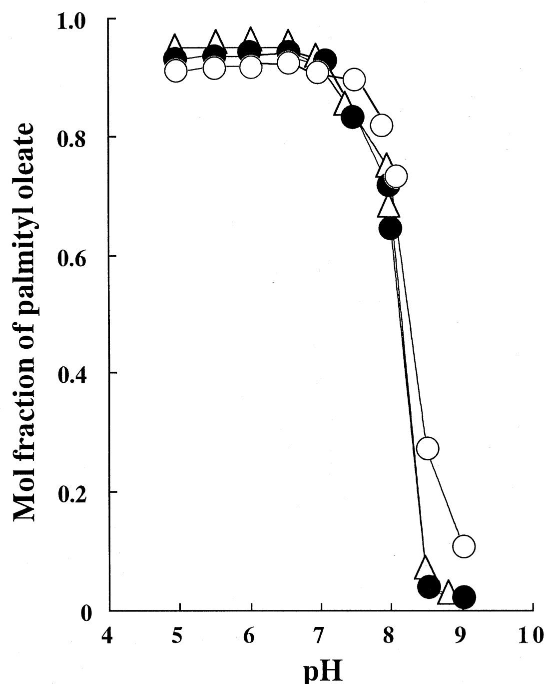


FIG. 5. Effect of pH on the synthesis of palmityl oleate, as determined using [^{14}C]oleic acid (3.75 mM) and hexadecanol (3.75 mM) as substrates. After incubation at 37°C for 1 h, lipids were extracted and the molar fraction of palmityl oleate was determined. Acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0–8.0), and Tris buffer (pH 8.0–9.0) were used. The ionic strength of reaction mixture was 0.1. Symbols and lipase concentration are as in Figure 4.

of palmityl oleate/free oleic acid was 0.22:0.78 for synthesis and 0.46:0.54 for hydrolysis (data not shown).

Wax ester synthesis by other lipases. Other lipases obtained from rat tongues, *M. lipolyticus*, *C. cylindracea,* and *Rhizopus* species also synthesized wax ester dose-dependently from oleic acid and hexadecanol (data not shown). However, the enzyme concentrations at which equilibrium was reached were not the same: the apparent equilibrium ratio (0.89:0.11 for palmityl oleate/oleic acid) was reached by 0.6 U/mL of lingual lipase, 3 U/mL *C. cylindracea* lipase and 5 U/mL *Rhizopus* sp. lipase, but the equilibrium ratio was not reached by 5 U/mL of *M. lipolyticus* lipase (0.51:0.49 for palmityl oleate/oleic acid); the equilibrium ratio (0.92:0.08 for palmityl oleate/oleic acid) was reached by 20 U/mL of *M. lipolyticus* lipase. No wax ester-synthesizing activity from oleic acid and hexadecanol was detected when carboxylesterases from rat adipose and kidney were used as enzymes (data not shown).

Wax ester synthesis from triacylglycerol. Figure 6 shows the effect of lipase concentrations on palmityl oleate formation from trioleylglycerol with 2.5% BSA. When [^{14}C]trioleylglycerol/hexadecanol (molar ratio, 1:1) emulsion was incubated with pancreatic lipase, carboxylester lipase, or *P. fluorescens* lipase, the formation of oleic acid and palmityl oleate increased with increasing lipase concentration. At a high concentration of pancreatic lipase (84.5 µg/mL), the molar ratio of wax ester, oleic acid, and monooleoylglycerol was about 1:1.2:1. At a high concentration of *P. fluorescens* lipase (over 20 µg/mL), the molar ratio of wax ester to oleic acid was about 1:2. Rat lingual lipase and microbial lipases also synthesized wax ester from trioleoylglycerol (Table 1). The effect of saturated fatty alcohol acyl chain length on wax ester formation from trioleoylglycerol by pancreatic lipase, carboxylester lipase, and *P. fluorescens* lipase is shown in Figure 7. Trioleoylglycerol was almost completely degraded by high concentrations of lipases. The patterns of oleic acid formation were roughly the inverse of wax ester formation: free oleic acid formation decreased with an increase in the acyl chain length of fatty alcohol, whereas wax ester formation increased. Wax ester formation was not detected when fatty alcohols with acyl chains shorter than hexanol were used. Figure 8 shows the effect of pH on palmityl oleate synthesis from trioleoylglycerol by three lipases. Trioleylglycerol degradation and oleic acid formation increased with rising pH up to 9.0. However, palmityl oleate synthesis decreased sharply at alkaline pH. Similar results were observed using other lipases: palmityl oleate synthesis decreased at pH 9.0 compared to pH 6.8 (Table 1).

DISCUSSION

The typical substrates for lipase(s) are long-chain triacylglycerols, which are separated from the aqueous medium by the surface phase. Thus, lipase must be adsorbed to the lipid surface, and the quality of the surface is an important factor for lipase activity. At the lipid surface, the adsorbed lipase can

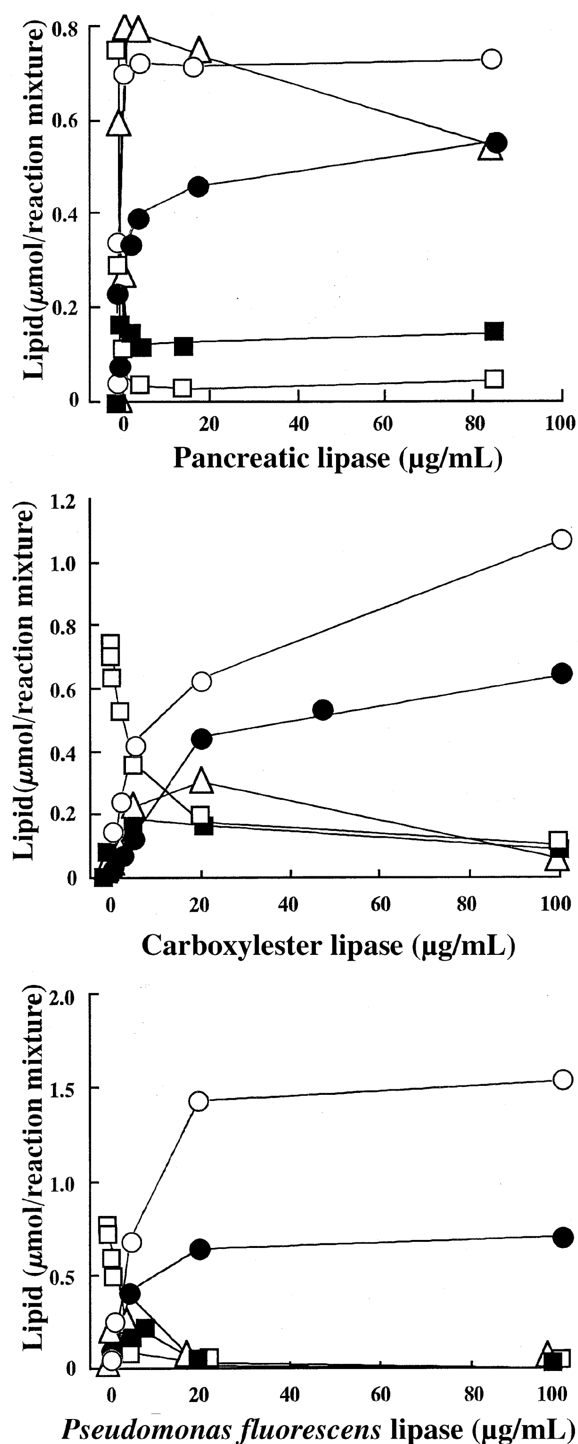


FIG. 6. Effect of lipase concentration on synthesis of palmityl oleate from trioleylglycerol, as determined using [^{14}C]trioleoylglycerol (3.75 mM) and hexadecanol (3.75 mM) as substrates with BSA (25 mg/mL). After incubation at pH 6.8 and 37°C for 1 h, lipids were extracted and their molar concentrations determined. ●, Palmityl oleate; □, trioleylglycerol; ○, oleic acid; ■, dioleoylglycerol; △, monooleoylglycerol. For abbreviation see Figure 3.

TABLE 1
Palmityl Oleate Synthesis from Trioleoylglycerol by Various Lipases[a]

Lipase	pH	PO	TO	DO	MO	OA
		(μmol/reaction mixture)				
Lingual	5.0	0.370	0.346	0.266	0.054	0.236
	6.8	0.263	0.221	0.335	0.150	0.512
Mucor lipolyticus	6.8	0.718	0.162	0.107	0.026	0.812
	9.0	0.156	0.002	0.015	0.456	1.611
Candida cylindracea	6.8	0.767	0.026	0.005	0.000	1.402
	9.0	0.367	0.429	0.088	0.066	0.361
Rhizopus sp.	6.8	0.445	0.049	0.211	0.577	0.665
	9.0	0.083	0.138	0.171	0.585	0.832

[a]Wax ester synthesis was determined using [^{14}C]trioleoylglycerol (3.75 mM) and hexadecanol (3.75 mM) as substrates with bovine serum albumin (25 mg/mL). After incubation at 37°C for 1 h, lipids were extracted and the molar concentrations of palmityl oleate (PO), trioleoylglycerol (TO), dioleoylglycerol (DO), monooleoylglycerol (MO), and oleic acid (OA) were determined. Lingual lipase concentration was 0.45 U/mL, and *M. lipolyticus, C. cylindracea,* and *Rhizopus* sp. lipase concentrations were 10 U/mL.

catalyze various reactions such as hydrolysis, esterification and transesterification. Therefore, substances that affect the surface would be expected to influence the lipase reaction rate. Fatty alcohols affect lipase activity in this way. Ferreira and Patton (19) suggested that the inhibition of lipase activity by fatty alcohols was due to inhibition of interfacial activation of the lipase by dilution of the high surface concentration of substrate. Mattson *et al.* (20) reported that the inhibitory effect of long-chain fatty alcohol is due to its adsorption on the substrate, thus blocking the interaction with enzyme. Both of these groups suggested that the slight inhibition of lipolysis was due to the esterification of fatty acid by fatty alcohols. However, they assayed lipase activities at alkaline pH (pH 8.0 and 9.0). If the lipase activities had been assayed at neutral pH, the contribution of esterification to the inhibition of lipolysis (fatty acid release) might have been greater, because the equilibrium favored ester formation at neutral pH.

Pancreatic lipase (21,22), carboxylester lipase (23,24), and *P. fluorescens* lipase (25) contain the common active site sequence (Gly-Xaa-Ser-Xaa-Gly) and Asp-His-Ser triad, which are characteristics of serine proteinase. Its catalytic mechanism resembles that of serine proteinases, proceeding *via* an acyl-enzyme intermediate. In this study, we demonstrated that lipases catalyze the synthesis of wax ester from fatty acid/triacylglycerol and long-chain fatty acyl alcohols by the mechanism shown in Scheme 1. Incubation of lipases with free fatty acid or triacylglycerol results in the formation of an acyl-enzyme intermediate, which is deacylated by nucleophilic attack of water or alcohol. When water is the fatty acid acceptor, hydrolysis occurs, but when alcohol is the acceptor, wax ester synthesis (alcoholysis) occurs. Long-chain fatty acyl alcohols such as hexadecanol are more efficient acyl acceptors than water. One explanation for this phenomenon is that alcohol is much more hydrophobic than water. Therefore, the long-chain alcohols may transfer to the lipid–water interface more easily than to water.

At neutral pH, only 10% of the wax esters were hydrolyzed by a high concentration of lipase during long-term incubation (48 h). Conversely, about 90% of oleic acid was esterified by lipase at high concentration. Therefore, the ap-

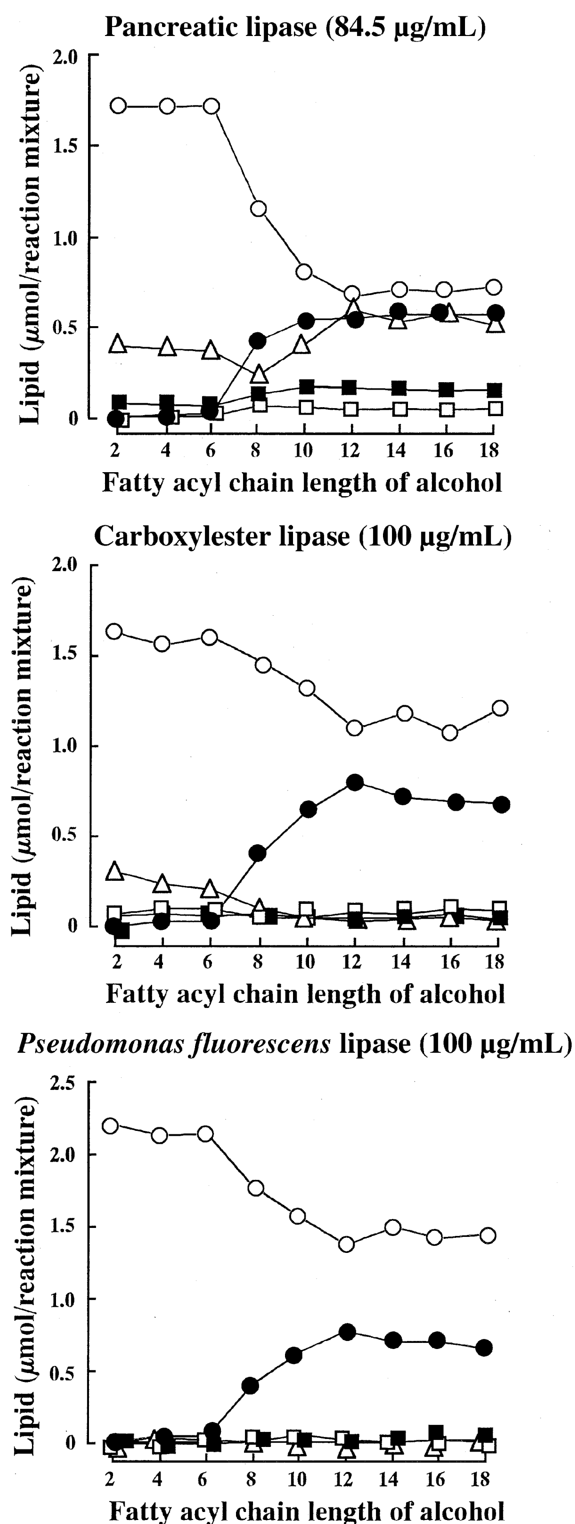


FIG. 7. Effect of fatty alcohol acyl chain length on the synthesis of wax ester from trioleoylglycerol, as determined using [^{14}C]trioleoylglycerol (3.75 mM) and saturated alcohols with various chain lengths (3.75 mM) as substrates with BSA (25 mg/mL). After incubation at pH 6.8 and 37°C for 1 h, lipids were extracted and their molar concentrations determined. ●, Wax ester; □, trioleylglycerol; ○, oleic acid; ■, dioleoylglycerol; △, monooleoylglycerol. For abbreviation see Figure 3.

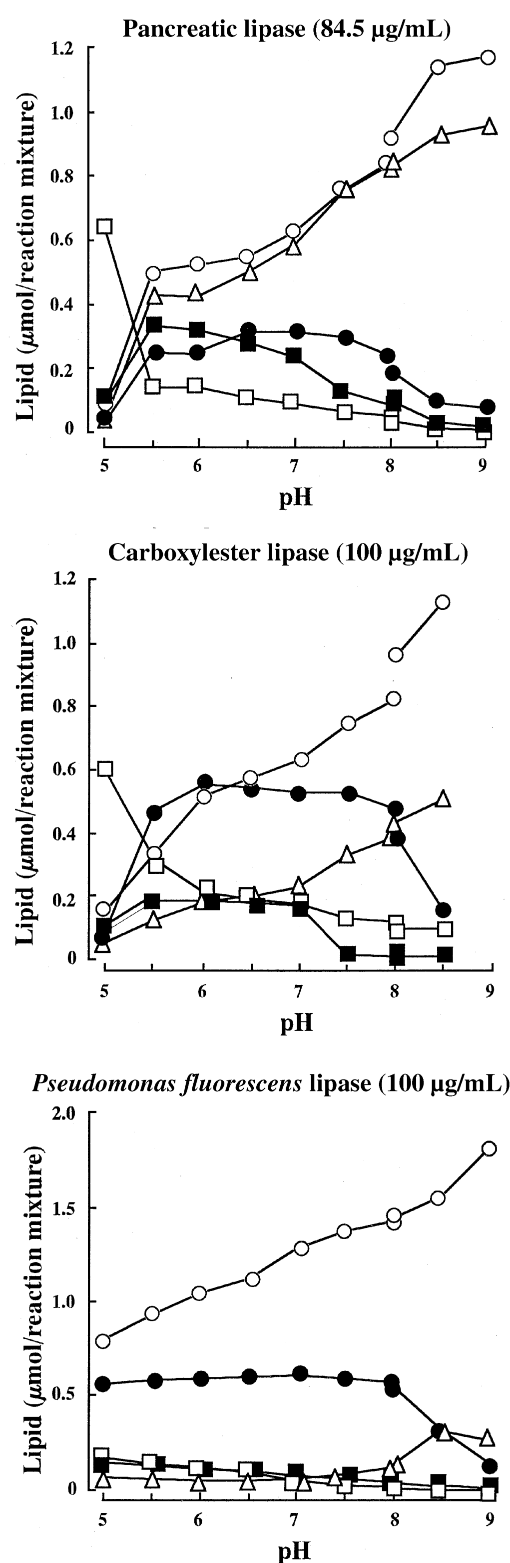
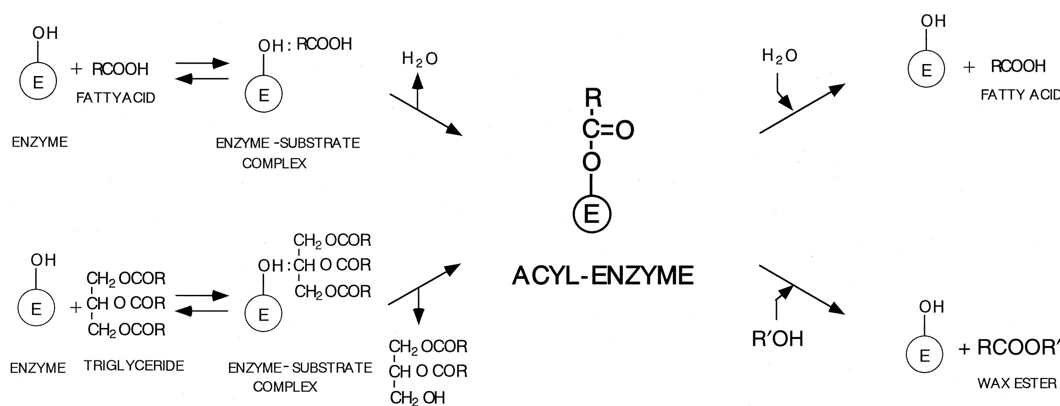


FIG. 8. Effect of pH on palmityl oleate synthesis from trioleylglycerol. Wax ester synthesis was determined using [^{14}C]trioleoylglycerol (3.75 mM) and hexadecanol (3.75 mM) as substrates with BSA (25 mg/mL). After incubation at 37°C for 1 h, lipids were extracted and their molar concentrations determined. Acetate buffer (pH 5.0 and 5.5), phosphate buffer (pH 6.0–8.0) and Tris buffer (pH 8.0–9.0) were used. The ionic strength of reaction mixture was 0.1. Symbols are as in Figure 6. For abbreviation see Figure 3.



SCHEME 1

parent equilibrium ratio of ester/oleic acid reached 0.9:0.1 (Fig. 3). However, the equilibrium ratio was affected by addition of BSA: the equilibrium ratio was changed to around 0.5/0.5 in the presence of BSA (25 mg/mL) (Fig. 3). But when triacylglycerol was used as the acyl donor, the equilibrium ratio was not affected in the presence of BSA: the molar ratio of wax ester/oleic acid/monooleoylglycerol was about 1:1.2:1 at a high concentration of pancreatic lipase, and wax ester/oleic acid was 1:2 at a high concentration of *P. fluorescens* lipase (Fig. 6). With a fatty acid as substrate (upper portion, Scheme 1), the enzyme competes for acyl-enzyme intermediate formation with BSA, which traps free fatty acid. With triacylglycerol as a substrate (lower portion, Scheme 1), BSA cannot affect the process of acyl-enzyme intermediate formation and nucleophilic displacement.

Enzymatically catalyzed wax ester synthesis may proceed any of in three ways (26): (i) Free fatty acids are directly incorporated into wax esters. Wax ester is synthesized from palmitic acid and hexadecanol in the liver of both rat and dogfish, and this reaction is not influenced by ATP, CoA, or Mg^{2+} (27). (ii) Fatty acyl-CoA is used as the acyl donor for the esterification of alcohols. Wax ester synthesis in murine preputial gland tumor is catalyzed by an acyltransferase which uses fatty acyl-CoA as the acyl donor (6). Acyl-CoA:fatty alcohol acyltransferase in microsomal preparations from the meibomian gland catalyzes the synthesis of wax ester from acyl-CoA and long-chain fatty acyl alcohols (28). (iii) Acyl moieties are transferred from phospholipids to alcohols. Wax ester is synthesized by retinal pigment epithelial membranes *via* acyl transfer of a palmityl group from the *sn*-1 position of an endogenous lecithin to alcohols (29). The present findings suggest that lipases catalyze the synthesis of wax esters in two ways: direct esterification of free fatty acid and acyltransfer from triacylglycerol to alcohol.

In conclusion, various lipases can catalyze the synthesize wax ester from free fatty acid or triacylglycerol, independent of the presence of ATP, Mg^{2+}, and CoA. At neutral pH and by using long-chain fatty acyl alcohols, the equilibrium ratio of wax ester and free fatty acid favored ester formation: the ratio was about 0.9:0.1. Therefore, various lipases might catalyze the synthesis of wax esters in the various organs.

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Metabolism of n-3 and n-6 Fatty Acids in Atlantic Salmon Liver: Stimulation by Essential Fatty Acid Deficiency

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ABSTRACT: Oxidation, esterification, desaturation, and elongation of [1-^{14}C]18:2n-6 and [1-^{14}C]18:3n-3 were studied using hepatocytes from Atlantic salmon (*Salmo salar* L.) maintained on diets deficient in n-3 and n-6 polyunsaturated fatty acids (PUFA) or supplemented with n-3 PUFA. For both dietary groups, radioactivity from 18:3n-3 was incorporated into lipid fractions two to three times faster than from 18:2n-6, and essential fatty acids (EFA) deficiency doubled the incorporation. Oxidation to CO_2 was very low and was independent of substrate or diet, whereas oxidation to acid-soluble products was stimulated by EFA deficiency. Products from 18:2n-6 were mainly 18:3n-6, 20:3n-6, and 20:4n-6, with minor amounts of 20:2n-6 and 22:5n-6. Products from 18:3n-3 were mainly 18:4n-3, 20:5n-3, and 22:6n-3, with small amounts of 20:3n-3. The percentage of 22:6n-3 in the polar lipid fraction of EFA-deficient hepatocytes was fourfold higher than in n-3 PUFA-supplemented cells. This correlated well with our other results obtained after abdominal injection of [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6. In hepatocytes incubated with [4,5-^{3}H]-22:6n-3, 20:5n-3 was the main product. This retroconversion was increased by EFA deficiency, as was peroxisomal β-oxidation activity. This study shows that 18:2n-6 and 18:3n-3 can be elongated and desaturated in Atlantic salmon liver, and that this conversion and the activity of retroconversion of very long chain PUFA is markedly enhanced by EFA deficiency.

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Dietary fatty acids of the n-3 and n-6 families provide the polyunsaturated fatty acids (PUFA) that fish, like all animal species so far studied, cannot synthesize *de novo*. In general, the polar lipids of fish are characterized by high contents of the n-3 fatty acids 20:5 and 22:6 and a lower content of the analogous n-6 fatty acid 20:4, with only a very small amount of 22:5 (1). The C_{20} and C_{22} fatty acids of the n-6 and n-3 families are vital structural components of biological membranes and also act as precursors for a variety of short-lived regulating molecules including the prostaglandins and leukotrienes (2). To carry out their biological functions, dietary n-3 and n-6 PUFA must be effectively metabolized in the animal. Their metabolism depends on the ability of the fish to desaturate and elongate the dietary fatty acids for esterification in neutral and polar lipids, the level of oxidation for energy production, and the possibility for peroxisomal retroconversion in order to produce shorter-chain fatty acids (3). In liver cells, these metabolic pathways compete for fatty acid substrates, and the particular pathway used may depend on the nutritional status of the animal and the dietary level of fatty acids, as well as hormonal and physiological factors.

The ability to desaturate and elongate C_{18} PUFA to longer-chain C_{20} and C_{22} PUFA varies between fish species. Rainbow trout (*Onchorhynchus mykiss*), for instance, readily convert 18:3n-3 to 20:5n-3 and 22:6n-3 (4,5), whereas red sea bream (*Pagrus major*) seem to lack the Δ5-desaturase necessary for this conversion (6). Atlantic salmon (*Salmo salar* L.) are able to convert C_{18} PUFA to C_{22} PUFA (7); however, the rates of synthesis of C_{20} and C_{22} PUFA from 18:3n-3 may be insufficient to meet the Atlantic salmon's requirement for these very long chain PUFA (8). It is believed that 18:1n-9, 18:2n-6, and 18:3n-3 all compete for the same Δ6-desaturase, which limits the rate of conversion of C_{18} PUFA to C_{20} and C_{22} PUFA. Although not investigated in any detail in fish (9), the affinity of C_{18} PUFA for Δ6-desaturase seem to vary in the order 18:3 > 18:2 > 18:1, as in mammals (10). Recently it has been discovered that this Δ6-desaturase is also involved in the formation of 22:6n-3 and 22:5n-6 (11). Evidence for this pathway operating also in fish has recently been presented (12). In addition, the Δ6-desaturase may be subjected to retroinhibition by C_{20} and C_{22} PUFA (13). Thus, a balanced proportion in the diet of the three C_{18} fatty acids is required for the synthesis of C_{20} and C_{22} PUFA of the n-6 and n-3 families.

Although Atlantic salmon is an important species in fish farming, little is known about the importance of n-3 and n-6 PUFA in its diet. The aim of the present study was to determine how a deficiency of dietary essential fatty acids (EFA) affects the metabolism of n-3 and n-6 PUFA in Atlantic salmon liver. Our focus was on esterification in neutral and polar lipid fractions and on the desaturation, elongation, and oxidation of n-3 and n-6 PUFA. The results obtained with isolated hepatocytes have further been compared with results from *in vivo* injection of radiolabeled fatty acids, in order to evaluate the significance of the *in vitro* model.

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Abbreviations: ACO, acyl-CoA oxidase; BSA, bovine serum albumin; DG, diacylglycerol; EFA, essential fatty acids; FFA, free fatty acids; GC, gas chromatography; LDCF, 2′,7′-dichlorofluorescein diacetate; PHCO, partially hydrogenated coconut oil; PL, phospholipids; PUFA, polyunsaturated fatty acids; TG, triacylglycerols; TLC, thin layer chromatography, UV, ultraviolet.

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MATERIALS AND METHODS

Materials. The radiolabeled fatty acids [1-^{14}C]18:2n-6, [1-^{14}C] 18:3n-3, and [4,5-^{3}H]22:6n-3 were obtained from Amersham (Buckinghamshire, United Kingdom), and the nonlabeled fatty acids, palmitoyl-CoA, essentially fatty acid-free bovine serum albumin, HEPES, 2′,7′-dichlorofluorescein, and collagenase type 1, were obtained from Sigma Chemical Co. (St. Louis, MO). 2′7′-Dichlorofluorescein diacetate (LDCF) was obtained from Eastman Kodak (Rochester, NY). Acetic acid, chloroform, petroleum ether, diethyl ether, and methanol were all obtained from Merck (Darmstadt, Germany). Benzene was obtained from Rathburn Chemicals Ltd. (Wakerburn, Scotland). Methanolic HCl and 2,2-dimethoxypropane were purchased from Supelco Inc. (Bellfonte, PA), and partially hydrogenated coconut oil (PHCO) was a gift from Denofa (Fredrikstad, Norway). Fatty acid methyl esters were a gift from Hydro Martens (Bergen, Norway). Nycodenz was obtained from Nycomed (Oslo, Norway). Glass-baked silica gel K6 plates were obtained from Whatman International Ltd. (Maidstone, England). The gas chromatography (GC) capillary column was obtained from J&W Scientific (Folsom, CA), and the gas chromatograph from Dani Strumentazione Analitica (Monza, Italy). A radioactive flow detector A100 (Packard 1900TR Tri-Carb) was obtained from Radiomatic Instruments & Chemicals (Tampa, FL).

Experimental fish and diets. The experiment was performed at AKVAFORSK's research station at Sunndalsøra, Norway, with Atlantic salmon fry (*S. salar* L.) reared on a commercial diet to an average weight of 4 g. The fish were then divided into two dietary groups and maintained in 0.5-m^3 freshwater tanks for 18 mon at 10°C. Both groups were fed semisynthetic diets with PHCO added to give a fat content of 8 g per 100 g diet. One group was fed a diet which provided the experimental fish with the recommended dietary intake of n-3 PUFA (8), that is, a diet where 0.5 g of the PHCO was replaced by 0.5 g methyl esters of 20:5n-3 and 22:6n-3 (1:1). When measured by gas chromatography, 20:5n-3 and 22:6n-3 constituted 5.3% of the total fatty acids in the diet. The other group was fed a diet in which PHCO was the only lipid source, rendering it almost deficient in n-6 and n-3 PUFA (Table 1). Both groups were fed using automatic feeders that provided the complete estimated feed requirement based on the expected daily weight gain (14). The semisynthetic diet consisted of a casein–gelatin mixture supplemented with vitamins, minerals, and amino acids (8).

Intraperitoneal injection of radiolabeled fatty acids. Fish that had been starved for 48 h were injected intraperitoneally with 4 μCi radiolabeled 18:2n-6 or 18:3n-3 in an ethanol solution. Specific radioactivity was 0.02 μCi/g fish. The fish were killed 24 h after injection, whereupon liver, blood, and muscle were analyzed for radioactive fatty acids.

Preparation of salmon hepatocytes. Parenchymal cells were prepared using a two-step collagenase perfusion procedure as described by Seglen (15) and modified by Dannevig and Berg (16). The parenchymal cells were suspended in a buffer (300

TABLE 1
Fatty Acid Compositions of the Dietary Lipid and Total Lipid Fraction of Hepatocytes

	Dietary lipid		Hepatocyte lipid[b]	
Fatty acids	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD
12:0	62.4	61.2	41.5 ± 12.0	30.5 ± 5.6
14:0	14.4	7.5	5.8 ± 0.6	6.0 ± 0.6
16:0	6.7	8.8	7.5 ± 1.2	6.6 ± 0.6
16:1n-7	ND	ND	5.8 ± 1.2	7.4 ± 0.9
18:0	8.6	11.1	3.3 ± 0.7	2.6 ± 0.1
18:1n-7	ND	ND	1.8 ± 0.9	1.7 ± 0.9
18:1n-9	0.8	0.5	25.7 ± 5.9	31.3 ± 1.4
18:2n-6	0.2	0.2	ND	0.2 ± 0.1
18:3n-3	0.1	0.1	0.4 ± 0.2	0.9 ± 0.4
20:0	0.2	0.2	ND	ND
22:0	0.1	0.1	ND	ND
20:1n-9	ND	ND	1.3 ± 0.3	1.7 ± 0.2
20:3n-9	ND	ND	1.4 ± 0.4	5.2 ± 1.5*
20:3n-6	ND	ND	ND	ND
20:4n-6	ND	ND	0.7 ± 0.1	2.0 ± 0.5*
20:5n-3	2.4	ND	0.8 ± 0.0	ND
22:5n-6	ND	ND	ND	1.5 ± 0.3*
22:6n-3	2.9	ND	4.4 ± 0.9	1.5 ± 0.4*
DB index	30.9	1.2	69.0	62.2
Σ Saturates	92.4	99.0	58.1 ± 9.7	45.6 ± 4.9
Σ Monounsaturates	0.8	0.5	34.7 ± 8.1	42.1 ± 1.8
Σn-6	0.2	0.2	0.5 ± 0.2	3.0 ± 0.9*
Σn-3	5.4	0.1	4.8 ± 1.2	2.1 ± 0.7

[a]The quantity of each fatty acid is given in percentage of total fatty acids. Data are means ± SEM.
[b]Asterisk (*) denotes significant difference from n-3 supplemented diet, based on 95% confidence interval. ND = not detectable; *n* = 4; EFAD = essential fatty acid-deficient diet; DB index = number of double bonds per 100 moles fatty acid.

mOsm) containing 137 mM NaCl, 5.4 mM KCl, 0.34 mM Na_2HPO_4, 0.35 mM KH_2PO_4, 0.81 mM $MgSO_4$, 2 mM $CaCl_2$, 40 mM HEPES at pH 7.5, and 1% bovine serum albumin (BSA), and purified by conducting three 2-min 50 × *g* sedimentations. The hepatocytes were suspended and mixed with 24 mL Nycodenz (density 1.15 g/mL), overlaid with 5 mL buffer, and centrifuged for 15 min at 3,500 × *g*. Under these conditions, viable cells will float to the interface between Nycodenz and the buffer, whereas leaky and dead cells will settle. Cells from the interface were washed twice (2-min 50 × *g* sedimentations) and resuspended in buffer. Cell viability was assessed by Trypan blue (0.4%) staining. The protein content was determined according to Lowry *et al.* (17).

Incubations with radiolabeled fatty acids. Immediately after preparation, *ca.* 6×10^6 hepatocytes (20–25 mg of protein) were incubated in 25-mL Erlenmeyer flasks with 0.1 mM radiolabeled fatty acids and 10 mM lactate in a total volume of 2 mL. The specific radioactivity of the fatty acids was 7mCi/mmol in the incubation. The radiolabeled fatty acids were added to the medium in the form of their potassium salts bound to BSA. The incubation time with labeled fatty acids varied from 30 to 180 min at 15°C.

Lipid extraction and fatty acid analysis. Total lipids were extracted from homogenized tissues using a method described by Folch *et al.* (18). The solutions used for lipid extraction contained 2,6-di(*tert*-butyl)-*p*-cresol (50 mg/L) as an antioxidant, and the lipid extracts were stored under nitrogen in the dark at –50°C to prevent the oxidation of unsaturated fatty acids. The chloroform phase was dried under nitrogen and dissolved in hexane. Radioactivity in an aliquot of the total lipid was measured with a scintillation spectrometer.

Polar lipids were separated from neutral lipids by thin-layer chromatography (TLC) using a mixture of petroleum ether, diethyl ether, and acetic acid (113:20:2, by vol) as the mobile phase. The lipids were visualized by spraying the TLC plates with 0.2 % (w/v) 2′,7′- dichlorofluorescein in methanol and were identified by comparison with known standards under ultraviolet (UV) light. The spots corresponding to phospholipids (PL), triacylglycerols (TG), diacylglycerols (DG), and free fatty acids (FFA) were scraped into glass tubes and transmethylated overnight with 2,2-dimethoxypropane, methanolic HCl, and benzene at room temperature, as described by Mason and Waller (19). Radioactivity in each lipid fraction was determined with a scintillation spectrometer.

The radioactive fatty acid compositions of the PL and TG fractions were determined by reversed-phase high-pressure liquid chromatography as described by Narce *et al.* (20). The mobile phase was acetonitrile/H_2O (80:20, vol/vol). The flow rate was 1 mL/min, and the temperature 30°C. Radioactivity in the different fatty acids was measured in a radioactive flow detector A100 (Radiomatic Instruments & Chemicals, Tampa, FL). Absorbance of nonradioactive fatty acids was measured in a UV detector (SPD-6AV UV-visible spectrophotometric detector; Shimadzu, Kyoto, Japan). Peaks were identified by comparing the sample retention volumes with the retention volumes of known fatty acid standards.

The total fatty acid composition of hepatocytes was determined according to the method described by Røsjø *et al.* (21). The methyl esters were separated on a nonpolar fused-silica capillary column by gas chromatography, and the relative quantity of each fatty acid present was determined by measuring the area under the peak corresponding to a particular fatty acid.

Fatty acid oxidation. Radioactive acid-soluble products and radioactive CO_2 (as a measure of the rate of β-oxidation) were measured as described by Christiansen *et al.* (22).

Acyl-CoA (palmitoyl-CoA) oxidase was assayed by determination of H_2O_2 production coupled to the oxidation of LDCF, mainly as described by Small *et al.* (23). The oxidation of LDCF by H_2O_2 to 2′,7′-dichlorofluoroescein was followed spectrophotometrically at 502 nm. The reaction mixture contained 0.1 M Tris-HCl (pH 8.5), 0.05 mM LDCF, 50 µg horseradish peroxidase type II (EC 1.11.1.7), 0.015 mM FAD, 0.6 mg/mL BSA, and 0.02% Triton-X100, and the reaction was started with 60 µM palmitoyl-CoA. All concentrations are given as final values. The reaction mixture contained 10–40 µg of protein in a total volume of 1 mL at 20°C. The LDCF was prepared daily at 5.1 mM in 0.01 M NaOH and stored in a light-proof container under nitrogen.

RESULTS

Fish growth and performance. Atlantic salmon fed the diet supplemented with n-3 PUFA increased in weight from 4 g at the start of the 18-mon feeding period to 302 ± 19.4 g at the end, whereas salmon fed the n-3 and n-6 PUFA-deficient diet increased in weight to 207 ± 21.3 g. In contrast, salmon fed a regular commercial diet for 18 mon under similar growth conditions weighed approximately 350 g. At the end of the trial, *ca.* 30% of the fish fed the EFA-deficient diet had swollen, pale livers and showed fin erosion, whereas these symptoms were not observed in the n-3 supplemented fish. There were, however, no significant differences in numbers of viable hepatocytes prepared from fish in either dietary group, with the percentage of viable cells routinely exceeding 90%.

Fatty acid composition of dietary lipids and of total lipids in salmon hepatocytes. In the diet not supplemented with additional PUFA, n-3 and n-6 PUFA together constituted only 0.2% of the total fatty acids; thus the diet was practically deficient in EFA. In the n-3 supplemented diet, however, 20:5n-3 and 22:6n-3 together constituted 5.3% of the total fatty acids, whereas the n-6 fatty acids constituted only 0.2% (Table 1).

Percentages of 20:5n-3 and 22:6n-3 of the total lipid fraction of hepatocytes from salmon fed the EFA-deficient diet were about threefold lower compared with the corresponding values of hepatocytes from salmon fed the n-3 supplemented diet. Percentages of longer-chain n-6 fatty acids (20:4n-6 and 22:5n-6) and 20:3n-9 in hepatocytes from EFA-deficient fish were about five- and fourfold higher, respectively, compared with hepatocytes from n-3 supplemented fish.

Incorporation of [1-^{14}C] fatty acids into hepatocyte lipid classes and metabolism into oxidation products. Both the disappearance rate of fatty acid substrates and the incorporation

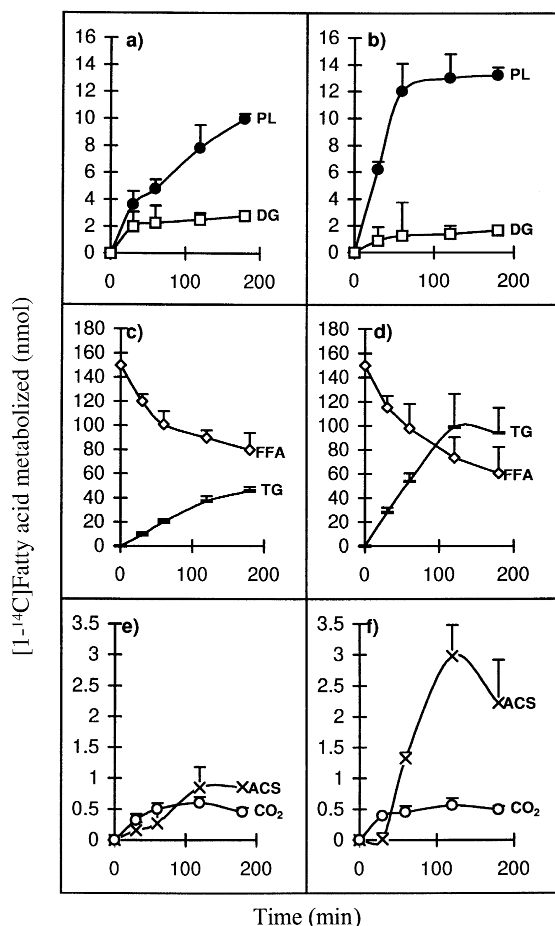

FIG. 1. Esterification in phospholipids (PL), triacylglycerols (TG), diacylglycerols (DG), and free fatty acids (FFA) and oxidation to acid-soluble products (ACS) and CO_2 from [1-^{14}C]18:3n-3 substrate in hepatocytes from fish fed an essential fatty acid (EFA)-deficient diet (b,d,f) and an n-3 supplemented diet (a,c,e). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of ^{14}C-labeled fatty acid esterified or oxidized. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given.

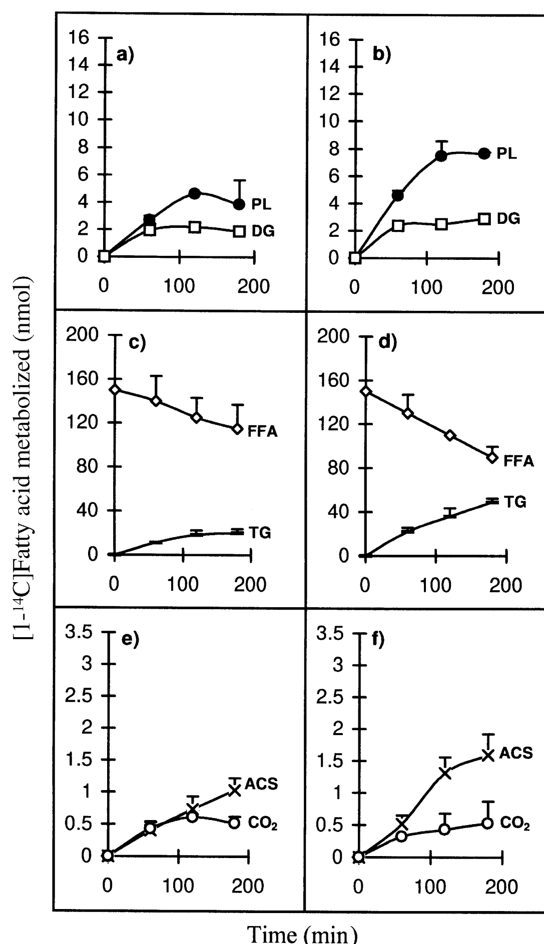

FIG. 2. Esterification in PL, TG, DG, and FFA and oxidation to ACS and CO_2 from [1-^{14}C]18:2n-6 substrate in hepatocytes from fish fed an EFA-deficient diet (b,d,f) and an n-3 supplemented diet (a,c,e). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of ^{14}C-labeled fatty acid esterified or oxidized. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given. For abbreviations see Figure 1.

of radioactivity into products proceeded fairly linearly for the first hour (Figs. 1,2). Later, reduced rates were observed, especially in the incorporation into CO_2 and PL, and most prominently in the hepatocytes from the EFA-deficient fish. When comparing the initial rates, a disappearance of free 18:3n-3 four to five times higher than of 18:2n-6 was observed in cells from n-3 supplemented fish. EFA deficiency increased these rates by a factor of 1.5–2, but the ratio was not significantly affected.

Most of the radioactivity from both substrates was recovered in the neutral lipids (60–80% during the first hour), less in PL (15–20%), with very little in oxidation products (2–5%). The initial rates of incorporation into TG and PL were found to be about twice as high for 18:3n-3 as for 18:2n-6, while no difference could be seen in the production of radiolabeled CO_2. For acid-soluble products there seemed to be a lag phase with 18:3n-3, making comparisons of the initial rates somewhat difficult. After 2 h, no difference was seen in acid-soluble products in hepatocytes from the n-3 supplemented fish, while in hepatocytes from the EFA-deficient fish about two times more acid-soluble products were observed with 18:3n-3. EFA deficiency also increased the rate of incorporation of radioactivity into TG and PL by a factor of two, while incorporation into DG was unaffected (18:2n-6) or slightly reduced (18:3n-3).

[1-^{14}C]PUFA in hepatocytes. [1-^{14}C]18:3n-3 was rapidly esterified into PL of EFA-deficient hepatocytes during the first 30 min of the incubation, but during prolonged incubation, the initial rapid increase in the 18:3n-3 content of the PL was followed by a gradual decrease. Concomitantly, the contents of 18:4n-3, 20:5n-3, 22:5n-3, and 22:6n-3 gradually increased (Fig. 3). The rate of incorporation of radiolabeled 18:3n-3 into PL and the desaturation and elongation rates of this substrate were higher in EFA-deficient hepatocytes than in cells from n-3 supplemented fish. In hepatocytes from both dietary groups, the main products formed during the 3-h incubation were

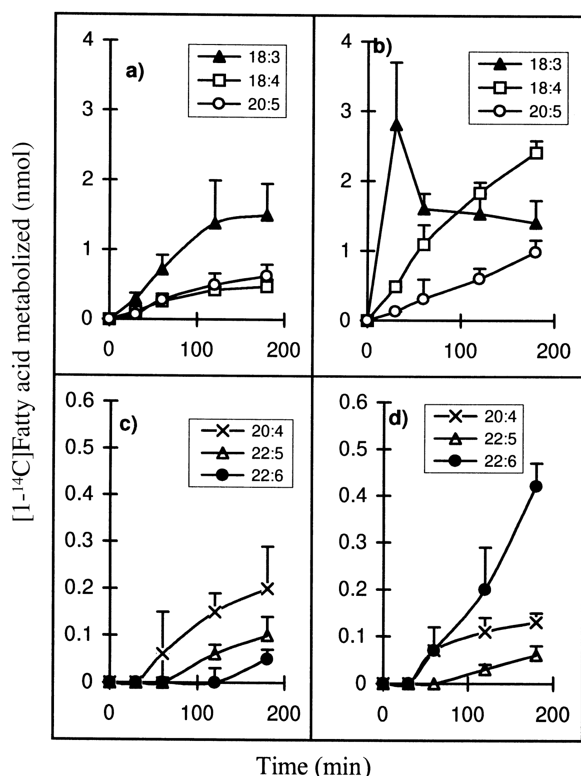

FIG. 3. Radiolabeled fatty acid metabolites found in PL of hepatocytes during a 3-h incubation with [1-^{14}C]18:3n-3 in hepatocytes from fish fed an EFA-deficient diet (b,d) and an n-3 supplemented diet (a,c). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of esterified ^{14}C-labeled fatty acid. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given. For abbreviations see Figure 1.

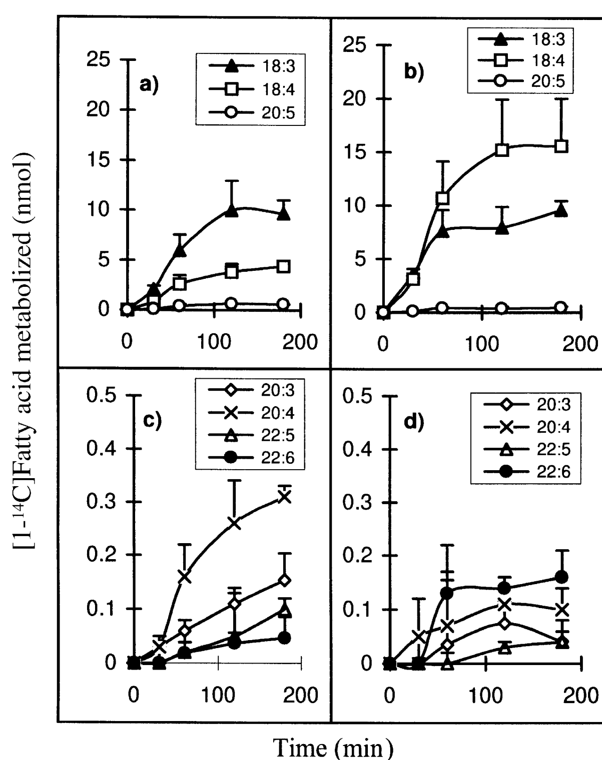

FIG. 4. Radiolabeled fatty acid metabolites found in TG of hepatocytes during a 3-h incubation with [1-^{14}C]18:3n-3 in hepatocytes from fish fed an EFA-deficient diet (b,d) and an n-3 supplemented diet (a,c). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of esterified ^{14}C-labeled fatty acid. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given. For abbreviations see Figure 1.

18:4n-3 and 20:5n-3 plus minor amounts of the n-3 20:4, 22:5, and 22:6 fatty acids. The C_{20} and C_{22} fatty acids resulting from the desaturation and elongation of 18:3n-3 were mainly incorporated into the polar lipid fraction, whereas the neutral lipids were the main recipients of the unmetabolized [1-^{14}C]18:3n-3 substrate and the desaturation product 18:4n-3 (Fig. 4). The "dead end" elongation product of 18:3n-3, i.e. 20:3n-3, also appeared mainly in the neutral lipid fraction. The amount of radioactivity recovered in 20:3n-3 and 20:4n-3 was greater in cells from the n-3 supplemented fish than in hepatocytes from EFA-deficient fish, whereas the incorporation into 22:6n-3 was substantially enhanced by EFA deficiency.

Figure 6 illustrates that for both dietary groups [1-^{14}C]18:2n-6 was incorporated into neutral lipids of hepatocytes primarily in unmetabolized form, and the incorporation rate was higher in EFA-deficient cells than in cells from n-3 supplemented fish. The main product formed from 18:2n-6 in the neutral lipid fraction was the desaturation product 18:3n-6, whereas in the polar lipids 18:2n-6 was primarily converted to longer-chain PUFA such as 20:3n-6, 20:4n-6 and 22:5n-6 (Fig. 5). The amounts of radiolabeled desaturation and elongation products formed from [1-^{14}C]18:2n-6 during the 3-h incubation were higher in EFA-deficient cells than in cells from n-3 supplemented fish.

Retroconversion of [4,5-^{3}H]22:6n-3 and peroxisomal β-oxidation of palmitoyl-CoA by hepatocytes. In addition to incubation studies with the 18-carbon fatty acids, hepatocytes from fish in both dietary groups were incubated with tritiated 22:6n-3 in order to investigate retroconversion ability and any possible influence of dietary n-3 fatty acids on this system. As shown in Table 2, most of the tritiated 22:6n-3 was esterified unchanged in polar and neutral lipids, but 4.1% had been chain- shortened to 20:5n-3 in hepatocytes from n-3 supplemented fish incubated for 3h, while in EFA-deficient cells, 10.7% of the substrate was retroconverted to 20:5n-3. The specific activity of acyl-CoA oxidase (ACO) was also measured. In hepatocytes from n-3 supplemented fish, this activity was found to be 30% less than in hepatocytes from fish fed the EFA-deficient diet, at 0.39 ± 0.03 mU/mg and 0.56 ± 0.06 mU/mg, respectively.

In vivo *metabolism of [1-^{14}C]PUFA.* Fish from both dietary groups were injected abdominally with either [1-^{14}C]18:2 n-6 or [1-^{14}C]18:3n-3. Twenty-four hours after injection, 20–30% of the injected radioactivity was recovered in the total lipid fraction of the carcass, *ca.* 0.5% in the total blood lipid fraction, and 3–8% in the total liver lipid fraction. There were no significant differences between the two dietary groups in the amount of radioactive fatty acids incorporated in carcass and

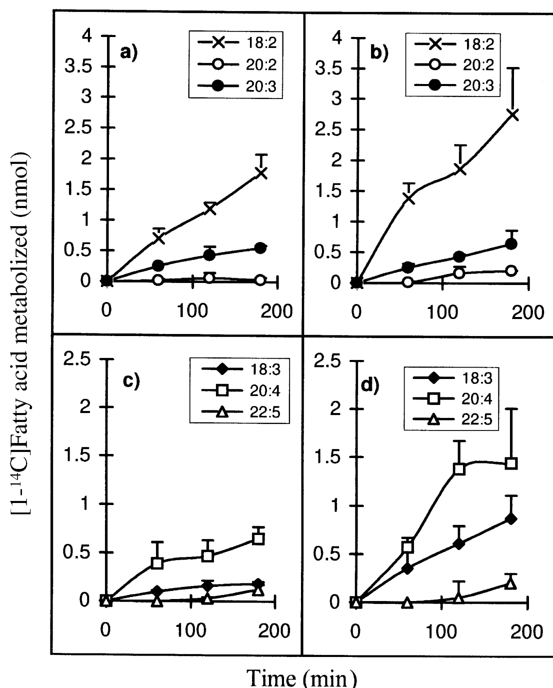

FIG. 5. Radiolabeled fatty acid metabolites found in PL of hepatocytes during a 3-h incubation with [1-^{14}C]18:2n-6 in hepatocytes from fish fed an EFA-deficient diet (b,d) and an n-3 supplemented diet (a,c). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of esterified ^{14}C-labeled fatty acid. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given. For abbreviations see Figure 1.

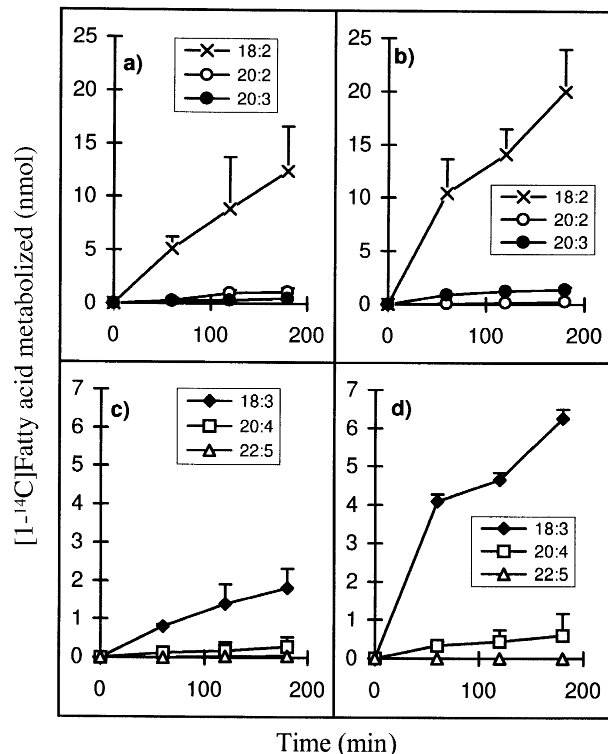

FIG. 6. Radiolabeled fatty acid metabolites found in TG of hepatocytes during a 3-h incubation with [1-^{14}C]18:2n-6 in hepatocytes from fish fed an EFA-deficient diet (b,d) and an n-3 supplemented diet (a,c). The incubation conditions are described in the Materials and Methods section. The results are expressed as nmol of esterified ^{14}C-labeled fatty acid. Mean values and SEM of two parallel incubations of hepatocytes from three livers are given. For abbreviations see Figure 1.

blood lipids. However, the amount of radioactivity recovered from the total liver lipids fraction was twice as high for fish fed the EFA-deficient diet as for fish fed the n-3 supplemented diet.

A comparison between the proportional distributions of radioactivity from [1-^{14}C]18:3n-3 in PUFA of hepatocytes (*in vitro*) and of liver (*in vivo*) is presented in Table 3. Of the total radioactivity esterified in the salmon liver *in vivo*, *ca.* 90% was recovered in the PL fraction and 10% in the TG fraction. By contrast, in isolated hepatocytes, the corresponding recoveries were near 80% in TG and 20% in PL. Each ^{14}C-labeled fatty acid metabolite formed was, however, distributed in a characteristic way between the PL and TG fractions. Both *in vivo* and *in vitro*, the C_{20} and C_{22} desaturation and elongation metabolites were primarily found in the polar lipids, whereas unaltered 18:3n-3 and its desaturation product 18:4n-3 were found mainly in the neutral lipids. The main products found in the polar lipids were the desaturation product 18:4n-3 and the desaturation and elongation products 20:5n-3, 22:5n-3, and 22:6n-3. Minor amounts of the "dead end" elongation product 20:3n-3 were also found. In the n-3 supplemented fish, the proportion of desaturated and chain-elongated fatty acids esterified in the PL fraction was higher in the liver *in vivo* (71%) than in isolated hepatocytes *in vitro* (49%). In cases of EFA deficiency, corresponding values were higher both *in vivo* (82%) and *in vitro* (74%). The percentage of radioactivity recovered in 22:6n-3 was fourfold higher in EFA-deficient hepatocytes than in cells from n-3 supplemented fish, whereas in EFA-deficient liver *in vivo,* the recovery was twice as high compared with n-3 supplemented fish. The only significant difference in the distribution of radioactivity from [1-^{14}C]18:3n-3 incorporated into the neutral lipid was an increased recovery in 18:4n-3 in EFA deficiency, both *in vivo* and *in vitro*.

Table 4 shows the proportional distributions of radioactivity from [1-^{14}C]18:2n-6 in PUFA in hepatocytes (*in vitro*) and liver (*in vivo*). More than 70% of the total radioactivity esterified in the salmon liver *in vivo* was recovered in the PL fraction, whereas less than 30% was recovered in the TG fraction. The opposite relationship was found in isolated hepatocytes, with 85% of the total esterified radioactivity recovered in TG and 15% recovered in PL. The percentage of desaturated and chain-elongated fatty acids was increased by ca. 10% by EFA deficiency both *in vivo* and *in vitro*. The main radiolabeled fatty acids esterified in the PL fraction from radiolabeled 18:2n-6, both *in vivo* and *in vitro*, were the desaturation and elongation products 20:3n-6 and 20:4n-6 and the desaturation product 18:3n-6. The longer-chain 22:4n-6 and 22:5n-6 were only found in the studies with isolated hepatocytes. In the TG fraction of n-3 supplemented fish *in vivo*, the substrate was almost exclusively esterified unchanged, whereas in EFA-deficient fish, the desaturation product 18:3n-6 was found. The content

TABLE 2
Percentage Distribution of Radioactivity into PUFA of Hepatocytes Incubated with [4,5-^{3}H]22:6n-3[a]

Diet	PL		TG	
	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD
Activity[b]	0.15 ± 0.04	0.18 ± 0.06	0.41 ± 0.11	0.43 ± 0.09
Fatty acids				
22:6n-3	91.3 ± 15.3	86.6 ± 8.5	95.4 ± 9.9	89.9 ± 1.2
22:5n-3	1.7 ± 1.0	1.4 ± 0.8	1.6 ± 0.3	3.8 ± 1.3
20:5n-3	7.0 ± 2.3	12.0 ± 2.3*	3.0 ± 0.7	6.3 ± 1.0*

[a]Each entry in the table is the average of four experiments ± SEM. Values marked with * are significantly different from the n-3 supplemented diet based on 95% confidence interval.
[b]Activity corresponds to nmol mg^{-1} of ^{14}C-fatty acid incorporated into lipid fractions of isolated hepatocytes during 3-h incubation.

of the elongation product 20:2n-6 in the neutral fraction of hepatocytes was higher in cells from n-3 supplemented fish than in EFA-deficient hepatocytes.

DISCUSSION

Fatty acid composition. The use of PHCO as the only lipid source in the experimental diet almost deprived the Atlantic salmon of a dietary supply of n-3 and n-6 PUFA. It was clear that the fatty acid composition of liver tissue was affected by the fatty acid composition of dietary lipids. In the total lipid fraction of hepatocytes from fish fed the EFA-deficient diet, the percentage of n-3 fatty acids, especially 22:6, was substantially lower, and the percentage of the long-chain n-6 fatty acids 20:4 and 22:5 was markedly higher as compared to the fatty acid composition of hepatocytes from fish fed the n-3 supplemented diet. Thus, in the hepatocyte lipid fraction of fish fed the EFA-deficient diet the ratio of n-3 to n-6 PUFA was tenfold lower, and the percentage of 20:3n-9 was fourfold higher than in hepatocytes from fish fed the n-3 supplemented diet. Under EFA-deficient conditions n-9 PUFA, and 20:3n-9 in particular, are synthesized instead of the normal products of Δ5-desaturation in tissue lipids (24–26). Consequently, 20:3n-9 is widely used as an indicator of EFA deficiency. For instance, if the ratio of 20:3n-9 to 22:6n-3 in rainbow trout liver exceeds 0.4, the fish is considered EFA-deficient (24). In the present study, the high ratio (3.5) of 20:3n-9 to 22:6n-3 in the total lipid fraction of hepatocytes from salmon fed the EFA-deficient diet, together with the appearance of swollen pale livers and fin erosion, indicates an EFA-deficient status. The latter two symptoms are known signs of EFA deficiency in fish (27,28). Even though no dietary n-6 fatty acids were available to fish from either of the two dietary groups, the percentage of the n-6 fatty acids 20:4 and 22:5 was higher in hepatocytes from fish fed the EFA-deficient diet as compared to hepatocytes from fish fed the n-3 supplemented diet. The higher percentage could have been due in part to mobilization of 18:2n-6 PUFA from lipid reserves in other parts of the body. Furthermore, because of the lack of dietary 18:3n-3, which is normally considered to be the preferred substrate for Δ6-desaturase, 18:2n-6 may readily be chain-elongated and desaturated to 20:4n-6 and 22:5n-6. These results are in agreement with what has previously been found in mammals, where the conversion of 20:4n-6 to 22:5n-6 has been demonstrated only in animals fed a diet deficient in EFA (29–31).

TABLE 3
Percentage Distribution of Radioactivity from [1-^{14}C]18:3n-3 in PUFA of PL and TG of Hepatocytes (*in vitro*) or Liver (*in vivo*)[a]

Diet	PL, *in vitro*		PL, *in vivo*		TG, *in vitro*		TG, *in vivo*	
	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD
Activity[b]	0.53 ± 0.11	0.90 ± 0.25	2.19 ± 0.40	4.44 ± 1.30*	2.10 ± 0.43	3.60 ± 0.52*	0.42 ± 0.12	0.13 ± 0.03
Fatty acids								
18:3n-3	50.4 ± 5.8	25.9 ± 5.8*	28.0 ± 6.0	18.0 ± 6.5	63.2 ± 8.8	36.8 ± 3.3*	77.5 ± 4.5	51.5 ± 3.8*
20:3n-3	0.6 ± 0.1	0.2 ± 0.2	3.6 ± 1.4	1.3 ± 0.4	1.0 ± 0.7	0.2 ± 0.2	ND	ND
18:4n-3	16.1 ± 3.4	44.5 ± 12.7*	3.5 ± 0.8	11.4 ± 0.5*	28.9 ± 3.4	60.1 ± 16.9*	22.6 ± 4.6	48.5 ± 3.8*
20:4n-3	6.7 ± 3.1	2.4 ± 0.4*	3.9 ± 1.6	12.0 ± 4.5	2.0 ± 0.1	0.4 ± 0.2	ND	ND
20:5n-3	21.2 ± 5.4	18.1 ± 3.1	42.1 ± 0.8	25.2 ± 1.2*	3.9 ± 0.7	1.7 ± 0.4	ND	ND
22:5n-3	3.4 ± 1.4	1.1 ± 0.4	5.1 ± 3.4	8.0 ± 0.5	0.7 ± 0.3	0.2 ± 0.1	ND	ND
22:6n-3	1.7 ± 0.7	7.8 ± 0.9*	14.2 ± 1.1	24.9 ± 1.2*	0.3 ± 0.3	0.6 ± 0.2	ND	ND
DI	0.49 ± 0.13	0.74 ± 0.08*	0.71 ± 0.05	0.82 ± 0.03*	0.36 ± 0.06	0.63 ± 0.09*	0.22 ± 0.06	0.51 ± 0.13

[a]Each entry in the table is the average of four experiments ± SEM. Values marked with * are significantly different from the n-3 supplemented diet based on 95% confidence interval.
[b]Activity corresponds to nmol mg^{-1} of ^{14}C-fatty acid incorporated into lipid fractions of isolated hepatocytes during 3-h incubation, or nmol g^{-1} liver of ^{14}C-fatty acid incorporated during 24-h *in vivo*. The desaturation index (DI) is given as (18:4 + 20:4 + 20:5 + 22:5 + 22:6)/(18:3 + 20:4 + 20:5 + 22:5 + 22:6). nd= not detectable. EFAD = essential fatty acid deficient

TABLE 4
Percentage Distribution of Radioactivity from [1-^{14}C]18:2n-6 in PUFA of PL and TG of Hepatocytes (*in vitro*) or Liver (*in vivo*)[a]

	PL, *in vitro*		PL, *in vivo*		TG, *in vitro*		TG, *in vivo*	
Diet	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD	n-3 Supplemented	EFAD
Activity[b]	0.14 ± 0.09	0.21 ± 0.05	1.70 ± 0.23	5.71 ± 1.45*	0.80 ± 0.23	1.20 ± 0.32	0.09 ± 0.01	2.26 ± 0.37*
Fatty acids								
18:2n-6	53.8 ± 9.1	44.9 ± 12.6	51.8 ± 0.4	37.5 ± 6.5*	76.9 ± 26.1	70.0 ± 14.0	100 ± 0.0	81.2 ± 9.3*
20:2n-6	1.0 ± 0.9	3.3 ± 1.3	ND	ND	6.7 ± 2.2	0.9 ± 0.1*	ND	ND
18:3n-6	5.4 ± 0.6	14.2 ± 3.9*	2.9 ± 1.3	9.6 ± 1.5*	11.1 ± 3.1	21.9 ± 3.8*	ND	16.2 ± 9.4*
20:3n-6	16.6 ± 1.2	10.4 ± 3.6	26.1 ± 6.8	26.4 ± 6.5	2.9 ± 2.8	4.9 ± 0.8	ND	1.9 ± 0.9
20:4n-6	19.6 ± 3.6	23.5 ± 9.3	19.2 ± 0.89	26.5 ± 1.2*	1.7 ± 1.5	2.1 ± 2.0	ND	ND
22:4n-6	ND	0.5 ± 0.2	ND	ND	0.2 ± 0.2	0.1 ± 0.1	ND	ND
22:5n-6	3.6 ± 1.5	3.3 ± 1.6	ND	ND	0.2 ± 0.1	ND	ND	ND
DI	0.46 ± 0.12	0.54 ± 0.08*	0.48 ± 0.15	0.63 ± 0.14*	0.17 ± 0.05	0.29 ± 0.08*(	ND	0.18 ± 0.05

[a]Each entry in the table is the average of four experiments ± SEM. Values marked with * are significantly different from the n-3 supplemented diet based on 95% confidence interval.
[b]Activity corresponds to nmol mg^{-1} of ^{14}C-fatty acid incorporated into lipid fractions of isolated hepatocytes during 3-h incubation, or nmol g^{-1} liver of ^{14}C-fatty acid incorporated during 24-h *in vivo*. The desaturation index (DI) is given as (18:4 + 20:4 + 20:5 + 22:5 + 22:6)/(18:3 + 20:4 + 20:5 + 22:5 + 22:6). nd= not detectable. EFAD = essential fatty acid deficient

Oxidation vs. incorporation into lipids. Nearly all of the metabolized radioactivity was esterified into neutral and polar lipids in cells from fish fed both the EFA-deficient and the n-3 supplemented diet, whereas only 2–4% of the radiolabeled substrate was oxidized to acid-soluble products and CO_2. These results are in agreement with findings in isolated rainbow trout hepatocytes incubated with radiolabeled 18:2n-6 or 18:3n-3 where only trace amounts of oxidation products were found, showing a substantially lower oxidation than observed in rat hepatocytes (32).

The initial disappearance rate of [1-^{14}C]18:3n-3 was found to be four to five times higher than for [1-^{14}C]18:2n-6, and the rate of esterification two to three times higher. This suggests that the acyl-CoA synthetase in these fish has a higher activity toward 18:3n-3 than toward 18:2n-6, which is, again, in agreement with what is found in mammals (33). In EFA deficiency these rates increased by a factor of two, indicating that the acyl-CoA synthetase activity may be increased by the lack of dietary PUFA. These results are also consistent with the observed increase in radioactivity recovered in the livers of EFA-deficient salmon.

In isolated hepatocytes, radioactivity from both [1-^{14}C]18:3n-3 and [1-^{14}C]18:2n-6 was preferentially esterified into the TG fraction, whereas in the liver lipids of Atlantic salmon *in vivo,* it was preferentially incorporated into the PL fraction irrespective of diet. This discrepancy between the *in vivo* and the *in vitro* results may be partly due to the time difference between the *in vivo* and the *in vitro* exposure to radiolabeled fatty acids. The liver lipids were extracted 24 h after intraperitoneal injection of radiolabeled fatty acids, whereas the hepatocyte lipids were extracted after only 3 h of exposure of the cells to radiolabeled fatty acids. One would expect the majority of the PUFA to be initially incorporated into TG and then to be transesterified and modified into PL. A difference in the concentration of fatty acid substrate available to the liver cells in the two experimental situations may also influence the distribution of radiolabeled fatty acids in PL and TG. Excess fatty acids in the *in vitro* system are probably preferentially stored as depot lipids in TG. These results agree with previous findings in rainbow trout. Almost 70% of the total radioactivity from [1-^{14}C]18:3n-3 was recovered in hepatocyte TG in the study of Buzzi *et al.* (5), whereas rainbow trout receiving radiolabeled 18:3n-3 in their feed, incorporated most of the radioactivity into the PL fraction (4).

Desaturation and elongation. In keeping with the studies of Tocher and Dick (36) and Bell *et al.* (7), our results show that Atlantic salmon hepatocytes possess the ability to desaturate and elongate C_{18} n-6 and n-3 fatty acids to the longer-chain PUFA. Radioactivity from [1-^{14}C]18:3n-3 was mainly found in the Δ6- and Δ5-desaturase products 18:4 and 20:5, and from [1-^{14}C]18:2 n-6 in 20:3 and 20:4. Very similar distributions were observed in liver lipids after *in vivo* injection, confirming the relevance of the *in vitro* hepatocyte system as a model for studies of fatty acid metabolism in fish. Both systems further revealed that the percentage of overall desaturated and chain-elongated radiolabeled fatty acids was increased by EFA deficiency, thus indicating that Δ6-desaturase activity was higher in EFA-deficient cells than in cells from fish fed n-3 supplemented diets. These results are consistent with previous findings in rainbow trout, where the rate of the first step in the conversion of 18:3n-3 to 22:6n-3 by Δ6-desaturation to form 18:4n-3 was increased by a deficiency of n-3 EFA (5). The same has been shown for mammals (34,35).

It has traditionally been accepted that 18:3n-3 is converted to 22:6n-3 *via* a pathway combining the sequential action of Δ6, Δ5-, and Δ4-desaturases with chain-elongation reactions. However, recent studies with rat hepatocytes have established that 22:5n-3 is elongated to 24:5n-3, further Δ6-desaturated to 24:6n-3, and then chain-shortened to 22:6n-3 by partial β-oxidation (peroxisomal retroconversion) (36,37). In both dietary groups of our trial, the products 22:5n-6 and 22:6n-3 were seen, but substantially more 22:6n-3 was produced, and especially in cells from EFA-deficient fish. Why this pathway seems to be several times more active with the n-3 fatty acids as compared

to n-6 fatty acids is not fully elucidated. Since the Δ6-desaturase most probably is involved in this pathway, the higher affinity for n-3 observed with the C-18 fatty acids may perhaps also apply to the C-24 intermediates. Possible differences in the affinity of peroxisomal chain-shortening system should, however, also be considered. Recently Buzzi *et al.* (12) have provided supporting evidence for a similar metabolism of n-3 fatty acid by the "Sprecher pathway" in rainbow trout hepatocytes. Thus, an increased production of 22:6n-3 from 18:3n-3 in our EFA-deficient hepatocytes may be due to increased Δ6-desaturation of both 18:3 to 18:4 and 24:4 to 24:5, but perhaps there is also an increased retroconversion rate of 24:6 to 22:6. In the present study, we found that the retroconversion of [4,5-^{3}H]22:6n-3 to [4,5-^{3}H]20:5n-3 was twice as high in hepatocytes from fish fed the EFA-deficient diet, compared with cells from fish fed the n-3 supplemented diet. The retroconversion is known to take place in peroxisomes (38), and the increased retroconversion in our trial was in keeping with the high activity of the peroxisomal enzyme ACO found in EFA-deficient hepatocytes.

In summary, we found that among the dietary fatty acids available to hepatocytes, the proportion of n-3 fatty acids metabolized was greater than that of n-6 fatty acids. The results demonstrate that the rates of conversion of 18:3n-3 and 18:2n-6 to 22:6n-3 and 22:5n-6 were enhanced by EFA deficiency as well as their incorporation into polar and neutral lipids and also the rate of retroconversion by peroxisomal β-oxidation.

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Effects of Postnatal Ethanol Exposure on Brain Growth and Lipid Composition in n-3 Fatty Acid-Deficient and -Adequate Rats

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ABSTRACT: The artificial rearing model was used to investigate the effects of short-term exposure to ethanol on growth and fatty acid composition of forebrain (FB) and cerebellum (CB) during the brain growth spurt in either n-3 fatty acid-adequate (AD) or n-3 deficient (DEF) rat pups. On postnatal day 5, offspring of female rats that had been fed AD or DEF diets from day 5 of life were assigned to three groups: members of two groups were gastrostomized and artificially fed formulas appropriate for their maternal history, and the third group (suckled control) was fostered to lactating dams of a similar dietary history. Half of the artificially reared pups in each dietary condition were fed ethanol in their formula (7% vol/vol) in one-quarter of their daily feedings, while the others received maltose-dextrin substituted isocalorically for ethanol. Blood alcohol concentrations did not differ betwen the dietary groups. FB weight on postnatal day 9 was lower in ethanol-exposed offspring in both dietary conditions. Brain fatty acid composition reflected dietary history in that, compared with AD pups, DEF pups had lower percentages of docosahexaenoic acid, higher percentages of 22:5n-6, and a higher n-6/n-3 fatty acid ratio. However, the effects of ethanol exposure were inconsistent, lowering the n-6/n-3 ratio in the phosphatidylethanolamine (PE) fraction in FB but not in CB, while increasing this ratio in the phosphatidylcholine (PC) fraction in FB of the DEF pups only. Thus, while ethanol had some effects on lipid composition, there was no difference between the dietary groups in their vulnerability to the effects of early short-term ethanol exposure on brain growth.

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Ethanol exerts powerful effects on brain development, as well as on subsequent physiological and behavioral regulation (1). In the search for mechanisms underlying these effects, diet and, in particular, the possible interaction of ethanol with dietary lipids have been implicated as contributing factors (2–5). Docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (AA, 20:4n-6), the predominant polyunsaturated fatty acids (PUFA) in the mammalian brain, are found primarily in the phospholipid fractions of neural membranes and accrue rapidly in the mammalian brain during development (6). These long-chain PUFA must be obtained either directly through dietary sources or derived from their precursor dietary fatty acids, linolenic acid (LNA, 18:3n-3) and linoleic acid (LA, 18:2n-6), through a series of desaturation and elongation reactions. It has been suggested that ethanol may alter membrane PUFA composition by altering the activity of Δ-6 and Δ-5 desaturases, two of the desaturation enzymes important in this pathway (7,8). Several studies report changes in brain fatty acid composition in adult rats and mice following several weeks of ethanol administration (9,10), although these findings have been inconsistent (11). In our laboratory, we have shown that feeding ethanol to mice during gestation lowered the n-3/n-6 ratio in pup brains, particularly by lowering the concentrations of DHA in brain phosphatidylcholine (PC) fraction (12). Similarly, a study in guinea pigs reported that administration of ethanol during pregnancy, while having complex effects on various phospholipid molecular species, was also associated with impaired accumulation of DHA in fetal brain phospholipids (13).

The essential role of AA in growth and development is well documented, and its functional role in the brain may be related to its contributions to regulatory function by serving as a precursor to the 2-series eicosanoids (14) as well as possibly to its action as a second messenger (15). The importance of DHA for retinal function is supported by studies in which laboratory animals, such as rats (16,17), guinea pigs (18), and monkeys (19), made deficient in n-3 fatty acids exhibit alterations in their electroretinograms. These alterations have been extended to functional visual outcomes in n-3 deficient monkeys (19) and in both preterm and term human infants fed formula without long-chain n-3 PUFA (20–23). There are also reports of deficits in performance on learning tasks in n-3 deficient animals (24–30). However, these findings are not consistent solely with deficits in learning, but could also be due to nonspecific effects on behavioral regulation (14,31–33). Nonetheless, they support the importance of understanding the effects of exposure to agents such as ethanol during the developmental period on brain long-chain PUFA, since it is possible that some of the neurobehavioral effects of ethanol may be mediated through such changes.

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Abbreviations: AA, arachidonic acid; AD, n-3 adequate; ANOVA, analysis of variance; BAC, blood alcohol concentration; CB, cerebellum; DEF, n-3 deficient; DHA, docosahexaenoic acid; ETOH, ethanol; FB, forebrain; LA, linoleic acid; LNA, linolenic acid; MD, maltose-dextrin; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PUFA, polyunsaturated fatty acid; SC, suckled control.

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Because ethanol, administered during pregnancy can alter maternal lipid metabolism, subsequent changes in offspring lipid composition may not be due to the direct effects of ethanol, but rather may reflect changes in maternal supply of fatty acids. The artificial rearing model in rats allows the direct administration of both dietary fatty acids and ethanol to the developing pup and thereby avoids the potential confounding effects of maternal changes due to the effects of ethanol, particularly at high doses. By feeding ethanol during the early postnatal period, which encompasses the time of rapid brain growth in the rat (34), this model has been useful in describing ethanol-induced developmental abnormalities (35,36). The effects of ethanol during this period are robust, and exposure for periods even as short as 1–2 d has been found to produce deficits in brain growth (37) and in later behavior (38,39). We therefore used this approach in the present study to examine the effects of ethanol on brain development and lipid composition in rats. If the harmful effects of ethanol on brain development are associated in part with reductions in PUFA concentrations in the brain, ethanol exposure during postnatal days 5–9, which is known to produce deficits in brain growth, should also produce alterations in brain PUFA composition.

In addition to the ethanol treatment, this study incorporated a dietary manipulation by including rats that were already deficient in n-3 fatty acids. It has been shown previously that the feeding of n-3 deficient formula in the artificial rearing model is capable of producing substantial increases in the n-6/n-3 ratio of the rat brain (40). When mated, female rats that have been raised in this way and maintained on deficient diets give rise to offspring that are also n-3 deficient (27). Because previous work suggested that rats and mice made n-3 deficient are more susceptible to the effects of pentobarbital (41) and to the effects of toxic doses of triethyltin (24), we were interested in whether the effects of ethanol during the early postnatal period on brain growth and fatty acid composition would be more pronounced in n-3 deficient, compared with n-3 adequate, rat pups.

EXPERIMENTAL PROCEDURES

Animals and diets. The maternal generation in this study consisted of female offspring of Sprague-Dawley rats from a colony maintained at the University of Waterloo. These animals were fed, beginning on postnatal day 5, one of two diets differing only in their fatty acid composition. One group received a diet containing both LA and LNA in what was considered to be adequate levels (n-3 adequate or AD group), while the other was fed a diet containing adequate amounts of LA but only negligible amounts of LNA (n-3 deficient or DEF group). Until day 19, these pups were fed their appropriate diets in the form of a synthetic rat milk substitute *via* a gastrostomy tube, as described below. They were then weaned onto either AD- or DEF-modified AIN-93M chow diets (Dyets, Inc., Bethlehem, PA). Beginning at approximately 28 wk of age, these females were bred with ACI male rats purchased from Harlan Teklad (Madison, WI), and the pups used in this study were offspring of that mating.

TABLE 1
Composition of Rat Milk Substitutes

Ingredient	Form	g/kg diet
Protein		
	Casein extract (SO79841)[a]	46.5
	Whey extract (SO84826)[a]	46.5
	Amino acid mixture[b]	0.85
Carbohydrate		
	Lactose	28.6
Custom fat mixture		
	Fat mixture[c]	123.2
Vitamins		
	Teklad vitamin mixture #40060[d]	3.52
	Supplemental vitamin mixture[e]	0.48
Minerals		
	Calcium mixture[f]	7.92
	Noncalcium mineral mixture[g]	5.30
	$CuSO_4$ solution (0.03 kg/L)	0.85
	$ZnSO_4$ solution (0.38 kg/L)	0.26
Other ingredients		
	Carnitine (0.1 kg/L)	0.35
	Creatine solution (0.01 kg/L)	6.16
	Ethanolamine	0.03

[a]Ross Laboratories, Columbus, OH.
[b]Arginine (512 g/kg), glycine (310 g/kg), taurine (156 g/kg), picolinic acid (22 g/kg).
[c]The fat mixture was prepared from the following oils (all quantities shown in g/kg of diet). For the n-3 adequate group, medium-chain triglyceride (MCT) oil (Ross Laboratories, Columbus, OH) (49.28 g/kg), safflower oil (President's Choice, Sunfresh, Ltd., Toronto, Canada) (6.16 g/kg), olive oil (Bertoli Canada, Inc., Laval, Canada) (61.6 g/kg), and linseed oil (NATUR, Bioforce Canada, Inc., St. Laurent, Canada) (6.16 g/kg). For the n-3 deficient group, MCT oil (45.58 g/kg), safflower oil (43.12 g/kg), and olive oil (34.50 g/kg).
[d]*p*-Aminobenzoic acid (11.01 g/kg), ascorbic acid (97.5%) (101.66 g/kg), biotin (0.044 g/kg), vitamin B_{12} (0.1%) (2.97 g/kg), calcium pantothenate (6.6 g/kg), choline dihydrogen citrate (349.69 g/kg), folic acid (0.20 g/kg), inositol (11.01 g/kg), menadione (4.96 g/kg), niacin (9.91 g/kg), pyridoxine HCl (2.20 g/kg), riboflavin (2.20 g/kg), thiamine HCl (2.20 g/kg), vitamin A palmitate (500,000 U/g) (3.96 g/kg) vitamin D (500,000 U/g) (0.44 g/kg), vitamin E acetate (500 U/g) (24.23 g/kg), corn starch (466.69 g/kg).
[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), $Al_2(SO_4)_3$ (0.156 g/kg), $MnSO_4$ (0.042 g/kg).

The rat milk substitute in these experiments consisted of a powdered protein base that was fortified with protein, carbohydrates, fat, vitamins and minerals so that it approximated the composition of rat milk (Table 1). Although the percentage fat content was the same for all pups, the fat composition differed among experimental groups in terms of the makeup of the essential fatty acids (Table 2). Specifically, whereas the AD diet contained both LA and LNA in a ratio of 3.18, the DEF diet contained approximately three times as much LA and very small amounts of n-3 fatty acids. The increase in LA in the DEF group was primarily at the expense of LNA, saturated fatty acids, and the monounsaturate 18:1n-9, which was reduced by 35% in the DEF group compared to the AD group. The protein powders also contained small amounts of fat, and the total amount of n-3 fatty acids contributed to the diet by the protein was calculated to be 0.028 g/kg of diet or 0.00023% of total di-

etary fatty acids. The n-3 PUFA in the greatest concentration in the protein powder was 22:5n-3 (0.00765 g/kg of diet), which constituted less than 0.0001% of all fatty acids in the diet

Experimental design. This study consisted of a 2 × 2 factorial design with two levels of n-3 PUFA status (either AD or DEF) and two levels of ethanol [either 7% (vol/vol) (ETOH) or 0% (MD)]. In the MD groups, an isocaloric amount of maltosedextrin (MD) was substituted for ethanol. Also, for each level of PUFA status, there was a third group of pups [suckled control (SC)] that was fostered to a dam from that level, and these two groups acted as reference groups to evaluate the effects of the artificial rearing and formula feeding. Furthermore, they acted as a measure of the effects of the dams' PUFA status, as mediated through the milk, on fatty acid composition in the pups.

Procedure. This study was conducted in compliance with the Animals for Research Act of Ontario (Revised Statutes of Ontario) and the Guide for the Care and Use of Experimental Animals from the Canadian Council on Animal Care, and all procedures were reviewed and approved by the Animal Care Committee at the University of Waterloo. For both the maternal and F1 generations, rat pups were anesthetized with methoxyfluorane (Metofane) and gastrostomized as described previously (42). Briefly, a length of polyethylene tubing (PE 10 Intramedic Tubing; Clay Adams, Parsippany, NJ) was inserted into the mouth, down the esophagus, and out through the lateral wall of the stomach. The tube was fitted with small plastic flanges to hold it in place, and was attached, *via* a larger tube, to a Harvard infusion pump. The pump was calibrated to deliver a volume of food equal to 33% of the pup's body weight each day, in 12 equally spaced feedings of 20 min each. The pups were housed in plastic cups floating in a tank of water maintained at 36 ± 1°C. Each morning, the pups were reweighed and the pumps recalibrated accordingly, and the pups were bathed and their anogenital region stimulated gently with a soft, wet tissue to facilitate urination and defecation.

For the F1 offspring, ethanol was administered by adding a 95% ethanol solution to the formula to make a 7% (vol/vol) ethanol solution. For the artificially reared control pups, the ethanol was replaced by an isocaloric solution of MD. In both cases, the experimental treatments were administered for four consecutive feedings each day. Other pups from each litter were fostered to lactating dams that were fed diets identical to their own mothers', and these pups were weighed daily but otherwise left undisturbed. The litter size of these SC litters was maintained at 10–12 pups.

Ninety minutes after the onset of the final ethanol feeding on day 9, all pups were anesthetized with an overdose of halothane and at least 0.1 mL of blood was removed by cardiac puncture for determination of blood alcohol concentration (BAC). The pups, still under anesthesia, were then killed by decapitation and their brains removed and stored at −80°C.

BAC. Blood was centrifuged immediately after collection and the plasma stored at −80°C until analysis. EtOH concentrations were subsequently determined by the ultraviolet NAD/NADH method (Sigma Technical Bulletin 332-UV; St. Louis, MO). All standards and reagents were obtained from Sigma, and all assays were run in duplicate. All samples obtained from ethanol-fed pups, as well as some from control pups, were compared to a standard curve.

Biochemical analysis. Lipids were extracted from the forebrain (FB) and cerebellum (CB) by the method of Folch *et al.* (43) and were separated into phospholipid fractions by thin-layer chromatography on silica gel plates using a chloroform/methanol/acetic acid/water (100:75:7:4, by vol) solvent system. Fatty acid methyl esters were produced from the phosphatidylethanolamine (PE) and PC fractions with 6% methanolic sulfuric acid and analyzed by capillary gas chromatography (Shimadzu GC-17A gas chromatograph; Shimadzu Corp., Kyoto, Japan). The instrument was equipped with a flame-ionization detector and a 15 m × 0.32 mm × 0.25 μm fused-silica capillary column (Supelcowax 10; Supelco Park, Bellefonte,

TABLE 2
Selected Fatty Acid Composition of Experimental Diets[a,b]

Fatty acid	Maternal chow diet		Rat milk substitute	
	Adequate	n-3 Deficient	Adequate	n-3 Deficient
12:0	1.07	5.59	11.76	8.39
14:0	0.60	0.60	0.51	0.55
14:1	ND	ND	0.01	0.12
16:0	14.02	13.02	11.44	9.80
16:1n-7	1.26	1.13	1.00	0.64
18:0	2.56	2.45	2.33	2.30
18:1n-9	38.82	37.47	53.55	34.32
18:2n-6	35.27	36.76	12.69	40.88
18:3n-3	5.08	0.53	3.99	0.35
Others	1.32	2.45	2.72	2.65
(n-6/n-3)	6.94	69.35	3.18	116.80

[a]Data expressed as weight percentage of total fatty acids
[b]The fat content of the casein powder was 0.25 g/100 g powder. Individual fatty acid values were as follows (g/100 g total fatty acids): saturated fatty acids (6:0–22:0), 45.19; monounsaturated fatty acids, 28.73; n-3 fatty acids, 2.06; n-6 fatty acids, 1.55. The fat content of the whey powder was 2.54 g/100 g powder, and the individual values were as follows: saturated fatty acids (6:0–24:0), 65.15; monounsaturated fatty acids, 27.58; n-3 fatty acids, 2.20; n-6 fatty acids, 4.22. ND, not detected.

TABLE 3
Morphological Variables of Pups on Postnatal Day 9[a]

	n-3 Adequate			n-3 Deficient			
	Ethanol (n = 11)	Maltose-dextrin (n = 10)	Suckled Control (n = 8)	Ethanol (n = 15)	Maltose-Dextrin (n = 13)	Suckled Control (n = 10)	Effects[b]
Body weight							
Day 5	12.71	12.78	12.58	12.57	12.52	11.98	
	(±0.35)	(±0.30)	(±0.46)	(±0.31)	(±0.30)	(±0.34)	
Day 9	16.81	17.78	20.61	16.34	16.71	17.34	
	(±0.48)	(±0.43)	(±1.42)	(±0.50)	(±0.43)	(±1.33)	
Forebrain weight	0.5449	0.5806	0.6662	0.5379	0.5617	0.6281	E, FF[A], FF[D]
	(±0.0111)	(±0.0147)	(±0.0201)	(±0.0131)	(±0.0129)	(±0.0247)	
Cerebellum weight	0.0502	0.0524	0.0638	0.0496	0.0530	0.0568	FF[A]
	(±0.0028)	(±0.0026)	(±0.0027)	(±0.0015)	(±0.0020)	(±0.0035)	

[a]Data shown as means ± SEM weight in grams.
[b]E, main effect of ethanol, $P < 0.05$; FF[A], significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of adequate maternal feeding; FF[D], significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of n-3 deficient maternal feeding.

PA). The column temperature was held at 150°C for 2 min, then increased to 210°C at 3°C/min and held for another 10 min. The carrier gas (helium) flow rate was 2 mL/min, with a split ratio of 50, and the injector and detector temperatures were maintained at 250°C. Fatty acids were identified by comparison with retention times of authentic standards (Nu-Chek-Prep, Elysian, MN).

Statistical analysis. Data were analyzed using the general linear models approach for analysis of variance (ANOVA) (SAS v. 6.0.9; SAS Institute, Cary, NC) to assess main and interactive effects of both ethanol and diet. Any significant interaction effects were further interpreted by comparing the individual groups using *post-hoc* Tukey's *t*-test. Similarly, Tukey's tests were used to assess the effect of diet between the two SC groups as well as to compare these SC groups with their respective formula-fed (MD) control groups.

RESULTS

Morphological variables. Pup body and brain weights are shown in Table 3. There were no significant differences in body, FB, or CB weight at the end of the treatment period that were due to the concentrations of n-3 fatty acids in the diet. Ethanol reduced FB weight but, despite a similar trend in the CB weight data, this was not significant, nor was there an effect on body weight. Furthermore, artificial rearing reduced both FB and CB weight in the AD groups, relative to the weights in the SC groups, and lowered FB weight in the DEF group.

BAC. Mean BAC of the ethanol-exposed pups were 287 ± 15.6 mg/DL and 251 ± 11.6 mg/DL for the AD and DEF groups, respectively, and this difference was not significant.

Brain fatty acid composition. Brain fatty acid compositions resulting from the experimental treatments are shown in Tables 4–7. For clarity, only the effects on n-3 and n-6 essential fatty acids are described.

(i) Effects of n-3 deficiency. SC pups. The effects of long-term feeding of n-3 deficient diets in the maternal generation can be seen by comparing the two groups of SC pups on day 9. There were consistent effects of diet in that the percentages of DHA in both PE and PC for both FB (Tables 4 and 6) and CB (Tables 5 and 7) were significantly lower in the DEF SC pups relative to the AD SC pups. Concentrations of 22:5n-3 in both PE and PC in FB and in PC in CB were also significantly lower. There were reciprocal effects on the long-chain n-6 PUFA 22:5n-6, in that percentages in FB and CB PE were higher in the SC DEF pups. Thus, in FB PE and CB PC, the ratio of all n-6/n-3 fatty acids was significantly higher in the DEF SC pups.

Artificially reared pups. The effects of formula feeding of the AD and DEF diets, beginning on postnatal day 5, in pups of AD and DEF dams are addressed by the main effects of diet in the 2 × 2 ANOVA in the artificially reared groups. In PE and PC fractions of both FB and CB, there were main effects for diet in that n-3 deficiency led to a higher n-6/n-3 ratio, consistently lowered DHA percentages, and increased the percentage of 22:5n-6. In FB PE, n-3 deficiency lowered the percentage of 22:5n-3 and increased the percentage of the individual n-6 PUFA AA, and 22:4n-6. In FB PC and in the CB, there were no significant effects of diet on any other long-chain PUFA.

(iii) Effects of ethanol. The effects of ethanol administration from postnatal days 5 to 9 were assessed through the main effects of ethanol in the 2 × 2 ANOVA in the artificially reared groups. Relative to those of n-3 deficiency, the main effects of ethanol were inconsistent. In the PE fraction of the FB, ethanol treatment interacted with diet in that ethanol led to increased percentages of DHA in pups fed the AD diet, but did not affect percentages in the pups fed the DEF diets. Although ethanol also led to higher percentages of the individual n-6 PUFA 22:4n-6 and 22:5n-6, these increases were less than that seen in DHA, so that the overall ratio of n-6/n-3 fatty acids was significantly lowered by the ethanol treatment. In the PC fraction of the FB, ethanol decreased percentages of AA. There were interactive effects of diet and ethanol on the 22-carbon PUFA DHA, 22:4n-6, and 22:5n-6, as well as on the n-6/n-3 ratio. In all cases, ethanol decreased PUFA concentrations only in the DEF group; there was no difference between EtOH and MD groups in the

TABLE 4
Forebrain Fatty Acid Composition of Selected Fatty Acids in the Phosphatidylethanolamine Fraction on Postnatal Day 9[a]

	n-3 Adequate			n-3 Deficient			
Fatty acid	Ethanol (n = 11)	Maltose-Dextrin (n = 10)	Suckled Control (n = 8)	Ethanol (n = 15)	Maltose-dextrin (n = 13)	Suckled control (n = 10)	Effects[b]
12:0	0.73	0.97	1.05	0.70	1.02	1.51	
	(±0.16)	(±0.35)	(±0.32)	(±0.13)	(±0.29)	(±0.37)	
14:0	1.45	1.49	1.20	0.80	1.57	1.66	
	(±0.44)	(+0.47)	(±0.28)	(±0.08)	(±0.44)	(±0.24)	
16:0	6.01	5.29	5.65	6.06	5.83	5.55	E
	(±0.13)	(±0.14)	(±0.10)	(±0.20)	(±0.15)		
16:1n-7	10.11	13.09	11.77	10.38	10.81	12.92	INT
	(±0.15)	(±1.33)	(±0.90)	(±0.29)	(±0.21)	(±1.95)	
16:1n-9	0.89	0.96	0.88	0.91	1.02	1.08	
	(±0.05)	(±0.05)	(±0.08)	(±0.05)	(±0.03)	(±0.24)	
18:0	17.74	17.83	18.76	18.27	18.39	18.24	
	(±0.15)	(±0.34)	(±0.21)	(±0.11)	(±0.39)	(±0.63)	
18:1n-9	6.23	6.66	5.69	5.22	5.38	5.38	D
	(±0.17)	(±0.48)	(±0.55)	(±0.07)	(±0.12)	(±0.50)	
18:1n-7	1.32	1.34	1.17	1.30	1.44	1.43	E, MF
	(±0.03)	(±0.05)	(±0.02)	(±0.03)	(±0.04)	(±0.11)	
18:2n-6	0.77	0.80	0.56	0.56	0.75	0.45	
	(±0.06)	(±0.13)	(±0.09)	(±0.04)	(±0.07)	(±0.07)	
20:1n-9	0.17	0.25	0.25	0.26	0.29	0.40	
	(±0.03)	(±0.04)	(±0.07)	(±0.05)	(±0.07)	(±0.17)	
20:2n-6	0.60	0.58	0.47	0.51	0.53	0.31	FF^{D}
	(±0.03)	(±0.06)	(±0.06)	(±0.04)	(±0.03)	(0.04)	
20:3n-6	0.50	0.51	0.46	0.44	0.39	0.33	D
	(±0.03)	(±0.05)	(±0.04)	(±0.04)	(±0.02)	(±0.03)	
20:4n-6	18.22	17.78	18.26	20.05	19.80	18.86	D
	(±0.25)	(±0.72)	(±0.04)	(±0.18)	(±0.35)	(±0.69)	
22:4n-6	5.27	4.76	5.37	6.23	6.70	5.84	D, E
	(±0.08)	(±0.20)	(±0.14)	(±0.13)	(±0.10)	(±0.26)	
22:5n-6	3.87	3.48	3.31	14.93	14.02	13.49	D, E, MF
	(±0.10)	(±0.11)	(±0.07)	(±0.26)	(±0.17)	(±0.61)	
22:5n-3	0.51	0.49	0.56	0.23	0.20	0.23	D, MP
	(±0.03)	(±0.05)	(±0.04)	(±0.04)	(±0.02)	(±0.02)	
22:6n-3	16.82x	14.77y	16.36	4.48z	4.11z	4.53	D, E, INT, MF, FF^{A}
	(± 0.32)	(±0.63)	(±0.38)	(±0.10)	(±0.11)	(±0.26)	
(n-6)/n-3)	1.69	1.83	1.68	9.11	9.71	8.35	D, E, MF, FF^{D}
	(±0.04)	(±0.04)	(±0.03)	(±0.16)	(±0.21)	(±0.29)	

[a]Data shown as means ± SEM weight percentage of total fatty acids.
[b]D, main effect of diet, $P < 0.05$; E, main effect of ethanol, $P < 0.05$; INT, interaction effect, $P < 0.05$. Means of artificially reared groups with different superscripts are significantly different (Tukey's *t*-test, $P < 0.05$); MF, significant difference (Tukey's, $P < 0.05$) between suckled control kpups of adequate and deficient maternal feeding; FF^{A}, significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of adequate maternal polyunsaturated fatty acid (PUFA) status; FF^{D}, significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of n-3 deficient maternal PUFA status.

adequate condition. In the case of the n-6/n-3 ratio, ethanol increased the ratio but, again, affected only the DEF group.

In the PE fraction of the CB, ethanol led to decreases in AA and increases in 22:5n-3. In the PC fraction, ethanol interacted with diet to decrease percentages of 22:5n-3, but only in the AD pups.

(iv) Effects of formula feeding. There were also some effects of formula feeding of the rat milk substitute on fatty acid composition, as shown by differences between the SC pups and the artificially reared control MD pups. Relative to those in the AD SC pups, percentages of DHA in FB PE and of AA in CB PE were significantly lower in the AD MD pups. The ratio of all n-6/n-3 fatty acids in FB PE were increased by formula-feeding in the DEF pups.

DISCUSSION

In this study, the use of the artificial rearing paradigm allowed us to produce a generation of dams that were deficient in n-3 fatty acids relative to the control dams. When mated, their offspring, in turn, were also deficient, with FB n-6/n-3 ratios about four times as great as those of the AD offspring and CB ratios between two and four times as great. The most noticeable difference was in the percentages of DHA, which were substantially lower in DEF pups. There were reciprocal increases in the long-chain n-6 fatty acids, particularly 22:5n-6. The effects of ethanol exposure were less consistent than those of diet. Generally, ethanol affected percentages of some long-chain PUFA in both PE and PC fractions in the developing FB, but less so in

TABLE 5
Cerebellum Fatty Acid Composition of Selected Fatty Acids in the Phosphatidylethanolamine Fraction on Postnatal Day 9[a]

	n-3 Adequate			n-3 Deficient			
Fatty acid	Ethanol (n = 11)	Maltose-Dextrin (n = 10)	Suckled Control (n = 8)	Ethanol (n = 15)	Maltose-dextrin (n = 13)	Suckled control (n = 10)	Effects[b]
12:0	0.25 (±0.03)	0.25 (±0.07)	0.32 (±0.09)	1.23 (±0.42)	0.35 (±0.10)	0.55 (±0.09)	
14:0	1.14 (±0.34)	1.65 (±0.39)	1.53 (±0.49)	2.88 (±1.01)	1.70 (±0.46)	1.14 (±0.29)	
16:0	12.41 (±2.92)	9.61 (±1.26)	7.35 (±0.66)	9.56 (±1.56)	8.95 (±1.12)	8.80 (±0.91)	
16:1n-7	7.88 (±1.05)	7.42 (±1.42)	8.87 (±1.31)	7.63 (±0.77)	8.66 (±0.81)	6.83 (±1.22)	
18:0	16.60 (±2.08)	22.18 (±3.11)	26.27 (±4.08)	18.11 (±1.73)	18.41 (±1.16)	15.56 (±0.25)	MF
18:1n-9	12.30 (±1.12)	10.92 (±0.60)	7.78 (±0.98)	9.70 (±0.51)	10.74 (±0.24)	9.94 (±0.22)	D, FF[A]
18:1n-7	2.65 (±0.27)	2.47 (±0.24)	1.84 (±0.14)	2.16 (±0.12)	2.67 (±0.25)	2.71 (±0.10)	
18:2n-6	1.55 (±0.26)	1.16 (±0.22)	1.30 (±0.35)	1.24 (±0.19)	1.19 (±0.29)	0.71 (±0.05)	
20:1n-9	0.51 (±0.08)	0.48 (±0.15)	0.24 (±0.09)	0.33 (±0.08)	0.50 (±0.19)	0.20 (±0.03)	
20:2n-6	0.68 (±0.13)	0.95 (±0.32)	0.48 (±0.10)	0.53 (±0.04)	0.58 (±0.12)	0.30 (±0.05)	
20:3n-6	0.72 (±0.10)	0.76 (±0.13)	0.43 (±0.09)	0.69 (±0.13)	0.68 (±0.23)	0.39 (±0.07)	
20:4n-6	16.07 (±1.31)	12.68 (±1.03)	20.62 (±2.47)	16.77 (±0.79)	15.88 (±0.97)	17.13 (±0.44)	E, FF[A]
22:4n-6	4.28 (±0.69)	4.90 (±0.57)	4.57 (±0.88)	5.99 (±0.69)	5.85 (±0.76)	7.46 (±0.24)	
22:5n-6	2.20 (±0.32)	2.51 (±0.17)	1.47 (±0.44)	9.19 (±1.08)	8.00 (±1.40)	11.02 (±0.30)	D, MF
22:5n-3	0.68 (±0.12)	1.05 (±0.21)	0.61 (±0.19)	0.28 (±0.06)	1.27 (±0.44)	0.33 (±0.03)	E
22:6n-3	11.58 (±2.02)	11.82 (±1.61)	10.87 (±2.51)	3.68 (±0.42)	3.28 (±0.39)	5.17 (±0.18)	D, MF
n-6/n-3	4.23 (±1.64)	2.06 (±0.33)	4.41 (±1.52)	10.68 (±1.55)	7.66 (±0.85)	6.81 (±0.29)	D

[a]Data expressed as means ± SEM weight percentage of total fatty acids.
[b]D, main effect of diet, $P < 0.05$; E, main effect of ethanol, $P < 0.05$; MF, significant difference (Tukey's, $P < 0.05$) between suckled control pups of adequate and deficient maternal feeding; FF[A], significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of adequate maternal polyunsaturated fatty acid (PUFA) status; FF[D], significant difference (Tukey's, $P < 0.05$) between suckled control pups and formula-fed pups of n-3 deficient maternal PUFA status.

the CB. The direction of the effects was complex, increasing the percentage of AA in CB PE but decreasing it in FB PC, and increasing 22:5n-6 in FB PE but not in any other fraction. Effects of ethanol on DHA in the FB interacted with those of diet, with ethanol increasing DHA percentages in PE, but in the AD group only, while decreasing DHA in PC, but this time only in the DEF group.

A recent study (44) reported that ethanol consumption may increase the rate of biosynthesis of long-chain PUFA, presumably in response to the ethanol-induced peroxidation of, and subsequent lowering of, tissue PUFA concentrations. It is interesting to note that, in the present study, ethanol's effects on PUFA concentrations in PE were usually in the direction of an increase in PUFA (i.e., 22:4n-6 and 22:5n-6 in FB, AA in CB). When the effects of ethanol interacted with those of dietary fatty acid composition, as was the case for DHA in FB PE, ethanol increased DHA in the AD group but had no effect in the DEF group. If ethanol were stimulating the synthesis of long-chain PUFA in this study, one would expect the concentrations of certain PUFA to increase only if there were adequate amounts of the precursor fatty acids. Although there would be adequate levels of n-6 precursors in both the AD and DEF groups in this study, the amounts of n-3 precursors would be expected to be very low in the DEF group, and any stimulatory effect of ethanol on the n-3 series would be expected to be minimal. Therefore, if ethanol can increase biosynthesis of long-chain PUFA to compensate for increased peroxidation of PUFA in the tissues, this mechanism would only be effective in cases where there were adequate amounts of the precursor fatty acids.

Within each of the dietary conditions, there were some dif-

TABLE 6
Forebrain Fatty Acid Composition of Selected Fatty Acids of the Phosphatidylethanolamine Fraction on Postnatal Day 9[a]

	n-3 Adequate			n-3 Deficient			
Fatty acid	Ethanol (n = 11)	Maltose-Dextrin (n = 10)	Suckled Control (n = 8)	Ethanol (n = 15)	Maltose-dextrin (n = 13)	Suckled control (n = 10)	Effects[b]
12:0	0.82	0.46	2.99	2.51	1.37	3.46	
	(±0.21)	(±0.07)	(±0.94)	(±1.08)	(±0.46)	(±1.49)	
14:0	3.16	2.94	4.24	3.95	3.79	5.13	
	(±0.27)	(±0.08)	(±0.73)	(±0.59)	(±0.33)	(±0.92)	
16:0	53.85	53.58	52.64	53.14	52.70	50.97	
	(±0.83)	(±0.33)	(±1.02)	(±1.07)	(±0.47)	(±1.59)	
16:1n-7	3.48	4.00	3.28	3.55	3.71	3.18	E, FFA, FFD
	(±0.16)	(±0.08)	(±0.10)	(±0.11)	(±0.09)	(±0.18)	
16:1n-9	1.74	2.10	1.96	1.82	2.08	1.87	E
	(±0.05)	(±0.05)	(±0.05)	(±0.06)	(±0.03)	(±0.11)	
18:0	5.78	5.32	5.53	5.74	5.64	5.51	E
	(±0.10)	(±0.06)	(±0.06)	(±0.11)	(±0.19)	(±0.10)	
18:1n-9	15.21	15.59	14.28	14.78	14.42	14.09	D
	(±0.51)	(±0.28)	(±0.19)	(±0.27)	(±0.17)	(±0.23)	
18:1n-7	3.84	3.87	3.52	3.78	3.68	3.46	
	(±0.06)	(±0.05)	(±0.10)	(±0.10)	(±0.07)	(±0.13)	
18:2n-6	1.12	1.26	0.91	1.29	1.32	0.92	D, FFA, FFD
	(±0.04)	(±0.07)	(±0.03)	(±0.05)	(±0.05)	(±0.07)	
20:1n-9	0.44	0.46	0.32	0.29	0.29	0.29	
	(±0.07)	(±0.16)	(±0.02)	(±0.05)	(±0.04)	(±0.03)	
20:2n-6	0.36	0.38	0.26	0.29	0.43	0.31	
	(±0.02)	(±0.04)	(±0.01)	(±0.02)	(±0.07)	(±0.06)	
20:3n-6	0.32	0.34	0.33	0.29	0.27	0.20	
	(±0.02)	(±0.03)	(±0.02)	(±0.04)	(±0.01)	(±0.04)	
20:4n-6	5.54	5.86	5.76	5.44	6.18	5.87	E
	(±0.30)	(±0.17)	(±0.24)	(±0.22)	(±0.15)	(±0.29)	
22:4n-6	0.57^x	0.45	0.45	0.40^y	0.59^x	0.56	INT
	(±0.04)	(±0.04)	(±0.02)	(±0.06)	(±0.01)	(±0.03)	
22:5n-6	0.40^x	0.36^x	0.41	0.90^y	1.49^z	1.38	D, E, INT, MF
	(±0.02)	(±0.04)	(±0.13)	(±0.11)	(±0.04)	(±0.08)	
22:6n-3	2.00^x	1.76^x	1.54	0.26^y	0.51^z	0.52	D, INT, MF
	(±0.11)	(±0.07)	(±0.07)	(±0.05)	(±0.02)	(±0.03)	
n-6/n-3	4.23^x	4.98^x	5.36	38.64^y	20.67^x	18.22	D, INT
	(±0.14)	(±0.19)	(±0.27)	(±3.62)	(±0.92)	(±1.03)	

[a]Data expressed as means ± SEM weight percentage of total fatty acids.
[b]For abbreviations see Table 4.

ferences between the concentrations of long-chain PUFA in the SC offspring, which were fed breast milk, and the artificially reared control pups, which received the synthetic formula. These differences may be due to the fatty acid composition of the formula. For example, in the AD condition, breast-fed AD pups exhibited higher percentages both of DHA in FB PE and of AA in CB PE than did the formula-fed pups, suggesting that the AD formula, while adequate in total amounts of both n-6 and n-3 fatty acids, was less than optimal during this stage of development because it did not contain long-chain PUFA. Furthermore, the fact that formula-feeding increased the ratio of fatty acids in DEF pups could be due to the fact that the n-6/n-3 ratio of 116.8 in the deficient formula was at extremely high levels.

There were no differences in body, FB, and CB growth between the suckled DEF and AD pups, supporting previous findings that n-3, unlike n-6, fatty acids are not necessary for normal growth. Furthermore, despite the trend in the data towards lower CB weights in the ethanol-treated animals, effects on CB were not significant, nor was there an effect on body weight. The lack of effect of ethanol on body growth is consistent with other studies on artificially reared rat pups (45,46), and supports the specific nature of ethanol's neuroteratogenic effects when maternal nutrition is not a mediating variable. On the other hand, the lack of effect on CB weight is surprising, particularly since other work has reported that the CB is particularly sensitive to the harmful effects of ethanol during this early postnatal period (47). It should be noted that, in the present study, both FB and CB weights were lower on day 9 in the MD than in the SC pups. This finding is specific to this particular study: in previous studies in our lab (42), and in work published by other labs (47), the CB weight of artificially reared pups raised on a formula did not differ significantly from that of SC pups. However, the pups in the other studies were born to dam-reared rats, not to rats which had themselves been artificially reared. While the suckled pups of the artificially reared dams in this study appeared to have FB and CB weights similar to those reported in the literature, they may have been more susceptible to the

TABLE 7
Cerebellum Fatty Acid Composition of Selected Fatty Acids in the Phosphatidylethanolamine Fraction on Postnatal Day 9[a]

	n-3 Adequate			n-3 Deficient			
Fatty acid	Ethanol (n = 11)	Maltose-Dextrin (n = 10)	Suckled Control (n = 8)	Ethanol (n = 15)	Maltose-dextrin (n = 13)	Suckled control (n = 10)	Effects[b]
12:0	1.08 (±0.46)	2.72 (±1.17)	0.38 (±0.19)	0.93 (±0.39)	2.44 (±0.87)	0.64 (±0.26)	E
14:0	2.88 (±0.46)	3.39 (±0.78)	2.49 (±0.19)	2.75 (±0.49)	3.26 (±0.51)	2.99 (±0.32)	
16:0	43.67 (±1.84)	37.79 (±3.05)	46.70 (±1.14)	42.84 (±1.64)	44.14 (±1.66)	46.56 (±0.80)	FF^A
16:1n-7	2.23 (±0.29)	2.24 (±0.26)	3.73 (±0.34)	2.45 (±0.25)	2.20 (±0.22)	3.41 (±0.31)	FF^A, FF^D
18:0	8.42 (±1.01)	8.81 (±0.99)	7.28 (±0.62)	6.70 (±0.39)	6.95 (±0.81)	8.41 (±0.79)	D
18:1n-9	16.38 (±1.87)	16.52 (±1.23)	18.13 (±0.72)	18.60 (±0.60)	16.67 (±0.66)	17.86 (±0.30)	
18:1n-7	4.07 (±0.48)	4.21 (±0.45)	4.55 (±0.30)	4.49 (±0.24)	4.30 (±0.35)	4.81 (±0.10)	
18:2n-6	1.73 (±0.17)	1.68 (±0.15)	1.38 (±0.06)	2.34 (±0.38)	1.95 (±0.14)	1.13 (±0.06)	
20:1n-9	0.52 (±0.08)	0.77 (±0.15)	0.31 (±0.06)	0.43 (±0.07)	0.44 (±0.09)	0.35 (±0.02)	D, FF^A
20:2n-6	0.51 (±0.09)	0.62 (±0.08)	0.34 (±0.04)	0.65 (±0.14)	0.38 (±0.03)	0.31 (±0.01)	
20:3n-6	0.51 (±0.09)	0.73 (±0.18)	0.60 (±0.07)	0.53 (±0.04)	0.48 (±0.08)	0.33 (±0.03)	
20:4n-6	10.64 (±2.23)	9.79 (±1.17)	7.89 (±0.65)	8.65 (±1.00)	8.23 (±0.72)	6.98 (±0.39)	
22:4n-6	1.24 (±0.12)	1.41 (±0.18)	1.27 (±0.22)	1.62 (±0.23)	1.68 (±0.28)	1.65 (±0.15)	
22:5n-6	0.62 (±0.11)	0.68 (±0.09)	0.44 (±0.04)	2.38 (±0.31)	2.61 (±0.49)	1.70 (±0.07)	D, MF
22:5n-3	0.09 x (±0.03)	0.27y (±0.09)	0.23 (±0.01)	0.00x (±0.00)	0.02x (±0.02)	0.02 (±0.01)	INT, MF
22:6n-3	3.25 (±0.46)	4.12 (±0.51)	2.97 (±0.21)	1.09 (±0.12)	1.31 (±0.21)	0.98 (±0.05)	D, MF
n-6/n-3	5.41 (±1.36)	3.47 (±0.11)	3.72 (±0.12)	15.42 (±0.67)	13.67 (±1.14)	12.22 (±0.51)	D, MF

[a]Data expressed as means ± SEM weight percentage of total fatty acids.
[b]INT, interaction effect, $P < 0.05$. Means of artificially reared groups with different superscripts are significantly different (Tukey's *t*-test, $P < 0.05$). For other abbreviations see Table 5.

stresses associated with artificial rearing. The suggestion that artificial rearing, while not having detrimental effects on reproduction *per se*, may have long-term effects on the vulnerability of subsequent generations warrants further study.

The fact that the BAC did not differ between the DEF and AD groups is not consistent with our previous findings in which the BAC of pregnant mice fed an n-3 deficient diet were significantly higher than those of dams fed a diet with relatively high levels of n-3 PUFA (12). However, it should be kept in mind that the present study was conducted directly in infant rats, in which enzyme function may still be developing. Thus, direct comparison of BAC in the two studies may not be appropriate.

The fact that the effects of ethanol did not occur exclusively or predominantly in the DEF group suggests that n-3 deficiency did not render the developing rat brain more susceptible to the effects of ethanol in this model. When the ethanol treatment interacted with diet, it was often in a direction opposite to that predicted. For example, the effects on DHA in FB PE and on 22:5n-6 in CB PC were seen only in the AD groups. One explanation for the effect on DHA could be a "floor" effect, in which the concentrations of n-3 PUFA in the DEF brains were already so low that ethanol effects were masked. However, it should be noted that the effects on 22:5n-6 were seen only in the AD group even though total amounts of this PUFA were much higher in the DEF group, suggesting that, if a floor effect was present, it was specific to n-3 PUFA.

The changes seen in lipid composition caused by ethanol in this study are smaller than those produced by dietary manipulation, which is consistent with our previous work using ethanol administration in a maternal feeding model in mice (5,12). In other work, we have evaluated the behavioral effects of such dietary treatments and found that the effects, when present, are quite small (27,48,49). Therefore, it seems unlikely that changes in brain membrane lipid composition can be contributing to the

functional concequences that have been associated previously in rats with exposure to ethanol during this period of rapid brain development.

In summary, the findings of this study support previous work in showing that exposure of artificially reared rat pups to high levels of ethanol on postnatal days 5–9 reduces FB weight, but are not consistent with the hypothesis of increased susceptibility to the effects of ethanol in the n-3 deficient pups. This short-term exposure of pups directly to ethanol during the brain growth spurt did lead to some alterations in brain membrane fatty acid composition. However, these effects, compared with those of diet, were small and somewhat inconsistent and are not supportive of a role for alterations in brain membrane lipid composition in the neurobehavioral effects of this regimen of ethanol exposure.

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Column and High-Performance Size Exclusion Chromatography Applications to the *in vivo* Digestibility Study of a Thermoxidized and Polymerized Olive Oil

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ABSTRACT: This study aimed (i) to design an *in vivo* model to study fat digestibility, and (ii) to apply this design to test the *in vivo* digestibility of a highly thermoxidized olive oil. True digestibility of unheated olive oil was tested 2, 4, 6, and 7 h after administering 1 g of olive oil/100 g body weight to young adult Wistar rats by means of esophageal probes. Remaining gastrointestinal lumen fat showed an inversely linear relationship ($r = -0.9932$; $P < 0.001$) with the length of the experiment. A 4-h test was considered adequate because after this period, half of the oil administer still remains in the lumen, making it possible to accurately measure the different nondigested, nonabsorbed thermoxidized compounds. In a second experiment, fresh olive oil (3.6 mg polar content/100 mg oil) was heated at 180°C for 50 h in the presence of air; the polar content in this oil rose to 46.0 mg/100 mg oil. After 4 h, the true digestibility coefficient of 50-h heated olive oil did not significantly change, although it tended to decrease (24%) with respect to the unheated oil. Silica gel column chromatography and high-performance size exclusion chromatography were used to quantify nonthermoxidized and thermoxidized products present in the oils and in the gastrointestinal lumen after these test periods. True digestibility of the different thermoxidized compounds from the heated oil was 30–40%, whereas that of thermoxidized compounds from the fresh oil was much higher (~80%). Nonoxidized triacylglycerol hydrolysis was negatively affected by the presence of large amounts of thermoxidized compounds. The present proposed model seems to be a useful tool for the study of thermoxidized oils. Data also show that thermoxidized compounds from abused olive oil are poorly but actively hydrolyzed and absorbed *in vivo*.

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The current diet of the population of Westernized countries contains substantial quantities of fat that has been subjected to different processing and heat treatments. Heating these fats in the presence of air produces oxidative and thermal degradation in the unsaturated acyl groups of triacylglycerols. These changes modify the nutritional properties of culinary fat and lead to the formation of many different oxidized and polymerized compounds with a higher polarity than that of the original triacylglycerols (1–4). Moreover, oils heated at frying temperature have been used as models for studying the thermoxidation produced in the frying process.

Stapräns *et al.* (5,6) have suggested that oxidized lipids in the diet are absorbed by the small intestine. Sánchez-Muniz *et al.* (7) found that hepatic thiobarbituric acid-reactive substances increased after feeding rats a diet containing thermoxidized sunflower oil used 75 times in frying potatoes.

Digestibility is generally believed to decrease when heated fats are consumed (8), although some authors do not report important changes (for reviews, see 9,10). Triacylglycerols containing at least one oxygenated function are absorbed quite well (11–13). Moreover, it appears that triacylglycerol monohydroperoxides are hydrolyzed by pancreatic lipase to almost the same degree as the original triacylglycerols (11,12). However, data concerning triacylglycerol oligomer digestibility are confusing (14,15).

Studies on altered fat digestibility often use radiolabeled markers, which do not always have the same composition as the altered compounds present in heated or used frying fats. Moreover, these markers are potentially harmful and rather expensive. In the present work, two different but complementary studies were designed. In the first one, the elapsed time required to test for *in vivo* oil hydrolysis was assessed. In the second study, a combination of *in vivo* short-term fat digestibility, column chromatography, and high-performance size-exclusion chromatography (HPSEC) techniques was employed to study the digestibility of 50-h heated olive oil, and of the different altered compounds formed during heating. The possible inhibitory role of some altered compounds, such as oligomers, on fat digestibility of nonthermoxidized triacylglycerols was also tested.

MATERIAL AND METHODS

Materials. Commercial olive oil (Koipe, Andujar, Jaén, Spain) was purchased in a local store and maintained at 15°C in darkness.

Experiment 1 Methods. (i) Animals and maintenance. The protocol used for this experiment was authorized by an Internal

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Abbreviation: HPSEC, high-performance size exclusion chromatography.

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Commission of the Pharmacy Faculty (Facultad de Farmacia) of the Universidad Complutense of Madrid (Spain). Male Wistar rats [Instituto de Nutrición y Bromatología (CSIC), Facultad de Farmacia, Universidad Complutense de Madrid], weighing approximately 200 g each, were used. Groups of animals were housed together in plastic cages under controlled temperature conditions (22.3 ± 1.81°C) and a 12-h light/dark cycle; they were fed standard commercial rat pellets (Panlab S.L., Barcelona, Spain). Food and drinking water were supplied *ad libitum.* The rats were starved the night before the experiment took place, but had free access to water until used. They were given a single dose (1 g/100 g body weight) of raw olive oil by means of an esophageal probe. Control rats were administered isotonic saline solution at the rate of 1 mL/100 g body weight. Experiments were started between 0800 and 0900 to avoid variations due to circadian cycles. After 2, 4, 6, and 7 h exposure to the fat, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt) and euthanized by blood extraction from the descending aorta using a syringe. Afterward, 50 mL of isotonic saline solution was slowly passed from the distal esophagus to the distal ileum in order to obtain the luminal fat.

To determine the fat recovery of the technique, 2 g of fat was added into the lumen of whole intestine fresh biopsies and gently distributed through it. Afterward, 50 mL of saline solution was slowly passed from the proximal duodenum to the distal ileum in order to obtain fat. The percentage recovery obtained ranged between 93 and 101%.

(ii) Fat extraction procedure. Luminal fat was separated from the saline solution using a chloroform/methanol mix according to the method of Bligh and Dyer (16), and then purified using the Folch *et al.* method (17).

(iii) Fat digestibility test. Luminal fat was corrected by taking into account the endogenous fat obtained from control rats and compared to the amount of fat administered.

Experiment 2 methods. (i). Heating procedure. Olive oil (200 mL) olive oil was heated for 50 h at 180°C on a thermostatied plate with free access to atmospheric air.

(ii) Analytical determinations. The polar fraction of the unheated and heated olive oil was evaluated using the column chromatographic method of Waltking and Wessels (18), modifying the proportion of petroleum ether/diethyl ether used to fill the column and to elute the nonpolar fraction. A sample of ≈1 g of olive oil was dissolved in 20 mL petroleum ether/diethyl ether 87:13 (vol/vol) when fresh olive oil was analyzed, and 90:10 (vol/vol) when heated oil was tested (19). The sample was then transferred to a silica gel chromatographic column. A final elution of the column with chloroform/methanol 1:1 (vol/vol) was performed to improve recovery of the sample (20). Two samples each of the unheated and 50-h heated olive oil were analyzed. The separation of nonpolar fractions was checked by thin-layer chromatography on 0.5-mm thick 60 F250 silica gel plates (20 × 20 cm glass). Polar and nonpolar fractions were diluted 50 times (wt/vol) in hexane/diethyl ether 80:20 (vol/vol). Samples were applied as 10 µL spots using a 705 Hamilton microsyringe. Plates were developed with hexane/ethyl ether/acetic acid 80:20:1 (by vol) in a lined tank for *ca.* 25 min (*ca.* 17 cm) and then removed for the solvent to evaporate. Spots were visualized by exposure to iodine vapors.

(iii) HPSEC. Hydrolytic and/or thermoxidative alterations which occurred in the olive oil during heating were analyzed by HPSEC, following the method of Dobarganes *et al.* (2). Isolated polar fractions were studied using a Waters 501 chromatograph (Milford, MA) with a 20-µL sample loop. A Waters 410 refractive index detector and two 300 × 7.5 mm i.d. (5 µm particle size), 0.01 µm and 0.05 µm Hewlett-Packard PLgel (polystyrene-divinylbenzene) columns, connected in series, were operated at 40°C. High-performance liquid chromatography grade tetrahydrofuran served as the mobile phase at a flow of 1 mL/min. The sample concentration was 10–15 mg/mL in tetrahydrofuran. All eluents and samples were precleaned by passage through a 2-µm filter. The quantity of each polar compound was calculated as previously indicated (20).

(iv) Animals and maintenance. The protocol used for this experiment was similar to the one explained in the first experiment, and it also was authorized by an Internal Commission of the Pharmacy Faculty (Facultad de Farmacia) of the Universidad Complutense of Madrid (Spain). Male Wistar rats were studied after 4 h administration of a single dose (1 g/100 g body weight) of raw olive oil or the same amount of heated olive oil by means of an esophageal probe. Control rats were administered isotonic saline solution at the rate of 1 mL/100 g body weight. Experiments were started between 0800 and 0900. After a 4-h exposure to the fat, the rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (60 mg/kg body wt) and euthanized by blood extraction from the descending aorta using a syringe. Afterward, 50 mL of isotonic saline solution was slowly passed from the distal esophagus to the distal ileum in order to obtain the luminal fat.

(v) Fat extraction procedure. After the wash-out with saline solution, luminal fat was separated using a chloroform/methanol mix according to (16) and then purified (17).

(vi) *Fat digestibility test.* The content and composition of luminal fat were corrected by taking into account the endogenous fat obtained from control rats. Purified samples of the luminal fat were transferred to a silica gel chromatographic column, polar and nonpolar fractions were separated, and purity was checked as indicated previously. Highly polar lipids, such as phospholipids, are retained by the silica gel stationary phase and therefore do not elute together with acylglycerols and free fatty acids. Moreover, lecithin (the most abundant bile phospholipid) is poorly soluble in tetrahydrofuran. Therefore, although lecithin has a molecular weight between oxidized triacylglycerols and diacylglycerols and thus might present an HPSEC retention time similar to these compounds, it does not appear in the HPSEC analysis because of these methodological reasons. Polar fractions were studied by means of HPSEC, as mentioned above. The amount of thermoxidized compounds (triacylglycerol polymers and dimers, and oxidized triacylglycerols) and hydrolytic compounds (diacylglycerols, monoacylglycerols, and free fatty acids) was corrected taking into account the amount present in control rats and comparing that with the

amount of polar compounds present in the oil administered to the rats. As cholesterol and bile acids are present in the intestinal tract and have apparent molecular weights, and thus HPSEC retention times, that are similar to those of free fatty acids, the influence of these compounds in HPSEC determinations was tested. High amounts of cholic acid resulted in only minor chromatographic peaks, for which reason the influence of bile acids can be considered inconsequential. However, the addition of cholesterol to the samples increased the peak area related to free fatty acids. Cholesterol content in the luminal fat samples was calculated using Boehringer Mannheim's enzymatic colorimetric method (Mannheim, Germany), and the HPSEC free fatty acid data were corrected.

(vii) Statistical analysis. Data from heated and nonheated olive oil were compared by unpaired Student's t -test.

RESULTS

First experiment. Figure 1 shows the relationship between the recovery ratio of fat administered (%) and the time-length of the experiment (h). According to these results this relationship presents a very significant linear equation ($r = -0.9932$; $P < 0.001$) that is described by:

$$y = 98.380 + 13.200\, x \qquad [1]$$

where y is the percentage of remaining fat and x the time (h) of the experiment.

According to this graphic representation, after 4 h, about 50% of the amount of administered fat is still present in the gastrointestinal lumen, thus 1 g fat is still available to be studied by gas chromatography and HPSEC.

Second experiment. Heating olive oil at 180°C for 50 h produced a notable increase in the content of polar and thermoxidized compounds (polymers and dimers of triacylglycerols and oxidized triacylglycerols) whereas the amount (g/100 mg oil) of hydrolytic compounds (diacylglycerols and free fatty acids) was significantly decreased (Table 1). Luminal fat from animals administered the heated olive oil contained higher amounts of thermoxidized compounds and lower quantities of hydrolytic compounds (Table 1).

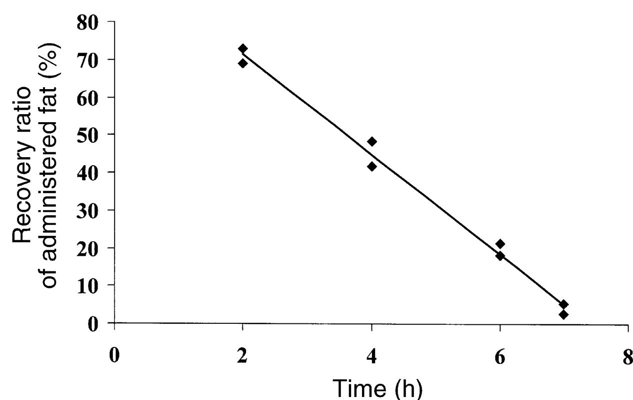

FIG. 1. Relationship between length of the experimental period (h) and luminal fat (recovery ratio of administered fat, %).

Table 2 indicates that in the case of the heated olive oil the percentage of luminal fat after the 4-h experiment tended to increase with respect to the fresh oil. Nonoxidized triacylglycerols, diacylglycerols, and free fatty acids were the major compounds present in the luminal fat after unheated olive oil was administered. However, in the heated oil, nonoxidized triacylglycerols, oxidized triacylglycerols, and polymers and dimers of triacylglycerols were the major compounds present. Thus, luminal fat displayed significantly higher amounts and percentages of triacylglycerol polymers and dimers and oxidized triacylglycerols (all $P < 0.0001$) and total triacylglycerols ($P < 0.05$). Nonoxidized triacylglycerols tended to increase. However, hydrolytic compounds (diacylglycerols, monoacylglycerols and free fatty acids) were significantly reduced in the luminal fat after the 4-h treatment (Tables 1 and 2).

Table 3 indicates the true digestibility coefficient of the whole unheated or heated oils and of thermoxidized compounds. Digestibility of dimers ($P < 0.001$), nonoxidized triacylglycerols ($P < 0.05$), and total triacylglycerols ($P < 0.001$) was significantly reduced with respect to the unused oil. The true digestibility coefficient was calculated using the ratio between injected fat minus remainder lumen fat and the injected fat after endogenous correction.

DISCUSSION

Heating olive oil at frying temperature during 50 h produces detectable changes in the oil composition, leading to the formation of new compounds. In fact the polar fraction of the olive oil increased about 13 times after the heating treatment. This increase is agreement with previous data published in frying oil (21–25) and heated oils (19,26).

According to Yoshida and Alexander (26), two typical groups of products—volatile breakdown derivatives and nonvolatile oxidation products—are produced by oil heating. The latter products include monomeric, dimeric, and polymeric compounds.

According to previous studies (1,20–22,27), HPSEC data show that after repeated frying uses, the amount of thermoxidized compounds clearly increases. In the present study, triacylglycerol polymers that were not detected in the unheated oil rose to be the third major product in the heated oil. Dimers and oxidized triacylglycerols increased in percentage of polar fraction (mg/100 mg polar fraction) and in absolute amount (mg/100 mg oil). This increase, together with the tendency of the number of products of hydrolytic alteration to decrease, confirms that thermoxidative reactions are more prevalent than hydrolytic ones when heating oil.

After the administration of unheated olive oil, the amount of polar components in the luminal fat increased *ca.* 19-fold. The percentage of monoacylglycerols and free fatty acids in the polar fraction increased noticeably, suggesting that after 4 h, active hydrolysis by the pancreatic lipase had taken place. However, data on monoacylglycerol and free fatty acid contents are

TABLE 1
Polar Content and Thermoxidized and Hydrolytic Compounds of Unheated and 50-h Heated Olive Oil, and Their Respective Luminal Fat After 40-h Experiment

	Unheated olive oil		Heated olive oil	
	Administered[a]	Luminal fat[b]	Administered[a]	Luminal fat[b]
Polar content (mg/100 mg oil)	3.6 ± 0.02	51.2 ± 11.4	46.0 ± 0.15	61.2 ± 12.8
Triacylglycerol polymers (mg/100 mg oil or fat)	ND	ND	10.9 ± 0.15	11.5 ± 2.4
Triacylglycerol polymers (mg/100 mg polar fraction)	ND	ND	23.6 ± 0.32	18.8 ± 3.6
Triacylglycerol dimers (mg/100 mg oil or fat)	0.2 ± 0.00	0.2 ± 0.1	11.5 ± 0.07***	11.4 ± 2.1***
Triacylglycerol dimers (mg/100 mg polar fraction)	4.2 ± 0.05	0.4 ± 0.2	24.9 ± 0.15***	18.7 ± 3.4***
Oxidized triacylglycerols (mg/100 mg oil or fat)	0.8 ± 0.03	1.3 ± .0.3	22.8 ± 0.22***	32.0 ± 4.0***
Oxidized triacylglycerols (mg/100 mg polar fraction)	22.6 ± 0.73	2.3 ± 0.5	49.6 ± 0.49***	52.3 ± 6.7***
Diacylglycerols (mg/100 mg oil or fat)	2.2 ± 0.00	22.3 ± 4.4	0.6 ± 0.01***	4.2 ± 1.1***
Diacylglycerols (mg/100 mg polar fraction)	60.6 ± 0.03	43.3 ± 8.2	1.3 ± 0.03***	6.8 ± 1.7***
Monoacylglycerols (mg/100 mg oil or fat)	ND	10.5 ± 2.6	ND	0.6 ± 0.2***
Monoacylglycerols (mg/100 mg polar fraction)	ND	20.5 ± 5.1	ND	0.9 ± 0.3***
Free fatty acids (mg/100 mg oil or fat)	0.5 ± 0.03	16.9 ± 3.7	0.3 ± 0.03*	1.5 ± 0.5***
Free fatty acids (mg/100 mg polar fraction)	12.6 ± 0.72	33.0 ± 8.4	0.6 ± 0.07***	2.5 ± 0.8***

[a]Data are the mean of two oil determinations.
[b]Mean of four animal samples.
*$P < 0.05$; *** $P < 0.001$, respect to the corresponding unheated oil. ND, not detected.

the result of a balance between hydrolysis of altered and nonaltered tri- and diacylglycerols by pancreatic lipase, and intestinal absorption. Polar content of the luminal fat from the heated oil was similar to that obtained from the unheated oil. However, a high proportion of oxidized triacylglycerols triacylglycerol polymers and dimers were still present in this polar fraction. The percentage of diacylglycerols and free fatty acids decreased. Thus, the nonpolar fraction/polar fraction ratio also tended to increase in the luminal fat of rats administered the heated oil.

Reduced digestibility and absorption is observed in all cases in which heated fats have been studied (9,10). In this study, the true digestibility coefficient of the heated olive oil (dimers and total triacylglycerols) decreased with respect to the unheated one, results that concur with previous data of other authors (28,29).

Some decades ago, Crampton *et al.* (30) reported that the principal reason for the lower nutritive value of diets that included linseed oil heated to 275°C was the presence of one or more dimer fatty-acid radicals in this polymerized fat. Deuel (31) considered that the extent to which frying fats were polymerized was one of the principal factors behind their diminished digestibility.

Potteau *et al.* (32,33) likewise reported that as polymerization of oil increased, its digestive utilization declined, as evidenced by elimination of part of the polymers were eliminated in the feces. Márquez-Ruiz *et al.* (34), studying the susceptibility to enzymatic hydrolysis of oxidized and polymeric triacylglycerols formed during frying, found a high hydrolysis rate of oxidized triacylglycerol monomers in contrast to the significant discrimination of pancreatic lipase against triacylglycerol dimers, and particularly triacylglycerol polymers.

According to the unheated olive oil data, true digestibility of triacylglycerol dimers was high (~80%). However, in the altered oil, the true digestibility ratio of dimers decreased noticeably (~30%). Henderson *et al.* (35) reported that in the case of oils containing low amounts (less than 4%) of triacylglycerol polymers, both triacylglycerols and polymers of triacylglycerols were almost completely hydrolyzed by pancreatic lipase after 1 h *in vitro*; but when highly oxidized oils, containing 20 or 30% of triacylglycerol polymers, were used some triacylglycerols re-

TABLE 2
Thermoxidative and Hydrolytic Compounds (g) of Either Unheated or 50-h Heated Olive Oil Administered to Rats and Present in the Luminal Gastrointestinal Fat After 4-h Experiment[a]

	Unheated olive oil		Heated olive oil	
	Administered	Luminal fat	Administered	Luminal fat
Oil	1.995 ± 0.089	1.050 ± 0.335	2.016 ± 0.068	1.310 ± 0.280
Triacylglycerol polymers	ND	ND	0.221 ± 0.007	0.156 ± 0.028
Triacylglycerol dimers	0.003 ± 0.001	0.002 ± 0.001	0.225 ± 0.154***	0.154 ± 0.032***
Total triacylglycerols	1.940 ± 0.086	0.450 ± 0.122	1.526 ± 0.051***	0.943 ± 0.269*
Nonoxidized triacylglycerols	1.924 ± 0.085	0.436 ± 0.118	1.076 ± 0.036	0.509 ± 0.145
Oxidized triacylglycerols	0.016 ± 0.000	0.014 ± 0.001	0.450 ± 0.015	0.434 ± 0.123
Diacylglycerols	0.043 ± 0.002	0.234 ± 0.122	0.040 ± 0.023	0.056 ± 0.023*
Monoacylglycerols	ND	0.110 ± 0.050	ND	0.007 ± 0.008**
Free fatty acids	0.009 ± 0.000	0.254 ± 0.080	0.004 ± 0.001***	0.021 ± 0.029**

[a]Data are the mean ± SD of four studied Wistar rats. *$P < 0.005$; **$P < 0.01$; ***$P < 0.001$, with respect to the same parameter in the unheated oil. ND, not detected.

TABLE 3
True Digestibility (g/g) of Either Unheated and 50-h Heated Olive Oil After 4-h *in vivo* Experiment[a]

	Unheated olive oil	Heated olive oil
Oil	0.474 ± 0.182	0.354 ± 0.137
Triacylglycerol polymers	ND	0.295 ± 0.124
Triacylglycerol dimers	0.780 ± 0.075	0.318 ± 0.140***
Total triacylglycerols	0.768 ± 0.072	0.381 ± 0.179***
Oxidized triacylglycerols	0.142 ± 0.040	0.036 ± 0.006**
Non oxidized triacylglycerols	0.773 ± 0.080	0.527 ± 0.184*

[a]Data are the mean ± SD of four studied Wistar rats. *$P < 0.005$, **$P < 0.01$, ***$P < 0.001$ with respect to the same parameter in the unheated oil. ND, not detected. True digestibility coefficient was calculated using the ratio (injected fat minus remainder lumen fat)/(injected fat), after endogenous correction.

mained intact. Yoshida and Alexander (26) found that dimeric compounds from thermally oxidized oils are absorbed by the rats. However, the cross-linking in the dimers is *via* a C–C bond (36), which is hydrolyzed with more difficulty by rat pancreatic lipase. With monomers, it is possible to hydrolyze readily two out of three of the ester bonds, whereas with the dimers which are larger, because of a number of different chemical entities and C–C linkages, internal ester groups would not be available for hydrolysis. Besides, much of the dimeric structure is more complex than just indicated (37), and intramolecular C–C linkages could form an addition to the intermolecular ones as a result of heating. This would inhibit hydrolysis even more, leading to the lower percentage values with longer heating periods. Yoshida and Alexander (26) found no appreciable enzymatic hydrolysis of polymeric compounds obtained from the oxidized oils. These compounds are rather complicated and more polar, capable of inactivating the enzyme. In the current study a higher proportion of oligomers (polymers and dimers) from the heated oil remain in the rat lumen suggesting hydrolytic inhibition by pancreatic lipase in relation to the unheated oil.

The present results suggest that, in our experimental conditions, hydrolysis of nonoxidized triacylglycerols by pancreatic lipase is inhibited or at least retarded by the presence of altered compounds. These data are in accordance with those of Márquez-Ruiz *et al.* (37) who found that hydrolysis of intact triacylglycerols can be affected by the presence of dimers and polymers in abused frying oils.

After a 4-h test the amount of oxidized triacylglycerols in the luminal fat from the unheated and the heated olive oils did not change significantly with respect to the heated oil. These data are not easy to explain, but suggest a kinetic balance between oxidized triacylglycerol formation from oligomers and hydrolysis to diacylglycerols, monoacylglycerols, and free fatty acids. Matsuchita (11) and Miyashita *et al.* (12) suggested that oxidized triacylglycerols are well absorbed and appear to be adequately hydrolyzed by pancreatic lipase because of the higher polarity and similar molecular weights of oxidized triacylglycerols compared to nonaltered triacylglycerols. As already indicated, luminal fat in the animals administered the heated oil tended to contain a lower percentage and amount of monoacylglycerols and free fatty acids (Table 2). This could be attributed to lower pancreatic lipase activity. However, the possibility of increased absorption of these compounds, because of their major polarity, should be not discarded, although the actual amount of luminal fat increased.

In conclusion, the present experimental design combining column chromatography, HPSEC, and 4-h *in vivo* digestibility seems to be a useful tool for the study of the enzymatic hydrolysis behavior of heated oils. After a 4-h experiment, true digestibility coefficients for triacylglycerol dimers and triacylglycerol monomers were significantly lower than those of the unheated oil. True digestibility of dimers was quite high but decreased as the alteration of the oil increased. Nonoxidized triacylglycerol hydrolysis was negatively affected by the presence of large amounts of thermoxidized compounds.

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Thioglycolate-Elicited Rat Macrophages Exhibit Alterations in Incorporation and Oxidation of Fatty Acids

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ABSTRACT: Incorporation and oxidation of fatty acids (FA) were investigated in resident and thioglycolate-elicited (TG-elicited) rat macrophages (Mϕ). Both cell types presented a time-dependent incorporation of [^{14}C]-labeled palmitic acid (PA), oleic acid (OA), linoleic acid (LA), and arachidonic acid (AA) up to 6 h. The total amount of [^{14}C]-FA incorporated by resident Mϕ after 6 h was: AA > PA = LA > OA. TG-elicited cells presented a 50% reduction in the incorporation of LA, PA, and AA, whereas that of OA remained unchanged as compared to resident Mϕ. The FA were oxidized by resident Mϕ as follows: LA > OA > PA > AA. TG elicitation promoted a reduction of 42% in LA oxidation and a marked increase in AA oxidation (280%). The increased oxidation of AA in TG-elicited cells may account for the lower production of prostaglandins in Mϕ under these conditions. The full significance of these findings for Mϕ function, however, remains to be examined.

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Unstimulated macrophages (Mϕ) are cells that have not encountered foreign materials and have low functional activities, e.g., low rates of secretion of neutral proteinases and/or low production of reactive metabolites of oxygen (1,2). Mϕ obtained from animals that have been submitted to nonmicrobial inflammatory stimuli (which do not stimulate lymphocytes to secrete cytokines), such as exposure to thioglycolate (TG), display increased phagocytic function and plasma membrane turnover, and enhanced activities of lysosomal enzymes (2). Previous studies on phagocytosis, respiratory burst, and fuel metabolism of elicited Mϕ indicated that these effects of TG are caused by changes in glucose, glutamine, and fatty acid (FA) metabolism (2,3). In fact, TG elicitation induces increases in hexokinase and citrate synthase activities, glucose consumption, and $^{14}CO_2$ production from [U-^{14}C]glucose and keeps unchanged the rate of production of lactate compared with resident Mϕ, indicating that elicited Mϕ have an increase in glycolysis and Krebs cycle activities (4).

FA play a key role in modulating cellular metabolism. FA can regulate glucose and glutamine metabolism in both resident and TG-elicited Mϕ (3). Indeed, FA-rich diets (with 20% by weight of hydrogenated coconut oil or unsaturated olive, safflower, or menhaden oil) can lead to changes in phagocytosis, enzyme activities (hexokinase, citrate synthase, glucose-6-phosphate dehydrogenase, and glutaminase), and production of cytokines (interleukin-1 and -6, tumor necrosis factor-α), eicosanoids (prostaglandins PGE_2 and 6-keto-$PGF_{1\alpha}$), and reactive oxygen species (superoxide, hydrogen peroxide, and nitric oxide) in resident and TG-elicited Mϕ (3,5).

FA can also be transferred from Mϕ to lymphocytes in coculture. First, [^{14}C]FA-labeled or triacylglycerol-loaded (TAG) Mϕ can export labeled lipid substances (6,7), [mainly FA themselves and phospholipids (PL) (6), to the supernatant and this process is decreased in TG-elicited Mϕ (6). After the co-culture of lymphocytes with Mϕ pretreated with labeled FA, radioactivity is found in the lipid fractions of the co-cultivated lymphocytes. TG elicitation induces a reduction of the transfer of FA from Mϕ to lymphocytes in culture (6).

In this study, the effect of TG elicitation on incorporation and oxidation of FA by rat peritoneal Mϕ was studied. To investigate the effect of chain length and degree of unsaturation of the FA, the following FA were considered: [U-^{14}C]palmitic (PA, 16:0), [1-^{14}C]oleic (OA, 18:1n-9), [1-^{14}C]linoleic (LA, 18:2n-6), and [1-^{14}C]arachidonic (AA, 20:4n-6) acids. Previous studies examined the incorporation and export of AA by Mϕ (8–10), but a comparative study with other FA has not been carried out yet.

MATERIALS AND METHODS

Animals. The experimental procedure used in this study was approved by the Ethical Committee of the Institute of Biomedical Sciences, University of São Paulo. Male Wistar rats (3–4 mon old, weighing 250 ± 25 g) obtained from the Department of Physiology and Biophysics (University of São Paulo, Brazil), were used. The animals were kept in cages of five rats each and housed under a light–dark cycle of 12/12 h (lights on at 0600) at 23 ± 2°C. The rats were fed *ad libitum* a diet containing 52% carbohydrate, 21% protein, and 4% lipid (NUVILAB CR1, Nuvital Nutrientes LTDA, Curitiba, PR, Brazil) and had free access to tap water.

Chemicals. Lipid solvents were obtained from Merck

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Abbreviations: AA, arachidonic acid; AG, mono- and diacylglycerols; CEST, cholesterol ester; CHOL, cholesterol; FA, fatty acid(s); FABP, fatty acid-binding protein; FCS, fetal calf serum; LA, linoleic acid; MEM, Eagle's minimum essential medium; Mϕ, macrophage(s); OA, oleic acid; PA, palmitic acid; PBS, phosphate-buffered saline; PC, phosphatidylcholine; PL, phospholipid(s); PG, prostaglandins; TAG, triacylglycerol(s); TG, thioglycolate.

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(Darmstadt, Germany). Penicillin (2.5 U/mL) and streptomycin (2.5 µg/mL) were purchased from Sigma (Sigma Chemical Co., St. Louis, MO). Eagle's minimum essential medium (MEM), pH 7.4, supplemented with 10% (by volume) heat-inactivated (56°C for 30 min) fetal calf serum (FCS) was obtained from Adolfo Lutz Institute (São Paulo, São Paulo, Brazil.

[U-^{14}C]PA (specific activity: 824 mCi/mmol), [1-^{14}C]OA (specific activity: 60 mCi/mmol), [1-^{14}C]LA (specific activity: 58.0 mCi/mmol), and [1-^{14}C]AA (specific activity: 55.0 mCi/mmol) were purchased from Amersham (Little Chalfont, Buckinghamshire, United Kingdom).

Preparation of Mϕ. For Mϕ isolation, eight rats per experiment were exsanguinated by decapitation and 20 mL of phosphate-buffered saline (PBS) was injected i.p. Resident Mϕ were collected from the peritoneal cavity using a Pasteur pipette. TG-elicited Mϕ were collected in a similar manner 4 d after an i.p. injection of 3 mL of a 40 g/L sterile Brewer's thioglycolate broth (Merck). Mϕ were centrifuged at 200 × *g* for 10 min. The supernatant fraction was then discarded, and the pellet was resuspended in 30 mL of a hemolysis solution (Tris-base, 0.017 M; and NH_4Cl, 0.0114 M, pH 7.4), with penicillin (2.5 U/mL) and streptomycin (2.5 µg/mL). The tube was then centrifuged after being kept in ice for 5 min. The supernatant was discarded and the pellet was resuspended in 30 mL of a PBS solution. This procedure was carried out three times with PBS. The Mϕ preparation was resuspended in MEM supplemented with FCS. Cells were diluted with culture medium to achieve a final suspension of 0.82×10^6 cells/0.5 mL/well. Mϕ were pre-incubated for 2 h at 37°C in an artificially humidified atmosphere of 5% CO_2 in air, under sterile conditions, in a Microprocessor CO_2 Incubator (LAB-LINE Instruments Inc., Melrose Park, IL). This process is essential to promote the adherence of Mϕ to the plate. After this time, the Mϕ supernatant fraction that was free of adhered cells was discarded. Less than 6% of resident or TG-elicited cells were discarded in the supernatant.

Time-course incorporation of [^{14}C]-labeled FA by Mϕ in culture. Adhered Mϕ were cultivated between 15 min and 6 h in 0.5 mL of MEM containing one of the [^{14}C]-labeled FA. The amount of radioactivity added to the cells was 0.013 µCi/well (0.5 mL) for OA, LA, and AA (due to the similarity in the specific activity), and 0.145 µCi/well for PA, which had the highest specific activity. At the end of each incubation period, the supernatant fluid was discarded and the wells were washed three times with PBS to remove any remaining labeled FA. All cells were scraped from the adhering surface, washed with PBS (3 times, 1 mL each), and transferred to microcentrifuge tubes. The tubes were centrifuged at 15,000 × *g* for 1 min (model Spin-I; INCIBRAS, São Paulo, Brazil). Mϕ were resuspended with 50 µL of ethanol, and then the total radioactivity incorporated was determined in Ecolume scintillation cocktail (ICN Biochemicals, Inc., Costa Mesa, CA) using a Beckman-LS 6000 IC liquid scintillation counter (Beckman Instruments, Fullerton, CA).

Oxidation of FA by Mϕ. Resident and TG-elicited Mϕ were incubated in siliconized Erlenmeyer flasks modified in the following manner: to the inside of a conventional flask was used a glass cylinder that effectively divided the flask into two compartments. To the main portion of the flask (Compartment 1) was added sample, which was incubated at 37°C with 0.82×10^6 Mϕ in a final solution of 2 mL MEM and 1.5% albumin–FA complex (0.1 µCi/mL of labeled FA and 0.9 mM of unlabeled FA). After 6 h the cells were disrupted using 400 µL of 25% perchloric acid solution. The labeled CO_2 was the collected over a period of 1 h in a solution of 1:1 (vol/vol) phenylethylamine/methanol contained in the inner glass cylinder (Compartment 2), and radioactivity was determined in a Beckman counter. A similar procedure has previously been used in our laboratory (11).

Statistical analysis. The results were assessed by using analysis of variance (ANOVA) at a significance level of $P < 0.05$.

RESULTS

Incorporation of PA, OA, LA, and AA by resident and TG-elicited macrophages. The incorporation of [^{14}C]-labeled PA, OA, LA, and AA during 6 h by resident Mϕ presented a time-dependent increase, which was modulated by TG elicitation (Fig. 1). For all FA, the incorporation was linear over a 6 h period. TG elicitation induced a decrease of the incorporation rate, keeping the linearity. The equations of the regression lines and Pearson's coefficients (r^2) are presented in the legend of Figure 1. AA showed the highest incorporation, even under TG elicitation. OA was the least incorporated, and PA and LA showed similar incorporation rates during the time-course, even under TG elicitation. The total radioactivity found in resident Mϕ after 6 h of culture was as follows: AA > PA = LA > OA (AA, 1,173 ± 136; PA, 492 ± 8; LA, 490 ± 7; and OA, 128 ± 13 nmol/10^{10} plated cells, as mean ± SEM, $n = 4$). TG elicitation induced a decrease of 50% in the rate of incorporation of PA, LA, and AA. OA was the only FA to remain unchanged after elicitation.

Oxidation of PA, OA, LA, and AA by resident and TG-elicited Mϕ. These experiments were carried out to evaluate the differences in FA oxidation by Mϕ and the modulation of this process by TG elicitation. The values of FA oxidation are presented in Table 1. During 6 h, resident and TG-elicited Mϕ were incubated in the presence of [^{14}C]-labeled FA, and $^{14}CO_2$ production was measured. For resident Mϕ, the oxidation was as follows: LA > OA > PA > AA (5,795 ± 267, 1,949 ± 263, 892 ± 113, and 442 ± 41 nmol/10^{10} Mϕ/6 h, respectively as mean ± SEM). LA had a reduction of 41.6% and AA an increase of 280% in the rate of oxidation after TG elicitation.

DISCUSSION

Monocyte (blood cells that are the original resident Mϕ) diameters range between 12 and 20 µm (12). Assuming a discoidal cell shape with average diameter of 15 µm and 5 µm height that exchanges FA through the upper and lateral sides but not through the bottom, we obtain an average cell volume of 8.8×10^{-10} cm^3 and average cell membrane exchange area of 4.12×10^{-6} cm^2. Applying data on FA permeability, derived from

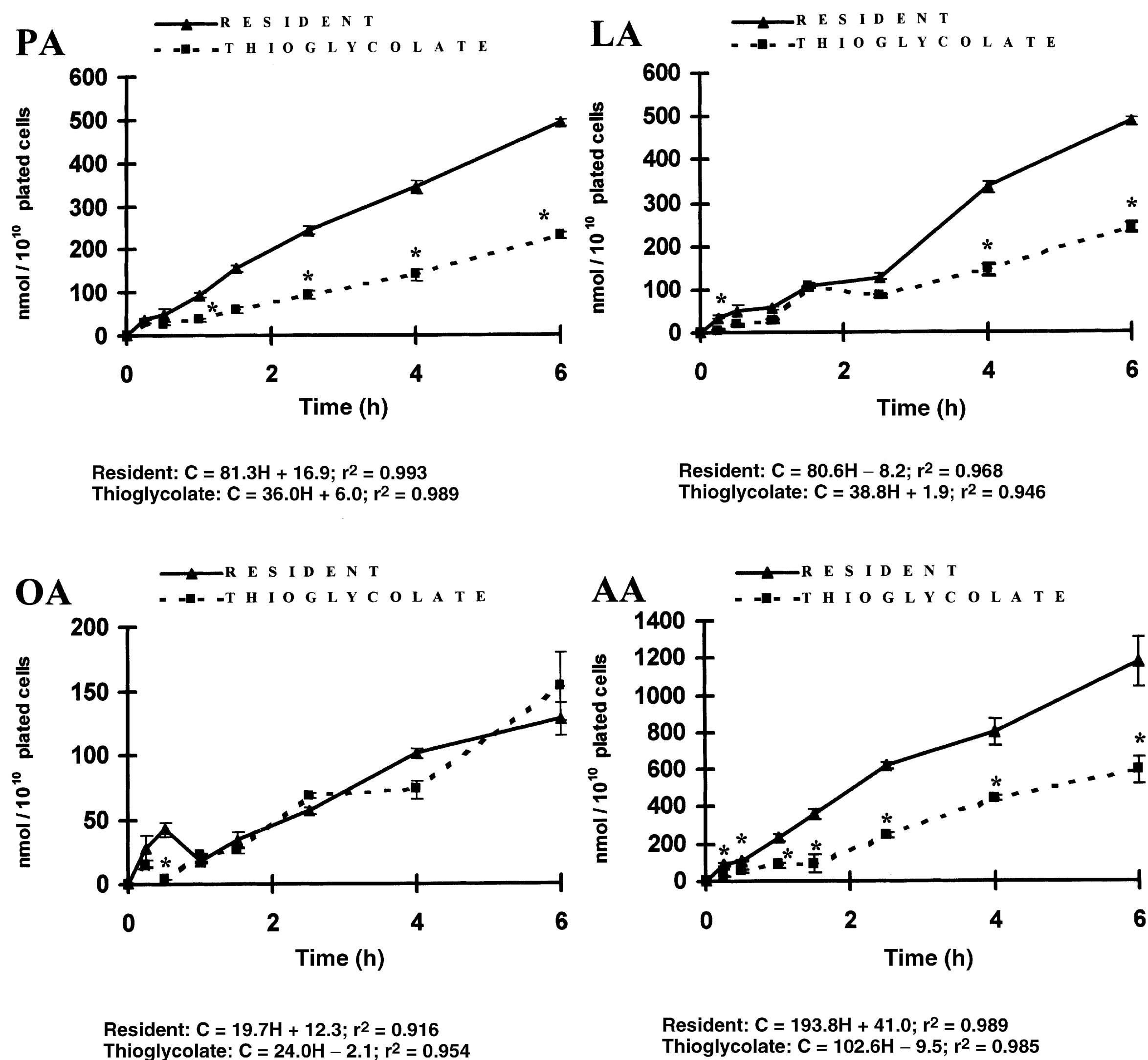

FIG. 1. Time-course incorporation of [U-^{14}C]palmitic acid (PA), [1-^{14}C]oleic acid (OA), [1-^{14}C]linoleic acid (LA), and [1-^{14}C]arachidonic acid (AA) by resident (▲) and thioglycolate-elicited (■) macrophages in culture during 6 h. The results are expressed as nmol/10^{10} plated cells as mean ± SEM of four experiments. Cells were cultivated with labeled fatty acids and after each time, collected and washed with phosphate-buffered saline. Radioactivity was determined as described in Materials and Methods section. Abbreviations C, radioactivity concentration of labeled fatty acids; r^2, Pearsons's coefficient; H, time (h). *, significantly different ($P < 0.05$) owing to thioglycolate elicitation.

vesicular systems (13) (P = 10^{-5} cm/s), to the geometrical parameters of Mϕ cells in our culture conditions, we obtained an "estimated half-life" ($t_{1/2}$) for FA entry of 15 s using Equation 1,

$$t_{1/2} = 0.693 \times \frac{\text{cell volume}}{\text{cell membrane permeability to FA} \times \text{cell membrane area}} \quad [1]$$

where cell volume is the estimated average cell volume, cell membrane permeability is the unmodified bilayer permeability for FA as derived from FA movement across unilamellar vesicles (13), and cell membrane areas is the estimated cell membrane area available for FA exchange (discounting cell bottom). This means that, in the time scale of our [^{14}C]FA uptake protocols (measured in hours), Mϕ can exchange FA many times between the cytosol and the supernatant. The existence of three FA pools can then be postulated with respect to the exchange time of FA: a very fast pool represented by the cell membrane compartment ($t_{1/2}$ = nano- to microseconds), a fast pool represented by the cytosol fluid compartment ($t_{1/2}$ = seconds), and a slow pool that constitutes FA incorporation into cell architecture during several hours. The resolution of our experimental protocol does not allow for obtaining information regarding the very fast and fast pools, as evidenced in Equation 1. Table 2 indicates that the amount of FA-derived radioactivity incorporated into the cell after 6 h is much (one order of magnitude) larger than the amount estimated from the uptake of FA into the free cytosolic compartment. This fact indicates that the radioactivity measured in the cell compartment after 6 h is essentially due to FA molecules that were incorporated and processed by the cell.

As seen in Table 2 and Figure 1, TG elicitation decreased

TABLE 1
$^{14}CO_2$ Production from [U-^{14}C]Palmitic, [1-^{14}C]Oleic, [1-^{14}C]Linoleic, and [1-^{14}C]Arachidonic Acids by Resident and Thioglycolate-Elicited Macrophages Incubated for 6 h[a]

	$^{14}CO_2$ production (nmol/10^{10} macrophages/6 h)			
	Palmitic acid	Oleic acid	Linoleic acid	Arachidonic acid
Resident macrophages	891.6 ± 113.1	1,949.3 ± 262.5	5,795.2 ± 267.0	441.9 ± 41.1
Thioglycolate-elicited macrophages	1,050.2 ± 138.7	2,544.0 ± 200.3	3,382.4 ± 251.9*	1,679.5 ± 141.9*

[a]For details see Materials and Methods Section. Results are expressed as mean ± SEM of seven experiments. *, Significantly different (P < 0.05) owing to thioglycolate elicitation.

TABLE 2
Calculated Estimate and Experimental Values of Radioactivity (dpm) in 6 h from Cultured Macrophages[a]

	Radioactivity (dpm) in total preparation (bath volume = 1 mL)	
Condition/period/pool	PA	OA, LA, and AA
Total cell compartment volume (*a*) (number of cells = 0.82×10^6) × (mean cell volume = 8.8×10^{-10} cm^3) = 7.24×10^{-4} cm^3		
Beginning of incubation, in bath (dpm/cm^3)	(*b*): 6.44×10^5	(c): 5.78×10^4
Estimated at equilibrium: inside total cell cytosolic compartment	(*a*) × (*b*): 466	(*a*) × (*c*): 42
Measured inside total cell compartment after 6 h (after rapidly washing out the bath)	73,794	1,398; 5,175; and 11,747
Measured inside total compartment of thioglycolate-elicited cells after 6 h	33,747	1,635; 2,482; and 5,777

[a]The quantities *(b)* and *(c)* are calculated from the bath concentration of fatty acid in µCi/cm^3. PA, palmitic acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid.

the incorporation of all tested FA into Mϕ except for OA. Since the rate of crossing the cell membrane is not a limiting factor for FA entry in a time scale of hours (as evidenced by the small estimated $t_{1/2}$), thioglycolate might affect the process of incorporation of FA into cell elements such as PL, acylglycerols (AG), cholesterol (CHOL), cholesterol ester (CEST) and TAG rather than their uptake from the medium to the cytosol. As shown previously (6), the incorporation of AA and LA into total lipid fraction of TG-elicited Mϕ is increased by 28 and 67%, respectively, as compared with resident cells. On the other hand, PA and OA incorporation into the total lipid fraction was not different between the two cell types. In both cell types the four FA were incorporated mainly into PL (≥ 62.8%), and most of that was in phosphatidylcholine (PC) (≥ 51.5%). In fact, the distribution of [^{14}C] from the FA into lipid fractions (PL, TAG, CHOL, CEST and AG) was not markedly changed by TG elicitation. The same occurred with respect to the distribution of radioactivity into the PL classes tested: PC, phosphatidylethanolamine, phosphatidic acid, phosphatidylserine, and phosphatidylinositol.

FA oxidation was also measured to explore further the effect of thioglycolate. LA was less incorporated and less oxidized after elicitation. Concerning OA, TG elicitation did not induce significant changes in incorporation and oxidation. However, the decreased incorporation of AA (Fig. 1) occurred concomitantly with a marked increase (280%) in oxidation of this FA. These findings may be implicated in the lower production of PG by TG-elicited as compared to resident cells (1,14,15). In fact, mouse TG-elicited Mϕ produce and secrete lower amounts of PGE_2 than resident Mϕ, even when exposed to zymosan (8).

The reduction in the incorporation of PA by Mϕ due to TG elicitation was not caused by increased oxidation. The reduced incorporation of PA may be a consequence of a higher export of this FA to the medium. In fact, 24-h-cultured Mϕ incorporate [3-^{14}C]pyruvate and [1-^{14}C]acetate into lipid fractions, and these lipid molecules (mostly in the form of PL and FA) are actively exported to the supernatant (16). The same exporting process is observed when resident or TG-elicited Mϕ are labeled with [^{14}C]FA, and the radioactivity exported to supernatant can be transferred to co-cultivated lymphocytes (6). Therefore, the export of lipids to the medium may also play an important role for the findings described, since the radioactivity found in co-cultivated lymphocytes was also changed when these latter cells were obtained from TG-injected rats (6).

It remains controversial how FA can cross PL membranes inward and become an intracellular component. There is evidence that nonionized FA can cross the membrane quickly by the flip-flop mechanism in vesicles (13,17), adipocytes (18), and pancreatic β-cells (19). Some authors have described that fatty acid-binding proteins (FABP) can be isolated from membranes (mFABP) and may improve transmembrane FA transport (20,21). On the other hand, the presence of FABP in the cytoplasm could play a role in intracellular FA transport, but it could also facilitate the inward flux (22,23) and metabolism of FA (24) including oxidation (25). Therefore, further studies should be performed to investigate the effect of TG elicitation on the content of FABP in these cells.

The findings presented herein and those previously published (6) led us to conclude that: (i) AA is the main FA incorporated by Mϕ, (ii) PL is the main lipid fraction of FA incorporation (PC is the major class), (iii) TG elicitation reduces PA, LA, and AA incorporation but does not change OA incorporation; (iv) TG elicitation reduces LA oxidation and raises the oxidation of AA. The significance of these findings for the function of Mϕ during inflammatory and immune responses remains to be elucidated.

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Fatty Acid Δ^9-Desaturation in the *Triatoma infestans* Fat Body: Response to Food and Trehalose Administrations

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ABSTRACT: The effects of food intake and carbohydrate administration on fatty acid Δ^9-desaturation were investigated in isolated microsomes from *Triatoma infestans* fat body. Fifth instar nymphs, which were solely blood-fed just after the molt and then fasted, were used as controls and for determination of optimal assay conditions. Both [1-^{14}C]palmitic and [1-^{14}C]stearic acids in a medium containing ATP, CoA, $MgCl_2$, NADH, NaF, and O_2 were tested. For the control group, optimal conditions were a pH of 6.8–7.2, an incubation temperature of 29°C, and an incubation time of 10 min. Δ^9-Desaturation depended on the presence of reduced pyridine dinucleotides; NADH and NADPH were equally efficient. Stearic acid showed a higher apparent V_{max} than palmitic acid, but the apparent K_m were very similar. When fifth instar nymphs were blood-fed weekly, a marked increase of Δ^9-desaturation was observed for both acids. Higher desaturation activity was also induced by injection of the carbohydrate trehalose into the hemolymph of fasted nymphs. These results indicate that insect Δ^9-desaturation, like the mammalian counterpart, is sensitive to dietary changes and carbohydrate administration.

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The major fatty acid components of insect tissues are usually, as in most other forms of life, the common C_{16} and C_{18} saturated and monounsaturated acids. Insect tissues convert acetate, glucose, and some amino acids to fatty acids, with palmitic acid as the major product. In several studies on whole insects, C_{16} and C_{18} saturated fatty acids were directly desaturated to their monoenoic derivatives (1–4). The oxidative introduction of the *cis*-double bond at the Δ^9 position in the fatty acyl-CoA substrates is catalyzed by stearoyl-CoA desaturase, probably the best-studied fatty acyl desaturase in mammalian and avian systems (5–8). The preferred substrates, palmitoyl- and stearoyl-CoA, are converted into palmitoleoyl- and oleoyl-CoA, respectively (6,7).

Most of the work on Δ^9-desaturation in insects has focused on the characterization of this process. In this regard, Tietz and Stern (9) showed that the fat body of *Locusta migratoria* is able to convert stearic into oleic acid. The desaturase activity resides within the microsomal fraction and has an absolute requirement for oxygen, in common with desaturases from higher animals. More recently, acyl-CoA Δ^9 desaturase activity in *Musca domestica* has been subjected to a detailed study on its properties and variations with insect age, sex, and development (10). The most dramatic difference occurred when the reproductive process was initiated in the female, at which time the activity decreased significantly.

The control mechanism that governs the conversion of saturated to monounsaturated fatty acids is important because the balance of the acids is one of the factors influencing the physical properties of cell membrane phospholipids (11). Thus, many investigators have attempted to elucidate the metabolic regulation of stearoyl-CoA desaturase; these studies, mainly in rats and mice, reveal that this enzyme is modulated by nutritional state (12–18). For example, enzyme activity is increased by a high carbohydrate diet (16) and decreased by extreme starvation (13,14) or administration of dietary polyunsaturated fatty acids (12,18).

To gain information about the regulation of fatty acid Δ^9-desaturation in an insect, we have studied the influence of different diets in *Triatoma infestans*, hematophagous hemipteran (i.e., true bug), the vector of the trypanosomiasis known as Chagas disease. *Triatoma infestans* nymphs ecdyse five times to develop into adults. In each stage, the insects need at least one blood meal for their normal development, metamorphosis, and reproduction. For this reason, to study the properties of Δ^9-desaturation, we used fat body microsomes obtained from fifth instar nymphs that were solely blood-fed 4 d after the molt and then fasted for 21 d (i.e., fasted control nymphs). We also evaluated this reaction when the nymphs were fed weekly and when trehalose was administered after a fast period. Trehalose was chosen for this study because it is the principal sugar in insect hemolymph. We found a strong dependence of fatty acid Δ^9-desaturation on the dietary conditions of the animal.

MATERIALS AND METHODS

Chemicals. [1-^{14}C]Palmitic acid (54 mCi/mmol, 98% radiochemically pure) and [1-^{14}C]stearic acid (57 mCi/mmol, 99% radiochemically pure) were purchased from Amersham Life Science (Amersham, England). Unlabeled fatty acids were

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provided by Nu-Chek-Prep (Elysian, MN). The cofactors used for enzymatic reactions and the trehalose for insect administration were obtained from Sigma Chemical Co. (St. Louis, MO). All chemicals were of analytical grade.

Insects. Nymphs of *T. infestans* were reared on hen blood and kept in our laboratory at 28°C under 70–80% humidity. Four days after the molt to fifth instar, the insects were fed and separated into two groups: one was fasted for 21 d and the other was fed weekly during the same period of time. Experiments were performed with 25-d-old nymphs from both groups.

Fat body microsomes. Insects were anesthetized on ice and fat bodies were removed, immediately rinsed with buffer (ice cold 0.25 M sucrose, 0.15 M KCl, 62 mM potassium phosphate buffer at pH 7.4, 5 mM $MgCl_2$, and 1.4 mM *N*-acetyl-L-cysteine), and then homogenized in this buffer at a rate of 4 mL/g tissue. The homogenization was performed at 4°C in a ground-glass tube by 10 up-and-down hand-driven strokes of a Teflon pestle. The homogenate was centrifuged sequentially at 4°C at 400 × *g* for 5 min, 10,000 × *g* for 20 min, and 110,000 × *g* for 60 min to obtain the microsomal pellet (19). The microsomes obtained were suspended in fresh homogenization buffer and stored at −80°C. Microsomal protein was estimated by the method of Lowry *et al.* (20) utilizing bovine serum albumin as standard.

Fatty acid desaturation assay. Unless otherwise indicated, microsomes were incubated with either 40 μM [1-^{14}C]palmitic or 60 μM [1-^{14}C]stearic acid in a final volume of 400 μL at 30°C . The reaction mixture consisted of 0.25 M sucrose, 0.15 M KCl, 40 mM potassium phosphate buffer at pH 7.4, 1.41 mM *N*-acetyl-L-cysteine, 40 mM NaF, 0.45 mM NADH, 1.3 mM ATP, 60 μM CoA (sodium salt), and 5 mM $MgCl_2$.The reaction was initiated by addition of 0.4 mg microsomal protein, and the mixture was incubated in open tubes in a thermoregulated shaking water-bath. Blank incubations without microsomes were done concurrently to assess the percentage of labeled palmitoleic or oleic acids already present in the original substrates.

Separation and identification of desaturation products. The desaturation reaction was stopped and CoA thioesters were hydrolyzed by addition of 1 mL 10% KOH in ethanol, followed by saponification at 80°C for 60 min under nitrogen. The unsaponifiable fraction was extracted twice with petroleum hydrocarbon (b.p. 30–40°C) and discarded. After acidification with HCl, the fatty acids were extracted three times with petroleum hydrocarbon. The solvent was evaporated under nitrogen, and the free fatty acids were dissolved in methanol/water/acetic acid (85:15:0.2, by vol).

Free fatty acids were then fractionated by reversed-phase high-performance liquid chromatography and identified on the basis of their retention times relative to appropriate standards (21). Separations were performed on an Econosil C_{18}, 10 μm particle size, reversed-phase column (250 × 4.6 mm) (Alltech Associates, Inc., Deerfield, IL) coupled to a guard column (10 × 4 mm) filled with pellicular C_{18} (Alltech); methanol/water/acetic acid (90:10:0.2, by vol) at a flow rate of 1 mL/min was the mobile phase. The column eluate was monitored by an ultraviolet spectrometer at 205 nm. Radioactivity was detected by a Radiomatic Instruments (Tampa, FL) Flo-one/Beta flow-through liquid scintillation counter fitted with a 0.5-mL cell. Ultima Flo-M scintillation cocktail (Packard Instruments, Downers Grove, IL) was used at a rate of 3 mL/min.

Hemolymph collection and carbohydrate estimation. For hemolymph collection, the insect legs were amputated and the insect was placed in a centrifuge tube with the head toward the pointed end and centrifuged at 120 × *g* for 1 min at 4°C. The resulting material was centrifuged at 12,000 × *g* for 2 min at 4°C to remove hemocytes. An aliquot of the hemocyte-free hemolymph was used for the enzymatic-colorimetric determination of glucose using a commercial kit (GOD-PAP; Wiener, Argentina). For the measurement of trehalose, an aliquot of hemocyte-free hemolymph was deproteinized with 5% trichloroacetic acid (22). After removal of denatured protein, the total hemolymph carbohydrates were determined using the anthrone reagent (23) with glucose as standard; from this value, the glucose concentration was subtracted and the data were converted to mg trehalose/mL hemolymph.

Trehalose administration. For trehalose treatment, 23- or 24-d-old nymphs fed once, 4 d after the molt to fifth instar, were injected through the abdominal intersegmental membrane with 100 μg trehalose dissolved in 0.15 M NaCl. The amount of trehalose administered was chosen in order to double its concentration in the hemolymph. At determined post-administration times, the insects were anesthetized on ice, and hemolymph and fat body samples were collected as described above. Control insects were injected with 0.15 M NaCl solution.

Changes in Δ^9-desaturation activity. To examine the effect of food intake patterns on Δ^9-desaturation, fifth instar nymphs were fed on day 4 after the molt and separated into two groups: one kept without food until they were sacrificed (fasted control nymphs) and the other fed weekly (continually fed nymphs). A third group was administered on day 23–24 after the molt with trehalose solution (trehalose nymphs). Δ^9-Desaturation was measured in fat body microsomes obtained from the three nymph groups using the optimal assay conditions established for the fasted group.

Statistical analysis. Data are presented as means ± SD. The significance of the effects of food and trehalose treatment was analyzed by analysis of variance using Instat v. 2.0 (Graph Pad Software, San Diego, CA). Where significant differences between the control fasted group and treatment occurred, a *post-hoc* Tukey's honest significant difference test was performed to identify the differing means.

RESULTS

Δ^9-Desaturation properties. For fat body microsomes obtained from fasted control nymphs and incubated with 0 to 100 μM [1-^{14}C]palmitic or [1-^{14}C]stearic acids, Figure 1 shows the Lineweaver-Burk double-reciprocal plot of the ini-

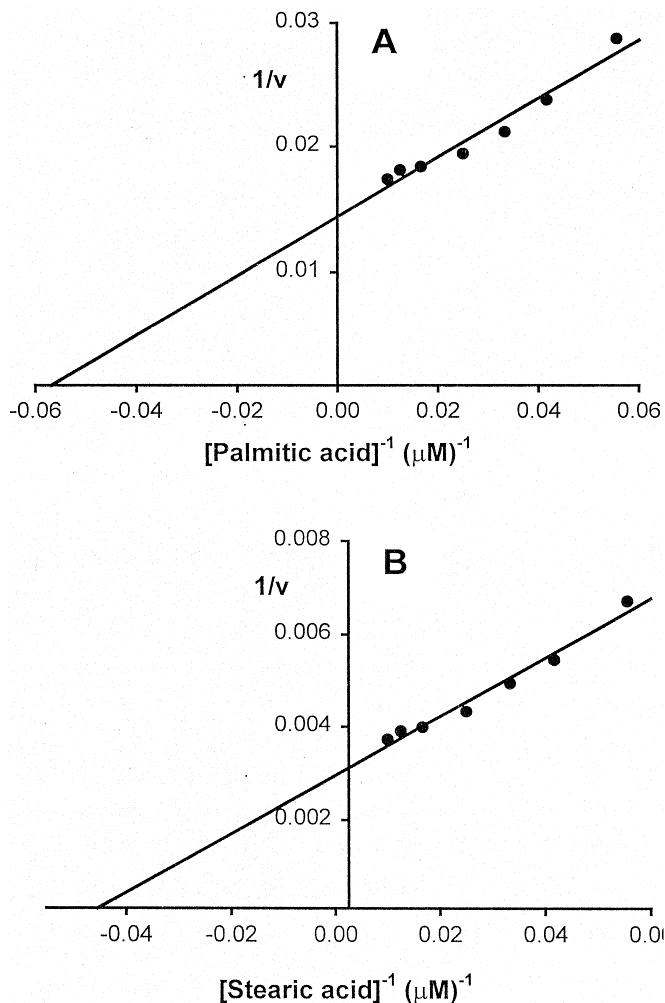

FIG. 1. Double-reciprocal plots of the rate of synthesis of palmitoleic acid and oleic acid as a function of varying concentrations of palmitic and stearic acids, respectively, using fat body microsomes from *Triatoma infestans.* Microsomal protein (400 μg) was incubated with increasing concentrations of either [1-^{14}C]palmitic acid (A) or [1-^{14}C]stearic acid (B). The assay mixture contained 60 μM CoA, 1.3 mM ATP, 0.45 mM NADH, 5 mM $MgCl_2$ in 40 mM potassium phosphate buffer, 0.25 M sucrose, 0.15 M KCl, 1.41 mM *N*-acetyl-L-cysteine, 40 mM NaF, final pH 7.0; final volume of 400 μL. Reactions were performed at 30°C for 10 min. Results are means of duplicate samples from three separate experiments, and the values are expressed as pmol palmitoleic acid formed/min·mg protein (A) or pmol oleic acid formed/min·mg protein.

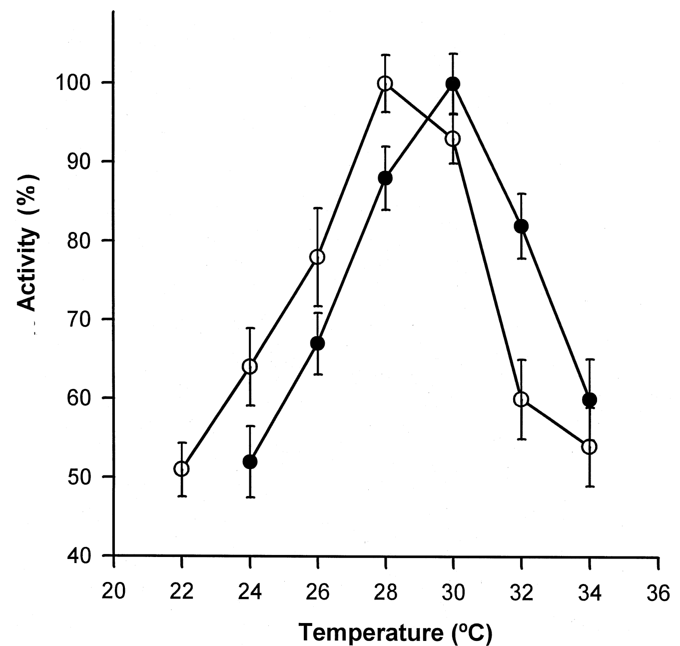

FIG. 2. Effect on fat body microsomal Δ^9-desaturation of incubation temperature. The assay medium contained either 40 μM palmitic acid (●) or 60 μM stearic acid (○) and 1 mg/mL microsomal protein. Reaction mixture and cofactors were as described in Figure 1. Incubation time: 10 min. Results are expressed as percentage of the maximal activities obtained: 65 pmol palmitoleic acid/min·mg protein or 275 pmol oleic acid/min·mg protein. Data represent the mean of triplicate determinations.

tial velocity vs. the substrate concentration. The apparent V_{max} for stearic acid (337 pmoles oleic acid/min·mg protein) was higher than that for palmitic acid (69 pmoles palmitoleic acid/min·mg protein). The effect of pH on the Δ^9-desaturation using either 40 μM palmitic acid or 60 μM stearic acid was investigated every two pH units in the pH range 5.8–8.0 using 40 mM potassium phosphate buffer in the reaction mixture. The optimal pH was about 6.8–7.2 (data not shown) for both acids. In incubation temperature–activity experiments, desaturation activities for palmitic and stearic acids had maxima between 28 and 30°C (Fig. 2). Time-course studies showed a linear increase in the formation of palmitoleic or oleic acids from palmitic and stearic acids, respectively, for the first 10 min of the reaction. Longer incubation times did not increase the amount of products formed (Fig. 3). Both NADH and NADPH were effective electron donors when they were supplied at concentrations of 0.45 mM. The omission of NADH (or NADPH), CoA, and ATP in the reaction mixture resulted in a lack of Δ^9-desaturation.

Trehalose administration. In fifth instar nymphs fed on day 4 after the molt and then fasted for 21 d, the concentrations of trehalose and glucose were 1.74 ± 0.25 and 0.18 ± 0.31 mg/mL hemolymph, respectively. When the fasted insects were injected with trehalose on day 25, trehalose levels increased to 3.57 ± 0.29 mg/mL 5 min after administration. This value was maintained for a further 2 h and then fell rather slowly to reach values (after 12 h) not significantly different from those in the fasted control group. Injection of the 0.15 M NaCl solution used as the vehicle for sugar administration did not significantly affect trehalose levels (1.65 ± 0.15 mg/mL hemolymph). The hemolymph concentration of trehalose in weekly-fed insects on day 25 after the molt was 4.38 ± 0.75 mg/mL.

Changes in Δ^9-desaturation activity. A highly significant ($P < 0.001$) increase in Δ^9-desaturation was observed in the continually fed nymphs when compared with the fasted control group when palmitic (Fig. 4A) or stearic acid (Fig. 4B) was substrate. For the first 12 h, the injection of trehalose had

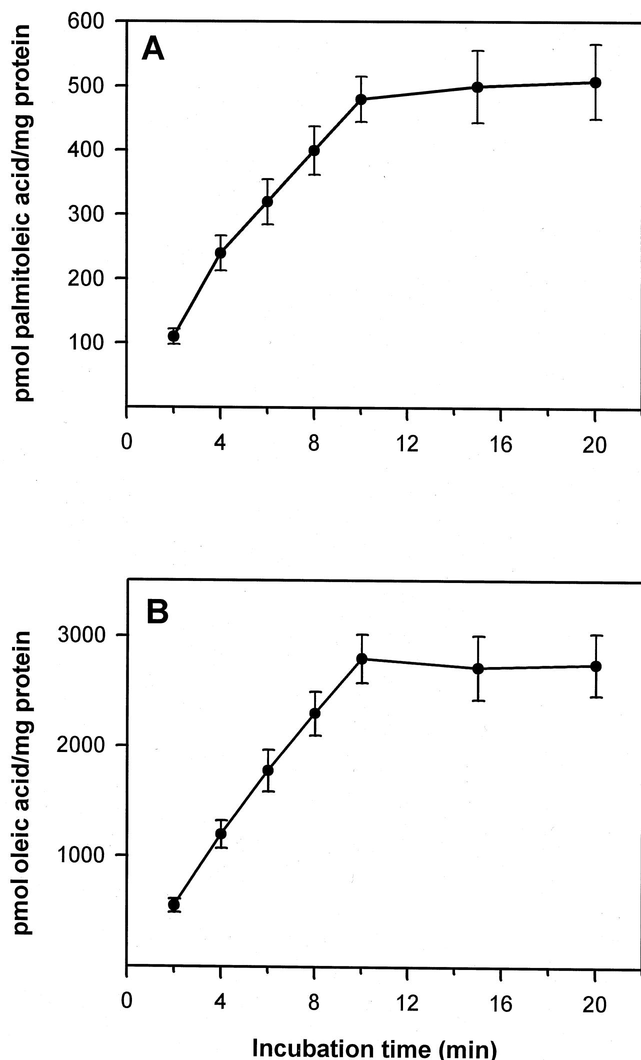

FIG. 3. Time course of fat body microsomal Δ^9-desaturation. The assay medium contained either 40 μM palmitic acid (A) or 60 μM stearic acid (B) and 1 mg/mL microsomal protein. The reactions were carried out at 29°C. Reaction mixture and cofactors were as described in Figure 1. Data represent the mean ± SD of triplicate determinations.

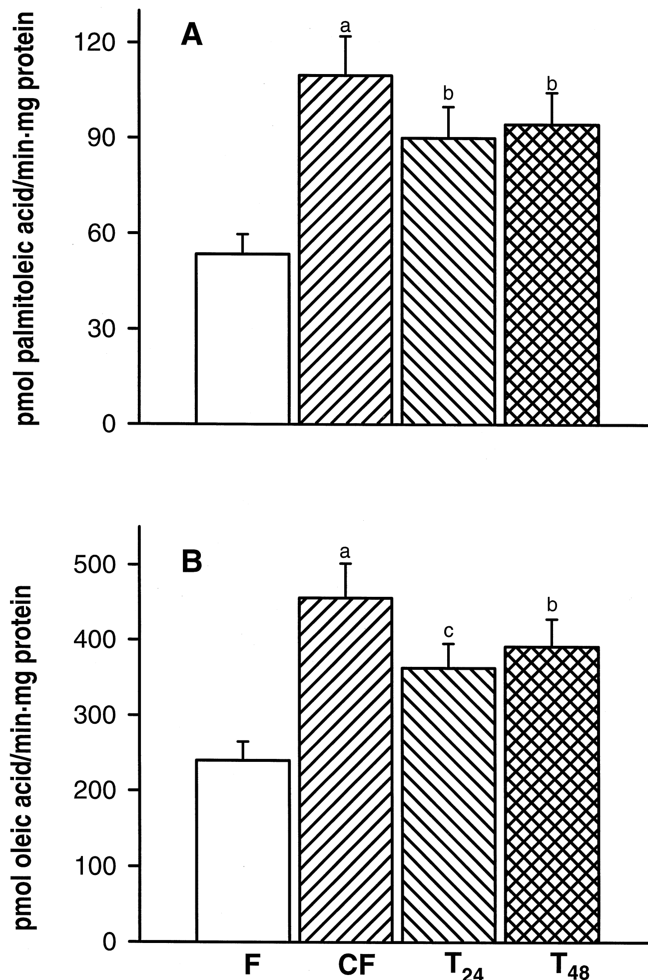

FIG. 4. Response of fatty acid Δ^9-desaturation to insect food intake and trehalose administration: (A) [1-^{14}C]palmitic acid; (B) [1-^{14}C]stearic acid. Reactions were performed under optimal assay conditions as described in the text. F: nymphs that received only one blood meal just after the molt (fasted control group); CF: nymphs continually fed; T_{24}: nymphs injected with trehalose and sacrificed 24 h later; T_{48}: nymphs injected with trehalose and sacrificed 48 h later. The significance of differences between fasted control group and treatments was analyzed by analysis of variance. Columns with different letters are significantly different from fasted control group; $^aP < 0.001$, $^bP < 0.005$, $^cP < 0.02$ as determined by Tukey's range test. Each column with a bar represents the mean ± SD of four samples.

no effect on Δ^9-desaturation (data not shown), but at 24 h very significant ($P < 0.005$) and significant ($P < 0.02$) increases were observed when palmitic (Fig. 4A) and stearic acids (Fig. 4B) were used. Interestingly, this 24-h requirement was at a time when hemolymph trehalose concentrations returned to normal values (see preceding paragraph). Forty-eight hours after trehalose administration, Δ^9-desaturation was similar to that found at 24 h. A fivefold higher trehalose dose slightly modified the Δ^9-desaturation obtained with 100 μg/insect, whereas a tenfold higher dose killed the insects. Insects injected with 0.15 M NaCl solution (used for trehalose administration) did not show any difference in Δ^9-desaturation when compared to the fasted ones. Taken together, the data from the three nymph groups suggest that feeding behavior and carbohydrate administration exert an inductive effect on Δ^9-desaturation of fatty acids.

DISCUSSION

In most organisms, palmitic and stearic acids are synthesized *de novo* from acetate and then converted to the required monounsaturated fatty acids by desaturation. A previous study demonstrated that *T. infestans* is also capable of synthesizing C_{16} and C_{18} saturated and monounsaturated fatty acids *de novo* (3). In addition, when the microsomal fraction from fat body was incubated in an appropriate medium to estimate Δ^9-, Δ^6-, and Δ^5-desaturations, only the first was detectable (19). These observations indicate that *T. infestans* is unable to synthesize linoleic acid (18:2n-6) and convert it to arachidonic (20:4n-6) acid and, therefore, depends upon the host blood

meal for their provision. Therefore, for the control of the saturated-unsaturated fatty acid ratio, the main endogenous regulatory system remaining in *T. infestans* is the biosynthesis of monounsaturated acids by Δ^9-desaturation. This fatty acid ratio plays an important role as a factor determining cellular membrane fluidity and membrane enzymatic activities.

In the present study, we optimized the conditions for Δ^9-desaturation measurement using microsomes from nymphs solely fed just after the molt. The desaturase system in *T. infestans* is quite similar to that found in higher vertebrates (5–8). Under our assay conditions, the Δ^9-desaturase accepted both 16- and 18-carbon saturated fatty acids as substrates. In the housefly, *M. domestica*, the substrate specificity for the Δ^9-desaturase microsomal fraction was 18:0-CoA > 16:0-CoA ≥ 14:0-CoA; and this fraction contained four to five times the desaturase activity found in the mitochondrial fraction (10). *Triatoma infestans* Δ^9-desaturation displayed maximal activity near neutrality (pH range 6.8–7.2), as was observed for *M. domestica* (10). The current study also shows that ATP, CoA, and NADPH or NADH are essential for desaturation of the free acids. Moreover, the replacement of NADPH by NADH resulted in a similar rate of desaturation for the saturated substrates. The Δ^9-desaturase from the locust, *L. migratoria* (9), was active with either NADH or NADPH; NADH is the most efficient source of reducing power for the majority of animal desaturases (24). Interestingly, the desaturase in *M. domestica* (10) prefers NADPH as the electron donor.

Earlier studies, primarily in mice and rats, revealed that Δ^9-desaturase activity was strongly sensitive to changes in diet (12–18) and was decreased by diabetes (25). The most obvious response to dietary changes occurred during the extreme situation of starvation (13,14), when Δ^9-desaturase activity fell to abnormally low levels. Hepatic Δ^9-desaturation in rats increases when a natural diet containing unsaturated triacylglycerides is replaced by a regimen of fasting followed by refeeding a fat-free, high-carbohydrate diet (16,26). The close correlation between diet and Δ^9-desaturation is reflected in the work of Actis Dato *et al.* (27), who showed the existence of a circadian rhythm in its activity and found the maximal activity around midnight, a time coinciding with the maximal food intake of a mouse feeding *ad libitum*. Similarly, the present study demonstrates that Δ^9-desaturation in *T. infestans* fat body is influenced by changes in the insect nutritional state; Δ^9-desaturation increased when the usual single blood meal just after the molt was replaced by a regimen of weekly food intake. The results also demonstrate that when insect fasting is prolonged for 21 d and then is followed by trehalose administration, the injected carbohydrate has disappeared from the hemolymph 12 h later (presumably as a result of rapid body tissues uptake) and the Δ^9-desaturation increase occurs 24 h after administration.

Trehalose was chosen for this study since in most insect species it is the predominant circulating saccharide and plays a central role in carbohydrate metabolism (28,29). Its hemolymph concentration ranges from about 4 to 20 mg/mL, whereas the glucose concentration is generally 10-fold lower. Furthermore, when ^{14}C-glucose is injected into insects, it is rapidly converted into blood trehalose, with the fat body as the principal site of synthesis (30). The lowest levels of hemolymph trehalose occur in coleopteran and hemipteran nymphs (31). The trehalose level in the *T. infestans* fasted nymphs (1.74 mg/mL hemolymph) is similar to that of nymphs of *Oncopeltus fasciatus* (2.5 mg/mL), another hemipteran (31). Hemolymph trehalose levels fall during flight or starvation. The glycogen energy reserve is mobilized from the fat body not as glucose but as trehalose (28–30).

Our study is the first to demonstrate that fatty acid Δ^9-desaturation in insects responds to dietary changes and to carbohydrate administration in a manner typical of higher animals. Early works showed that the activation of rat Δ^9-desaturation produced by carbohydrate feeding was suppressed when protein synthesis was interrupted by injection of actinomycin D (26). The increase in hepatic stearoyl-CoA desaturase activity in response to high carbohydrate diets is paralleled by an increase in stearoyl-CoA desaturase mRNA in total liver mRNA (16,32). This induction is due to an enhancement in the transcription of the stearoyl-CoA desaturase gene (33). The carbohydrate-mediated induction of stearoyl-CoA gene transcription in the fasted mouse can be explained by the stimulation of endogenous insulin release from the pancreas into the bloodstream. As already mentioned, diabetes decreases Δ^9-desaturation in rats and insulin administration restores enzyme levels to normal values (25,34). Furthermore, the insulin-mediated induction of stearoyl-CoA desaturase mRNA is inhibited by cycloheximide administration, indicating that protein synthesis is necessary for this induction (35).

Based on the foregoing reports, we hypothesize that the inductive effect of food and trehalose on *T. infestans* Δ^9-desaturation may result from the secretion of an insulin-like peptide that would be inhibited in the fasted insects. The secretion of this peptide would evoke an increase in Δ^9-desaturase mRNA. We have attempted to evaluate its presence in *T. infestans* hemolymph by an indirect ELISA (enzyme-linked immunosorbent assay) based on the technique of Engwall and Perlmann (36), but with a first antibody of commercial anti-insulin bovine serum. With this technique, we were unable to detect any insulin immunoreactivity in hemolymph samples, at least none greater than 20 pg/μL, the lowest limit of the method using bovine insulin. In spite of our results, the peptide was found in the hemolymph of the tobacco hornworm, *Manduca sexta*, and it modulates trehalose levels when injected into larvae (37). The authors reported that the total amount of immunoreactive insulin was less than that typically found in vertebrate serum. The affinity of mammalian antibodies for the insect peptide is probably less than that for the vertebrate hormone.

The similarity between arthropod and mammalian Δ^9-desaturases is striking in another manner. Recently, Wicker-Thomas *et al.* (38) and Luo *et al.* (39) reported the cloning and partial characterization of *Drosophila melanogaster* (fruit

fly) and *Amblyomma americanum* (tick) Δ^9-desaturase genes, respectively. Interestingly, the deduced amino acid sequence of the arthropod Δ^9-desaturase genes have strong homology with the Δ^9-desaturase gene from the rat (40).

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Lipid Composition of Eggs of an Oviparous Lizard (*Bassiana duperreyi*)

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ABSTRACT: Lipid analysis was performed on freshly ovulated eggs (n = 5) of the oviparous lizard *Bassiana duperreyi*. The fresh weight of the whole egg contents was 132.0 ± 4.3 mg (mean ± SE) of which lipid constituted 21.9 ± 1.1% (w/w). Triacylglycerol formed an exceptionally high proportion (85.4 ± 0.5%, w/w) of the total lipid, whereas phospholipid, free cholesterol, cholesteryl ester, and free fatty acid, respectively, contributed 11.2 ± 0.3, 1.4 ± 0.1, 1.3 ± 0.1, and 0.6 ± 0.1% of the total lipid mass. Linoleic and α-linolenic acids were the major polyunsaturates of the triacylglycerol fraction, respectively, forming 16.3 ± 0.1 and 8.3 ± 0.1% (w/w) of the fatty acids. Linoleic acid was the major fatty acid (29.0 ± 0.1%) of the total phospholipid, which also contained substantial amounts of arachidonic (6.4 ± 0.1%) and eicosapentaenoic (3.0 ± 0.1%) acids, but a relatively low proportion (1.6 ± 0.1%) of docosahexaenoic acid. Phosphatidylcholine formed the major phospholipid class (73.8 ± 2.3% w/w of total phospholipid) and was enriched in linoleic acid, whereas phosphatidylethanolamine, which formed 20.4 ± 1.9% (w/w) of total phospholipid, contained higher proportions of arachidonic and docosahexaenoic acids.

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Lipids are major nutritive components of the amniotic eggs of birds and reptiles, acting as a source of energy and providing substrates for cell membrane biosynthesis during the development of the embryo (1,2). A considerable amount of information is available on the lipid composition of avian eggs, albeit heavily biased by the plethora of detail obtained from eggs of the domestic chicken (3–6). Knowledge of the lipids of reptilian eggs is, by comparison, relatively limited (2). Although the composition and utilization of yolk lipids have been studied in some detail in crocodilians (2,7–13) and, to a lesser extent, in turtles (14,15), such information with regard to squamates (lizards and snakes) is currently limited to a single report (16). Moreover, as this sole example was concerned with eggs of the viviparous lizard, *Sceleropus jarrovi* (16), there is at present a total lack of information on the lipid composition of eggs from oviparous squamates.

In the present study, we describe the lipid and fatty acid profiles of eggs of the Australian scincid lizard *Bassiana duperreyi*, providing the first report of such data for an oviparous squamate.

MATERIALS AND METHODS

Sample collection and lipid analysis. The research was conducted under the University of Sydney Animal Care and Ethics protocol number L04/1-93/3/646. Freshly ovulated and incompletely shelled eggs (n = 5) were dissected from the oviduct of a female *Bassiana duperreyi* (snout–vent length 66 mm) captured on Flinders Island off the north coast of Tasmania (40°17′ S, 147°46′ E) in late October, 1997. Eggs were weighed and then frozen prior to analysis. Whole eggs were homogenized in a suitable excess of chloroform/methanol (2:1, vol/vol) with subsequent washing of the organic phase with KCl (0.88%, wt/vol) (17). The amount of total lipid in the samples was determined gravimetrically: half of the total chloroform extract was placed in a preweighed flask, the solvent was totally removed by evaporation, and the lipid mass was determined using an electronic balance accurate to 0.1 mg. The triacylglycerol (TAG), phospholipid (PL), free cholesterol (FC), cholesteryl ester (CE), and free fatty acid (FFA) components were isolated by thin-layer chromatography on silica gel G using a solvent system of hexane/diethyl ether/formic acid (80:20:1, by vol). Visualization of the bands and elution of the lipid classes from the silica were performed as previously described (12). The amount of FC recovered from the silica was determined using an enzymatic-colorimetric assay kit (Boehringer Mannheim, Lewes, United Kingdom). The isolated PL fraction was further subfractionated by thin-layer chromatography on silica gel D using a solvent system of chloroform/methanol/acetic acid/water (25:15:4:2, by vol). The major PL classes, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and sphingomyelin (Sph), were visualized and eluted from the silica (12).

Gas–liquid chromatography. The isolated acyl-containing lipid and PL classes were transmethylated to form fatty acid methyl esters as described previously (12). Analysis of the fatty acid methyl esters was performed by gas–liquid chromatography using a capillary column (Carbowax, 30 m × 0.25 mm, film thickness 0.25 µm; Alltech, Carnforth, United Kingdom) in a CP9001 Instrument (Chrompack, Middleburg, The Nether-

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Abbreviations: CE, cholesteryl ester; FC, free cholesterol; FFA, free fatty acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin; TAG, triacylglycerol.

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lands) connected to an EZ Chrom Data System (Scientific Software Inc., San Ramon, CA). The column was maintained at 185°C for 2 min following injection of the sample, then increased at 5°C/min for 9 min and maintained at 230°C for a further 24 min. The EZ Chrom Data System enabled the expression of the fatty acid compositions in terms of wt%. Peaks were identified by comparison with the retention times of standard fatty acid methyl ester mixtures (Sigma, Poole, United Kingdom) and were quantified by comparison with a 15:0 standard. The amounts of each lipid and PL class present in the extracts were calculated from the amount and composition of the fatty acyl groups derived from each class, together with the acyl group contribution to the molecular weights of these compounds.

RESULTS AND DISCUSSION

The wet mass of the whole eggs was 132.0 ± 4.3 mg of which 21.9 ± 1.1 % (w/w) was lipid (mean ± SE, $n = 5$). The proportions of the major lipid classes in the eggs of *B. duperreyi* (Table 1) differed markedly from those reported for the yolks of birds, crocodilians, and turtles. TAG formed approximately 85% (w/w) of the total lipid of the eggs of *B. duperreyi* compared with only 69% in yolk lipids of the alligator (2,9) and 68–73% in those of turtles (14,15). The proportion of TAG in the yolk lipid of birds such as the chicken, which is precocial (hatchlings active, capable of independent feeding and thermoregulation), is typically 67–70% (2,5), whereas TAG make a lesser contribution (about 58%) to the yolk lipid of birds such as the pigeon (18), which produces altricial hatchlings (inactive, dependent on parents for food and warmth). On the other hand, only about 11% of the total lipid of the *B. duperreyi* eggs consisted of PL compared with about 20% in the yolks of crocodilians and precocial birds (2,5) and 30% or more in the yolks of altricial birds (18). The proportion of FC was also relatively low in the lizard eggs, forming only 1.4% of total lipid compared with about 5% in the yolks of birds and about 7% in the alligator (2,5). CE, at around 1% of total lipid, was present in the lizard eggs in similar proportions to those found in the yolks of crocodilians and precocial birds (2,5).

Therefore, a major finding of the present work is that the contribution of TAG to the total lipid content of the yolk is greater in eggs of the oviparous lizard *B. duperreyi* than in eggs of birds, crocodilians, and turtles. Since TAG contain three fatty acyl groups, compared with two in PL and one in CE, the lipid of the lizard egg represents a more concentrated source of metabolizable energy than the yolk lipids of the other amniote groups. It should, however, be pointed out this energy difference is relatively modest: 1 mg of yolk lipid contains about 0.88 and 0.94 mg of fatty acid in eggs of the chicken and of *B. duperreyi,* respectively, an increase of only 7%. In birds, the higher proportion of TAG in the yolk lipids of precocial species compared with those of altricial species represents a greater reserve of metabolizable energy to help sustain the embryo through a longer incubation period resulting in a more advanced stage of development by the time of hatching (19,20). The relevance of the high proportion of TAG in the lizard egg in terms of the energetic requirements of the embryo (21,22) is not clear.

The proportions of the lipid classes in eggs of *B. duperreyi* are very similar to those reported for the viviparous lizard *S. jarrovi* (16) where TAG formed 87% and PL 9% of total egg lipid. It should, however, be noted that gestation in *S. jarrovi* utilizes a simple placental structure (Type I chorioallantoic placenta) involving respiratory exchange and the transport of water and inorganic ions but lacking the ability to deliver organic nutrients to the embryo (23). The absence of placentotrophy with regard to lipids in *S. jarrovi* implies that all the lipid components required for embryonic development must be derived from the egg, as is the case for the oviparous *B. duperreyi*. Comparative data on the lipid class profiles of the eggs of highly placentotrophic viviparous lizards are currently unavailable.

The major saturated fatty acid present in the lipid classes of the *B. duperreyi* eggs was palmitic acid (16:0) (Table 1). The monounsaturated fatty acid, oleic acid (18:1n-9) was the major acyl component of the TAG, CE, and FFA fractions. With regard to the polyunsaturates, linoleic acid (18:2n-6) was present

TABLE 1
Fatty Acid Compositions of the Major Lipid Classes of Eggs of *Bassiana duperreyi*[a]

Lipid class	Triacylglycerol	Phospholipid	Cholesteryl ester	Free fatty acid
% (w/w) of total lipid[b]	85.4 ± 0.5	11.2 ± 0.3	1.3 ± 0.1	0.6 ± 0.1
Fatty acid				
14:0	2.0 ± 0.1	ND[c]	ND	2.0 ± 0.6
16:0	17.5 ± 0.1	26.0 ± 0.2	9.7 ± 0.7	18.4 ± 1.3
16:1n-7	2.2 ± 0.1	0.7 ± 0.1	2.2 ± 0.2	1.4 ± 0.4
18:0	5.9 ± 0.1	4.5 ± 0.2	5.5 ± 0.6	9.6 ± 1.2
18:1n-6	42.5 ± 0.1	19.7 ± 0.1	53.1 ± 1.4	28.1 ± 1.7
18:2n-6	16.3 ± 0.1	29.0 ± 0.1	16.7 ± 0.9	14.0 ± 1.0
18:3n-3	8.3 ± 0.1	4.2 ± 0.1	4.9 ± 0.3	16.3 ± 3.6
20:4n-6	1.1 ± 0.1	6.4 ± 0.1	3.3 ± 0.4	1.3 ± 0.4
20:5n-3	0.8 ± 0.1	3.0 ± 0.1	ND	1.5 ± 0.4
22:5n-3	0.3 ± 0.1	0.4 ± 0.1	ND	ND
22:6n-3	0.6 ± 0.1	1.6 ± 0.1	ND	ND

[a]wt% of fatty acids (means ± SE, $n = 5$).
[b]Free cholesterol formed 1.4 ± 0.1% (w/w) of total lipid.
[c]ND, not detected (less than 0.1%).

TABLE 2
Fatty Acid Compositions of the PL Classes of Eggs of *Bassiana duperreyi*[a]

PL class	PC	PE	PS	Sph
% (w/w) of total PL	73.8 ± 2.3	20.4 ± 1.9	3.2 ± 0.9	2.6 ± 0.4
Fatty acid				
16:0	29.1 ± 0.9	8.3 ± 0.3	8.6 ± 0.6	17.8 ± 2.1
16:1n-7	0.8 ± 0.1	0.8 ± 0.3	1.0 ± 0.4	ND
18:0	3.7 ± 0.1	9.2 ± 0.3	22.4 ± 1.4	17.7 ± 1.0
18:1n-9	18.6 ± 0.2	30.1 ± 0.3	20.5 ± 1.5	12.3 ± 2.7
18:2n-6	30.1 ± 0.3	21.7 ± 0.4	13.3 ± 0.8	11.4 ± 0.3
18:3n-3	4.0 ± 0.1	5.4 ± 0.4	11.1 ± 4.1	9.1 ± 3.2
20:0	ND	ND	ND	14.3 ± 1.5
20:4n-6	5.0 ± 0.1	12.3 ± 0.4	14.1 ± 1.9	ND
20:5n-3	2.7 ± 0.1	2.9 ± 0.7	ND	ND
22:5n-3	0.3 ± 0.0	0.8 ± 0.1	ND	ND
22:6n-3	1.2 ± 0.1	3.4 ± 0.2	ND	2.0 ± 1.2

[a]wt% of fatty acids (means ± SE, $n = 5$). PL, phospholipid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; Sph, sphingomyelin. For other abbreviations see Table 1.

at high concentrations in all the lipid classes and was the major component of the PL fraction. Significant proportions of α-linolenic acid (18:3n-3) were also present in all the lipid classes. The profile of the $C_{20–22}$ polyunsaturates in the egg lipids of *B. duperreyi* differed from the pattern normally found in the yolks of crocodilians and domestic poultry. The proportion of arachidonic acid (20:4n-6) in the PL fraction was similar to the values reported for eggs of the domestic chicken but only about half that of alligator eggs (2,5). A notable feature was the very low proportion of docosahexaenoic acid (22:6n-3) in the PL of the lizard eggs, only 1.6% compared with about 5% in the chicken and 10% in the alligator (2,5). Since adequate levels of 22:6n-3 are essential for the differentiation of the neural tissues (24), the embryos of *B. duperreyi* must have some means of optimizing the provision of this fatty acid during development. It may be significant that, in contrast to the chicken and the alligator, the PL of the lizard eggs contained a relatively high proportion of eicosapentaenoic acid (20:5n-3) plus a lesser amount of docosapentaenoic acid (22:5n-3), both of which are potential precursors of docosahexaenoic acid and may therefore possibly be converted to 22:6n-3 by desaturation/elongation reactions in the yolk sac membrane or the embryonic tissues.

The small Australian scincid lizards are generally insectivorous (25,26). The polyunsaturate profile of the egg lipids of *B. duperreyi* may reflect the fatty acid composition of the insects eaten by the female lizard during the period of oocyte maturation. Although we were unable to collect samples of the relevant insects for analysis, the fatty acid profile of the lizard eggs was similar in some respects to the reported compositions of certain terrestrial invertebrates. The lipids of slugs and snails, for example, are rich in 20:4n-6 and 20:5n-3 but are deficient in 22:6n-3 (27).

PC was the major class of PL in the lizard eggs although a considerable proportion of PE and much lower proportions of PS and Sph were also present (Table 2). PC was characterized by high proportions of 16:0 and 18:2n-6 with relatively low proportions of the $C_{20–22}$ polyunsaturates. PE was rich in 18:1n-9 and and contained higher proportions of the $C_{20–22}$ polyunsaturates than did PC. The major saturated fatty acid of PS was stearic acid (18:0), and a high proportion of 20:4n-6 was also present. Sph contained primarily the saturated fatty acids 16:0, 18:0, and 20:0.

In summary, the egg lipids of *B. duperreyi* exhibit several distinct features, differing from those of birds and crocodilians both in the proportions of the lipid classes and in their fatty acid profiles. Since this species is oviparous, these lipids must provide the embryo with all the essential fatty acids required for membrane biogenesis, eicosanoid synthesis, and specific tissue differentiation as well as the requisite amount of fatty acids for energy production. It should be emphasized that the present study was based on a limited number of eggs collected from a single site in a single year. The possibility of geographical and annual variations in the fatty acid composition of yolk lipid as a result of dietary and climatic differences cannot be excluded.

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Competition Between Li^+ and Mg^{2+} for Red Blood Cell Membrane Phospholipids: A ^{31}P, ^7Li, and ^6Li Nuclear Magnetic Resonance Study

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ABSTRACT: The mode of action of the lithium ion (Li^+) in the treatment of manic depression or bipolar illness is still under investigation, although this inorganic drug has been in clinical use for 50 yr. Several research reports have provided evidence for Li^+/Mg^{2+} competition in biomolecules. We carried out this study to characterize the interactions of Li^+ and Mg^{2+} with red blood cell (RBC) membrane components to see whether Li^+/Mg^{2+} competition occurs. ^{31}P nuclear magnetic resonance chemical shift measurements of the phospholipids extracted from the RBC membranes indicated that the anionic phospholipids, phosphatidylserine and phosphatidylinositol, bind Li^+ and Mg^{2+} most strongly. From ^6Li relaxation measurements, the Li^+ binding constant to the phospholipid extract was found to be $45 \pm 5\ M^{-1}$. Thus, these studies showed that the phospholipids play a major role in metal ion binding. ^7Li spin-lattice relaxation measurements conducted on unsealed and cytoskeleton-depleted RBC membrane in the presence of magnesium indicated that the removal of the cytoskeleton increases lithium binding to the more exposed anionic phospholipids ($357 \pm 24\ M^{-1}$) when compared to lithium binding in the unsealed RBC membrane ($221 \pm 21\ M^{-1}$). Therefore, it can be seen that the cytoskeleton does not play a major role in Li^+ binding or in Li^+/Mg^{2+} competition.

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To test the Li^+/Mg^{2+} competition hypothesis for the pharmacological action of lithium, investigators have used small biomolecules or biomembranes as model systems (1–3). In the human body, blood transports Li^+ to the central nervous system; therefore, it is important to understand the interaction of Li^+ with the red blood cells (RBC) and their components. Rong *et al*. (3), using ^7Li nuclear magnetic resonance (NMR) relaxation measurements (T_1 and T_2), observed that the interactions of Li^+ with the RBC components ATP, 2,3-bisphosphoglycerate, spectrin, and Hb in different oxygenation forms were very weak. The RBC membrane did provide, however, most of the high-affinity Li^+ binding sites. The erythrocyte membrane displays very high mechanical stability and resilience, which comes from a partnership between the plasma membrane and an underlying meshwork called the membrane cytoskeleton. The major constituent of the membrane cytoskeleton that provides the infrastructure is spectrin, which binds indirectly to the RBC membrane *via* interactions with protein 4.1 and ankyrin (2).

In previous NMR studies based on Li^+ binding to agar gels (2) or on Na^+ binding to human RBC membranes measured by double-quantum experiments (4), investigators speculated that the cytoskeletal proteins provide binding sites for alkali metal ions in human RBC membranes. An NMR study with purified spectrin, however, did not show evidence of Li^+ binding to the major component of the cytoskeleton (3). In this study, we tested the contribution of the spectrin–actin network toward Li^+/Mg^{2+} competition by conducting ^7Li NMR relaxation measurements with unsealed and cytoskeleton-depleted RBC membrane suspensions.

The major class of lipids present in the RBC membrane is that of phospholipids. The most common in the RBC membrane are phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and sphingomyelin (SM). It is interesting to note that both of the anionic phospholipids, PI and PS, are found only in the inner leaflet of the RBC membrane (5,6). The intrinsic binding constants for interactions between some alkali and alkaline earth metal ions and PS have been reported (7). Evidence for Li^+ interactions with PS-containing liposomes was previously obtained from ^7Li and ^2H NMR relaxation data (8–10). Measurements conducted on inside-out and right-side-out vesicle suspensions clearly indicated that the inner leaflet of the RBC membrane provided the major Li^+ binding site (3). Significant differences between T_1 and T_2 values were also observed for suspensions of phospholipids extracted from the RBC membrane, suggesting that phospholipids, and not the proteins in or anchored to the membrane, provided the major Li^+ binding sites (3). Therefore, it is believed that the anionic

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Abbreviations: AA, atomic absorption; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEPLAS, plasmalogen PE; PI, phosphatidylinositol; PS, phosphatidylserine; RBC, red blood cell; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; SM, sphingomyelin; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time.

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phospholipids PS and PI, present in the inner leaflet of the RBC membrane, contribute to Li^+ binding.

Merchant and Glonek (11) used ^{31}P NMR spectroscopy to determine the relative interaction potentials of each of the phospholipids for various cations, which was determined by ^{31}P NMR chemical shift (δ) changes, signal broadening, signal quenching, or a combination of these. One of the useful features of NMR spectroscopy is that complex mixtures can be analyzed directly (11). Thus, for example, in examination of the spectrum of rat heart membrane phospholipids, all of the common phospholipids can be analyzed qualitatively and quantitatively in a single sample after extraction, without the need for a separation. Mota de Freitas *et al.* (12) reported an increase in the ^{31}P NMR chemical shift anisotropy parameter for increasing concentrations of either Li^+ or Mg^{2+} in the presence of human RBC membrane. These results suggested metal ion binding to the phosphate head groups in the membrane; this study (12), however, did not identify which phospholipids were predominantly involved in the Li^+/Mg^{2+} competition mechanism. This is because the ^{31}P NMR spectrum of the human RBC membranes is not resolved into resonances corresponding to specific phospholipids without extraction of phospholipids with a suitable organic solvent mixture. We have utilized the method of Merchant and Glonek (11) to test Li^+/Mg^{2+} competition for human RBC membrane phospholipids, and also to identify the phospholipids in the RBC membrane that interact most strongly with Li^+ and Mg^{2+} ions.

Investigating Li^+ binding to RBC anionic phospholipids is important because these interactions may affect the extent of lipid–protein interactions in the RBC membrane. The Na^+-Na^+ exchange protein, which mediates RBC Na^+-Li^+ countertransport (13), is a membrane protein. It is therefore possible that variable Li^+ binding to different phospholipids may lead to different interactions between anionic phospholipids and the membrane-bound Na^+-Na^+ exchange protein. These differences in phospholipid–protein interactions may be responsible for the variations in RBC Na^+-Li^+ countertransport rates reported for bipolar (14,15) and hypertensive (16–18) patients relative to normal individuals.

MATERIALS AND METHODS

Materials. Human RBC were obtained from a blood bank (Life Source, Glenview, IL) and were used prior to the expiration date. Inorganic salts and $CDCl_3$ were purchased from Aldrich Chemical Co. (Milwaukee, WI). Pure phospholipids were obtained from Sigma Chemical Co. (St. Louis, MO) or Avanti Polar Lipids (Alabaster, AL). D_2O was purchased from Cambridge Isotopes (Cambridge, MA). The dye reagent used for protein determination was from Bio-Rad Laboratories (Hercules, CA), and items used for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using the Phast system were purchased from Pharmacia Biotech (Uppsala, Sweden). ^6LiCl (95.7% isotope-enriched) was obtained from Oak Ridge National Laboratory (Oak Ridge, TN). All remaining chemicals were purchased from Sigma.

Preparation of unsealed RBC membranes. RBC were washed once in an isotonic choline wash solution and then lysed by 5 mM HEPES buffer, pH 8, as described previously (12,19,20). The membranes were stored at −20°C for no more than a week before they were used.

Preparation of cytoskeleton-depleted RBC membranes. Cytoskeleton-depleted unsealed RBC membranes were obtained by a procedure outlined by Fairbanks *et al.* (19), with a low ionic strength buffer. The supernatant was removed after centrifugation and checked for the presence of spectrin and actin, the major cytoskeletal proteins, by SDS-PAGE for confirmation of the removal of the cytoskeleton (4). We washed the pellet three to four times with 5 mM HEPES buffer, pH 8, to obtain cytoskeleton-depleted RBC membranes.

Extraction of phospholipids from human RBC membranes. RBC membranes (1–1.5 mL, membrane protein concentration 5.2 ± 0.4 mg/mL) were extracted using a procedure reported by Meneses and Glonek (21). The dried lipid film was suspended in a solvent mixture of chloroform/methanol/aqueous 0.2 M EDTA reagent at a ratio of 125:8:3 for ^{31}P NMR experiments. For Li^+ relaxation measurements, the extracted dried phospholipid film was dissolved in a chloroform/methanol solvent mixture at a ratio of 5:2. The aqueous EDTA phase removed the paramagnetic impurities which would otherwise have caused the ^{31}P NMR resonances to broaden; this would have interfered with the resolution of the various classes of phospholipids (22).

The ^{31}P NMR chemical shift values of the individual phospholipids are dependent on the amounts of water and methanol in the chloroform layer of the sample. Overlapping ^{31}P NMR resonances in a specific solvent system can be resolved by variation of the solvent composition (22). For all ^{31}P NMR experiments, the solvent composition was kept constant in order to prevent changes in the chemical shifts of the phospholipids associated with solvent effects (23,24). For all experiments described herein, concentrated stock solution (1M) of the metal ions was used, and the amount of water added to the sample was less than 1%. However, it should be noted that in these types of experiments, solvent effects upon the chemical shift cannot be prevented, especially for PS, which is more sensitive to solvent effects (23–25). The NMR sample tube was sealed to prevent changes in the solvent composition due to evaporation of such low-boiling solvents as methanol and chloroform. Instead of carrying out the NMR experiments at 37°C, we performed all measurements with phospholipids at 27°C to minimize the evaporation of the low-boiling solvents. However, prior to metal ion addition, as much of the aqueous EDTA phase was removed as possible to prevent EDTA chelation of the added metal ions. Before metal ion titration, ^{31}P NMR spectra were recorded before and after the removal of the aqueous EDTA layer, and we found no significant changes in δ values after the removal of the aqueous phase.

Phospholipid–metal ion titration. Prior to metal ion addition, we recorded a ^{31}P NMR spectrum of the sample by ad-

justing the spinning turbine so that only the organic phase of the sample was exposed to the receiver coil of the NMR spectrometer. The EDTA phase was then removed, and appropriate amounts of 1 M $MgCl_2$ or 1 M LiCl solution were added directly to the organic phase (11). The sample was mixed thoroughly, and the spectrum was recorded with the organic phase in the NMR window. Addition of 3 mM $MgCl_2$ or 20 mM LiCl to the phospholipid suspension caused precipitation. Metal ion titration was stopped when precipitation occurred or when one of the signals quenched.

Calculation of Li^+ and Mg^{2+} binding constants from ^7Li or ^6Li NMR T_1 measurements. The calculation of the apparent binding constants, K_{app}, of Li^+ to RBC membrane and phospholipid extracts from ^{7}Li or ^{6}Li T_1 measurements assumed a two-state (*f,* free; and *b,* bound metal ions) model undergoing fast exchange and a total Li^+ concentration, $[Li^+]_t$, that is large with respect to the binding site concentration, [B] (26,27):

$$\Delta R^{-1} = (R_{obs} - R_f)^{-1} = K_{app}^{-1}\{[B](R_b - R_f)\}^{-1} + [Li^+]_t\{[B](R_b - R_f)\}^{-1} \quad [1]$$

where R_{obs}, R_f, and R_b are the reciprocals of T_{1obs}, T_{1f}, and T_{1b}, respectively. K_{Mg} and K_{Li}, the binding constants of Mg^{2+} and Li^+ to biological membranes and phospholipids, were calculated from K_{app} values, which were in turn determined from ^{7}Li or ^{6}Li T_1 values measured in the presence of increasing Mg^{2+} concentrations:

$$1/K_{app} = 1/K_{Li}\,(1 + K_{Mg}\,[Mg^{2+}]) \quad [2]$$

These equations assume 1:1 stoichiometry for Li^+ and Mg^{2+} binding to sites in biomembranes and phospholipids.

Calculation of Mg^{2+} and Li^+ binding constants from ^{31}P NMR spectroscopy. The Mg^{2+}-binding constants, K_{Mg}, to individual phospholipids were calculated from the chemical shift changes, $\Delta\delta$, observed in Mg^{2+}-containing suspensions (no Li^+) of phospholipids extracted from human RBC membranes according to the following equation:

$$1/\Delta\delta = 1/(\delta_{obs} - \delta_{free}) = 1/\{K_{Mg}[Mg^{2+}](\delta_{bound} - \delta_{free})\} + 1/(\delta_{bound} - \delta_{free}) \quad [3]$$

where δ_{obs}, δ_{free}, and δ_{bound} are, respectively, the chemical shifts of the observed, Mg^{2+}-free, and Mg^{2+}-saturated ^{31}P NMR resonances of the individual phospholipids. From a linear plot of $1/\Delta\delta$ vs. $[Mg^{2+}]$, K_{Mg} can be determined by taking the ratio of the slope to the *y*-intercept. The Li^+ binding constants, K_{Li}, to individual phospholipids were calculated from the δ values observed in Li^+-containing suspensions (no Mg^{2+}) of phospholipid extract from an equation similar to Equation 3.

Limitations of the calculations of binding constants. The binding constants calculated from ^{31}P NMR data are approximate and can only be used to establish the observed trends of the affinities of the various phospholipids for Li^+ and Mg^{2+}. This is because the phospholipid extract sample used for metal ion titration contained two phases, the organic as well as the aqueous EDTA layer. Although, prior to metal ion addition, attempts were made to remove the EDTA layer, complete removal could not be achieved. Hence the amount of metal ion in the organic phase was different from the amount added, because some amount of metal ions was chelated by the EDTA or was more soluble in the aqueous phase.

Several methods were used in an attempt to obtain the amount of metal ions in the organic phase. Atomic absorption (AA) spectroscopy was used for quantifying the Mg^{2+} concentration in the organic layer by taking an aliquot from the organic layer of the NMR sample. Because the solvent system contained chloroform, the flame was not very stable and the readings obtained were not reliable. Attempts also were made to evaporate the solvent and dissolve the residue in water. The presence of the phospholipids resulted in a suspension instead of a solution. AA results, however, indicated that only about 50–60% of the Mg^{2+} added was going into the organic layer. By AA with a flame source, we were not able to detect Li^+ because aliquots from the NMR samples were too dilute to provide a sufficient sample.

Determination of protein and phospholipid concentrations. The protein concentration was determined by the Bio-Rad Bradford method, with bovine serum albumin used as a standard for generating the calibration curve (28). Because the samples contained membrane-bound protein, a modified procedure with detergent was used (29). The phospholipid concentration was determined from a revised procedure reported by Avanti Lipids (30).

NMR spectrometers. ^{31}P, ^{7}Li, and ^{6}Li NMR experiments were conducted with Varian VXR-300 or VXR-400 NMR spectrometers (Palo Alto, CA) equipped with multinuclear probes. A 5- or a 10-mm broad-band probe was used, depending on the availability of the sample. All samples were run by spinning of the sample at 16–18 Hz. A variable-temperature unit was used for maintaining the probe temperature at 27°C. T_1 relaxation measurements were conducted with the inversion recovery pulse sequence, using at least seven τ values for each T_1 value. Under our conditions, the relaxation measurements were accompanied by a uncertainty factor of less than 10%, and the reported values represent the range or the standard deviation obtained by averaging the values from two or three separately prepared samples. The changes in chemical shift of the ^{31}P NMR phospholipid resonances are reliable to 0.01 ppm because of instrument inaccuracy, whereas a change of greater than 10% in T_1 values is due to experimental error. Therefore, changes in δ values of 0.01 ppm or greater, and T_1 value changes of 10% or greater, are significant. Binding constants were calculated from T_1 values from at least five data points by use of James-Noggle plots (31).

RESULTS

This investigation was conducted by means of ^{31}P NMR chemical shift measurements and ^{31}P, ^{7}Li, and ^{6}Li NMR relaxation measurements. We used the ^{31}P NMR chemical shift variation upon metal ion addition to identify the phospholipids that interact most strongly with the metal ion added. ^{7}Li

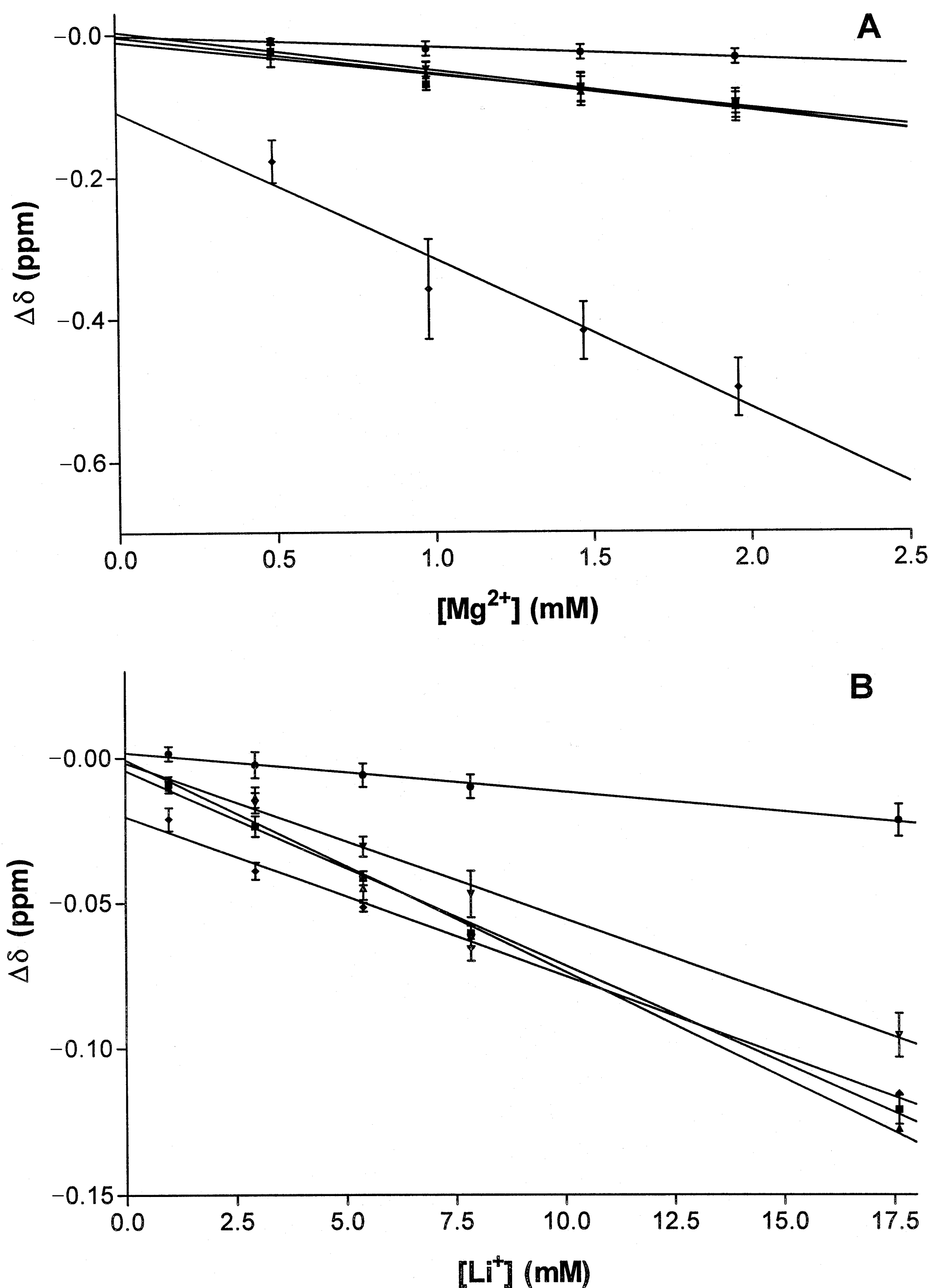

FIG. 1. Plot of the changes in ^{31}P nuclear magnetic resonance (NMR) chemical shifts of human red blood cell (RBC) membrane phospholipids upon addition of varying amounts of chloride salts of (A) Mg^{2+} and (B) Li^+. The phospholipids correspond to the following symbols: (●) phosphatidylcholine (PC); (▲) phosphatidylethanolamine (PE); (■) plasmalogen PE (PEPLAS); (◆) phosphatidylserine (PS); (▼) sphingomyelin (SM). The bars represent the range values obtained from two separate measurements.

and ^{6}Li T_1 measurements were used for computing the Li^+ association constant to the phospholipid extract as well as RBC membrane suspensions. We also used both approaches to investigate whether Li^+/Mg^{2+} competition was present for the phosphate head groups of the phospholipids.

^{31}P NMR δ measurements of phospholipid extracts. The phospholipids extracted from the human RBC membranes were titrated with varying amounts of $MgCl_2$. The variations of the δ values with Mg^{2+} addition are presented in Figure 1A. Upon Mg^{2+} addition, the ^{31}P NMR resonances of all of the

TABLE 1
^{31}P NMR δ of Human RBC Membrane Phospholipids Spiked with PI as a Function of Added $MgCl_2$[a,b]

	^{31}P NMR δ (ppm)					
$[Mg^{2+}]$/mM	PEPLAS	PE	SM	PS	PI	PC
0.00	0.29 ± 0.00	0.24 ± 0.00	0.04 ± 0.00	−0.22 ± 0.01	—	−0.84 ± 0.00
0.00/with PI[c]	0.25 ± 0.00	0.20 ± 0.01	0.02 ± 0.00	−0.29 ± 0.01	−0.59 ± 0.00	−0.84 ± 0.00
0.49	0.23 ± 0.00	0.18 ± 0.00	0.00 ± 0.00	−0.40 ± 0.01	−0.70 ± 0.00	−0.85 ± 0.00
0.98	0.20 ± 0.00	0.16 ± 0.00	−0.03 ± 0.00	−0.55 ± 0.00	−0.86 ± 0.00	−0.86 ± 0.00
1.47	0.18 ± 0.00	0.14 ± 0.00	−0.06 ± 0.01	−0.64 ± 0.01	−1.01 ± 0.04	−0.87 ± 0.01
1.96	0.15 ± 0.00	0.11 ± 0.00	−0.09 ± 0.00	−0.72 ± 0.00	−1.15 ± 0.00	−0.89 ± 0.00

[a]All samples were run at 27°C in a 10-mm nuclear magnetic resonance (NMR) probe with the sample spinning at 16–18 Hz. The chemical shifts (δ) were referenced to 85% phosphoric acid. At the beginning of each titration, the δ value of the phosphatidylcholine (PC) resonance was referenced to –0.84 ppm. Reported δ are average values obtained from two separate trials. The $[Mg^{2+}]$ concentrations are the amount added to the suspension and not the amount in the organic phase.
[b]PEPLAS, plasmalogen PE; PE, phosphatidylethanolamine; SM, sphingomyelin; PS, phosphatidylserine; PI, phosphatidylinositol; RBC, red blood cell.
[c]The phospholipid extract was spiked with PI so that the NMR sample contained 0.25 mg of PI/mL of the sample.

phospholipids shifted upfield, but the extent was different for the various phospholipids. The change in ^{31}P NMR chemical shift was largest for the anionic phospholipid PS when 1.96 mM Mg^{2+} was added (Fig. 1A); for the same Mg^{2+} concentrations, the PE, plasmalogen PE (PEPLAS), and SM resonances shifted upfield roughly 4.5 times less than that for PS. The PC resonance did not show any appreciable change in δ upon metal ion addition.

The effect of Mg^{2+} addition on PI could not be investigated in this manner because the percentage of PI in the RBC membranes is less than 2%, and its resonance was not observed clearly, even with overnight accumulations of the ^{31}P NMR spectrum (17,32). Moreover, because the interaction of PI with the metal ion caused broadening of the PI signal (11), PI with only a 2% abundance would make it difficult to observe the signal after metal ion addition. Hence, to the human RBC membrane phospholipid extract a known amount of PI was added, and Mg^{2+} titration was carried out. The amount of PI added to the extract was adjusted so that the mixture contained equal amounts of PI and PS. The addition of up to 1.96 mM Mg^{2+} caused the ^{31}P NMR resonances of PI and PS to move upfield by 0.56 and 0.43 ppm, respectively (Table 1). The K_{Mg} value calculated from Equation 3 for PS present in the phospholipid extract sample was 383 ± 18 M^{-1}. A pure PS sample under identical experimental conditions was found to bind Mg^{2+} with a binding constant of 385 M^{-1}. However, other phospholipids, such as PC, PE and SM, were found to bind to Mg^{2+} weakly (K_{Mg} in the range of 225–270 M^{-1}). The binding constants reported are subject to some error because they were calculated for the amount of Mg^{2+} added to the solvent mixture and not the amount present in the organic phase. The K_{Mg} to PI was calculated to be $(2.0 \pm 0.2) \times 10^3$ M^{-1}. The K_{Mg} value for PI is significantly larger than the value obtained for the other anionic phospholipid, PS. Thus, it was found that the anionic phospholipid, PI, interacted most strongly with Mg^{2+}; PS was found to bind Mg^{2+} with intermediate affinity, whereas SM, PE, and PC interacted weakly.

Figure 1B shows the changes in the ^{31}P NMR δ values as a function of added LiCl. Addition of up to 17.6 mM LiCl caused the δ values of PS, SM, PE, and PEPLAS to move upfield by approximately 0.1 ppm, whereas the δ value of the PC resonance did not change appreciably with Li^+ titration. When compared with the Mg^{2+} titration, larger amounts of Li^+ were needed for an effect on the ^{31}P NMR spectrum of human RBC membrane phospholipids. In order to characterize the interaction between PI and Li^+, we spiked the phospholipid extract from human RBC membranes with the same amount of pure PI as described above for the Mg^{2+} titration, and this sample was titrated with LiCl. Addition of up to 14.0 mM LiCl caused the PI resonance to move downfield (Table 2), whereas the SM resonance shifted upfield; the change in the δ value for all other resonances, including PS, was very small. The K_{Li} to PS was calculated to be 121 ± 17 M^{-1}, whereas for the other neutral phospholipids, the K_{Li} values were in the range of 55–65 M^{-1}. K_{Li} to PI and SM were calculated to be 102 ± 4 M^{-1} and 63 ± 1 M^{-1}, respectively.

^{31}P NMR δ changes of the phospholipid extracts containing both Li^+ and Mg^{2+} are shown in Figure 2. The addition of 0.50 mM $MgCl_2$ caused the ^{31}P NMR resonances to move upfield, whereas the ^7Li titration of this phospholipid sample caused the resonances to move downfield. This effect is clearly seen for the anionic phospholipid, PS, where addition of 0.50 mM $MgCl_2$ caused the resonance to move by 0.20 ppm upfield, as well as to broaden the PS signal. However, addition of 7.8 mM LiCl caused the PS resonance to move downfield by 0.03 ppm. A plot of the δ changes for the mixed ion titration is shown in Figure 3, with the PS signal displaying the greatest difference of the various phospholipids.

^{31}P NMR T_1 measurements of phospholipid extracts. We carried out ^{31}P NMR T_1 measurements of phospholipid extracts with and without metal ions in an effort to quantify metal ion binding to the phosphate head groups of the phospholipids. However, the T_1 variations within each class of phospholipids were too small (0.1 s) upon addition of either 0.50 mM $MgCl_2$ or 7.0 mM LiCl to be measured accurately (data not shown). The addition of metal ions did not cause any

TABLE 2
^{31}P NMR δ of Human RBC Membrane Phospholipids Spiked with PI as a Function of Added LiCl[a]

	^{31}P NMR δ (ppm)					
[Li^{+}]/mM	PEPLAS	PE	SM	PS	PI	PC
0.00	0.23 ± 0.03	0.19 ± 0.03	0.02 ± 0.01	−0.29 ± 0.03	—	0.84 ± 0.00
0.00/with PI	0.18 ± 0.01	0.13 ± 0.01	−0.001 ± 0.00	−0.34 ± 0.02	−0.62 ± 0.01	−0.84 ± 0.00
2.00	0.19 ± 0.04	0.14 ± 0.03	−0.02 ± 0.01	−0.36 ± 0.01	−0.59 ± 0.01	−0.85 ± 0.01
6.00	0.17 ± 0.01	0.12 ± 0.01	−0.03 ± 0.00	−0.32 ± 0.05	−0.56 ± 0.00	−0.85 ± 0.00
9.00	0.16 ± 0.00	0.11 ± 0.00	−0.05 ± 0.00	−0.35 ± 0.00	−0.53 ± 0.00	−0.85 ± 0.00
14.00	0.16 ± 0.00	0.11 ± 0.00	−0.07 ± 0.00	−0.33 ± 0.00	−0.48 ± 0.00	−0.87 ± 0.00

[a]Same experimental conditions as in Table 1. See Table 1 for abbreviations.

significant changes in the ^{31}P NMR T_1 values either, and hence this technique could not be used for quantitative measurements.

^{7}Li and ^{6}Li NMR T$_1$ *measurements of phospholipid extracts*. The phospholipid extract in a chloroform/methanol solvent system was titrated with increasing amounts of LiCl;

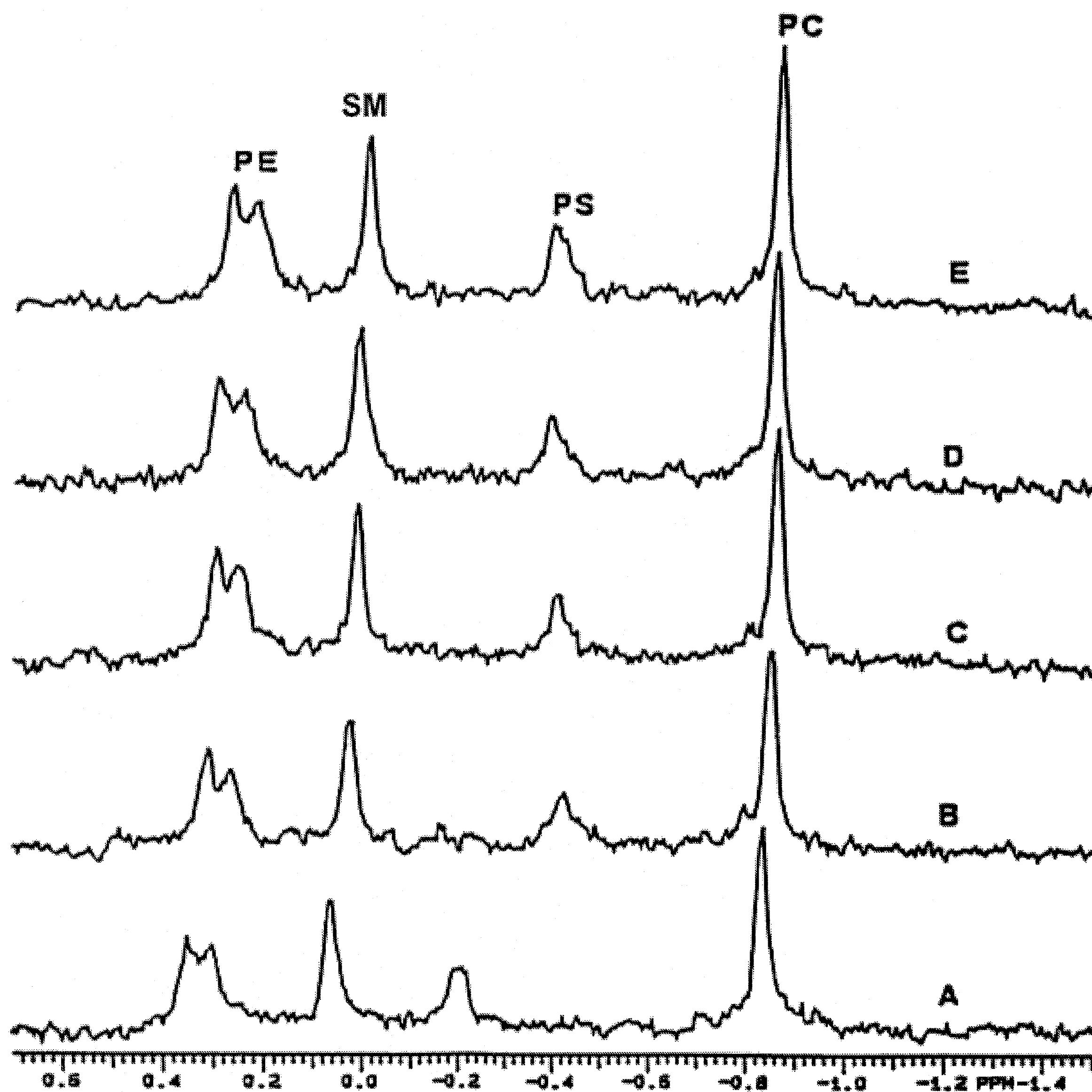

FIG. 2. ^{31}P NMR spectra of human RBC membrane phospholipids with (A) no metal ions added; (B) 0.50 mM Mg^{2+}; (C) 0.50 mM Mg^{2+} and 2.0 mM Li^{+}; (D) 0.50 mM Mg^{2+} and 3.9 mM Li^{+}; and (E) 0.50 mM Mg^{2+} and 7.8 mM Li^{+}. The peak assignments are indicated in spectrum E. See Figure 1 for abbreviations.

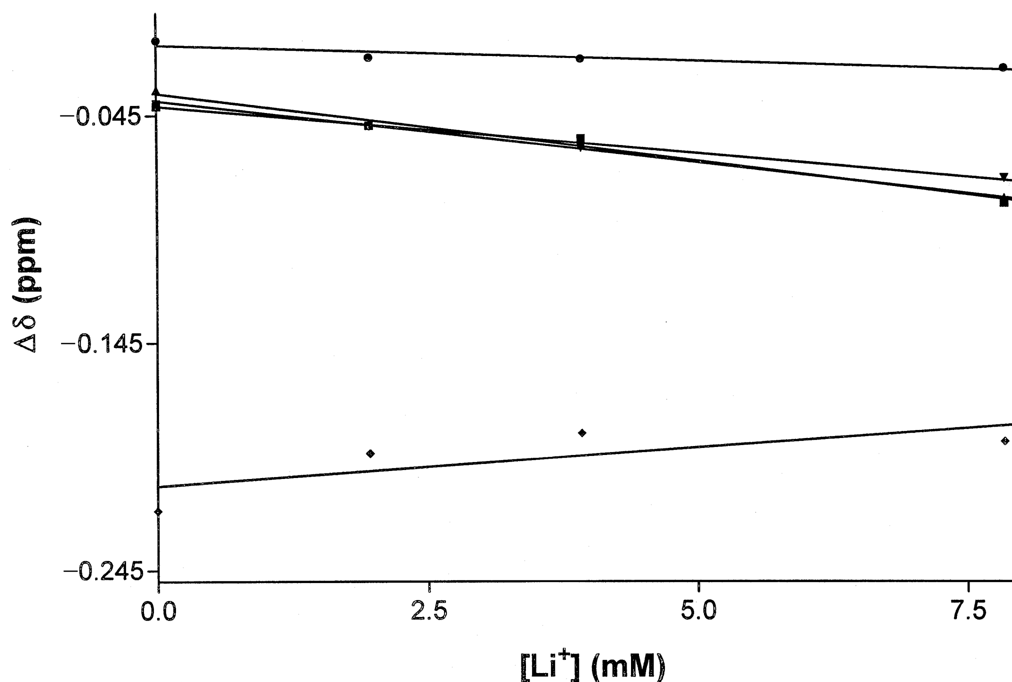

FIG. 3. Plot of the changes in ^{31}P NMR δ of human RBC membrane phospholipids containing 0.50 mM Mg^{2+} and varying amounts of LiCl. The phospholipids correspond to the following symbols: (●) PC; (▲) PE; (■) PEPLAS; (◆) PS; (▼) SM. For the regression line of PS, $r^2 = 0.74$, whereas for the other phospholipids, the r^2 for each regression line was greater than 0.94. See Figure 1 for abbreviations.

as the $^7Li^+$ concentration increased from 3.0 to 20 mM, the ^7Li T_1 values increased from 0.75 ± 0.16 s to 1.81 ± 0.13 s. However, for each Li^+ increment of 3.0 mM, the T_1 changes were very small (0.2 s) and only slightly sensitive to variations in Li^+ concentration. This method was therefore deemed unreliable for quantitative analysis. To see whether this effect was a mere ionic strength effect, we titrated the phospholipid extract containing 6.0 mM LiCl with $(Me)_4NCl$. Addition of up to 20 mM $(Me)_4NCl$ caused the ^7Li T_1 values to increase by only 0.2 s; therefore, the changes noticed in the ^7Li T_1 values are due to binding of the Li^+ to the phospholipids and are not due to ionic strength effects.

The ^6Li nucleus exhibits much larger relaxation time values than the ^7Li nucleus (27). Hence, we used ^6Li T_1 measurements to quantify Li^+ binding to the phospholipids. The ^6Li T_1 values increased from 7.63 ± 0.76 s to 13.13 ± 1.30 s upon addition of 5.0 to 30 mM ^6LiCl. The K_{Li} to the phospholipid extract was calculated to be 45 ± 5 M^{-1} ($n = 2$, $r^2 > 0.90$) using Equation 1. The phospholipid extract was titrated with ^6LiCl in the presence and absence of 0.1 mM Mg^{2+}. Addition of $^6Li^+$ caused an increase in the ^6Li T_1 values for both samples (Fig. 4), indicating Li^+ binding. However, at a given $^6Li^+$ concentration, the T_1 values were much higher for the sample containing 0.1 mM $MgCl_2$.

^7Li and ^6Li NMR T_1 measurements of RBC membranes. ^7Li T_1 measurements were conducted with samples of unsealed and cytoskeleton-depleted RBC membranes. As the LiCl concentration increased from 2.0 mM to 12.0 mM, the cytoskeleton-depleted membrane sample consistently gave higher T_1 values when compared to the unsealed-membrane sample. From Equation 1, the K_{app} for the cytoskeleton-depleted RBC membrane sample was 304 ± 30 M^{-1}, and for the unsealed RBC membrane sample, K_{app} was 202 ± 20 M^{-1}.

^7Li T_1 measurements were also conducted on the unsealed and cytoskeleton-depleted RBC membranes containing 4.0 mM LiCl and titrated with increasing amounts of $MgCl_2$. These results showed that, as the Mg^{2+} concentration increased from 0.0 to 0.6 mM, the ^7Li T_1 values for the unsealed membranes increased from 8.41 ± 0.04 to 13.16 ± 1.17 s, whereas for the cytoskeleton-depleted membranes the ^7Li T_1 values increased from 8.94 ± 0.06 to 14.15 ± 0.63 s.

In order to determine the actual Li^+ and Mg^{2+} binding constants (Eq. 2) for the unsealed and cytoskeleton-depleted RBC, we titrated the respective samples with increasing amounts of LiCl in the presence of either 0.05, 0.10, 0.15, or 0.20 mM $MgCl_2$. As Table 3 shows, the K_{app} for the unsealed RBC membrane was consistently lower when compared to values obtained for the cytoskeleton-depleted RBC membrane. The actual K_{Li} for the unsealed RBC was 221 ± 21 M^{-1} ($n = 3$), and for the cytoskeleton-depleted, the actual K_{Li} was 357 ± 24 M^{-1} ($n = 3$), whereas the K_{Mg} for the unsealed and cytoskeleton-depleted RBC membranes were 4397 ± 252 M^{-1} ($n = 3$) and 6996 ± 870 M^{-1} ($n = 3$), respectively.

^6Li T_1 measurements were also conducted on the two membrane samples. As the ^6Li concentration increased from 10 to 40 mM, the T_1 values were again larger for the cytoskeleton-depleted RBC membrane samples in comparison to the un-

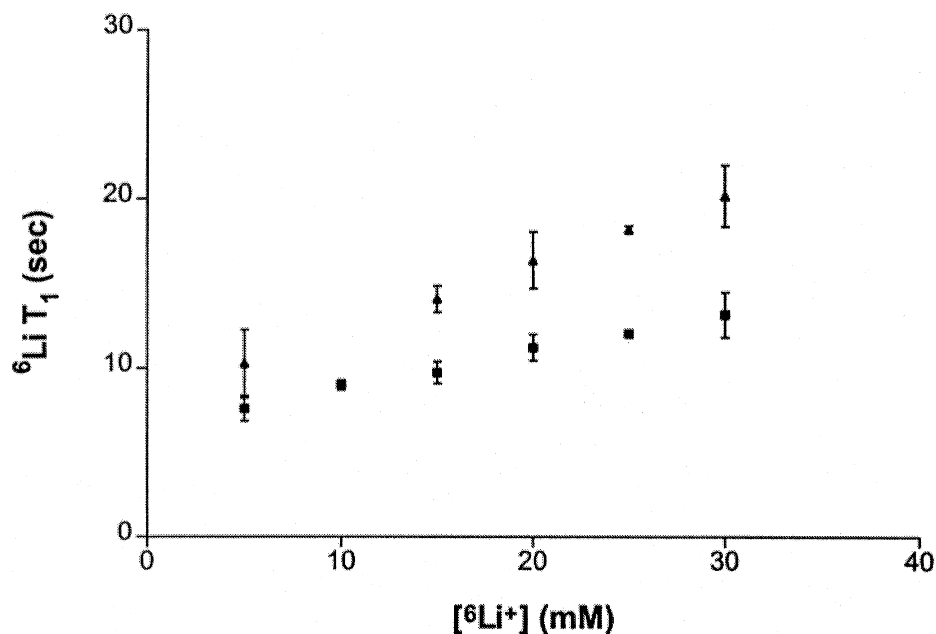

FIG. 4. Variations in ^{6}Li spin-lattice relaxation time values of the phospholipids extracted from human RBC membranes in the (■) absence and (▲) presence of 0.1 mM Mg^{2+}. The extracted dried phospholipids were suspended in a chloroform/methanol solvent mixture at a ratio of 5:2. The reported values are average values obtained from two separate measurements. See Figure 1 for abbreviation.

sealed RBC membrane samples. Using Equation 1, the calculated K_{app} value for the cytoskeleton-depleted RBC membrane samples was larger (259 ± 90 M^{-1}), when compared to the unsealed RBC membrane samples (115 ± 33 M^{-1}).

DISCUSSION

Li^+ and Mg^{2+} have similar ionic radii, and a "diagonal relationship" exists between these elements in the periodic table. As a result, the chemistry of Li^+ often resembles that of Mg^+. Hence, competition between these two ions could occur for biological ligands. Previous reports from our research group have shown evidence for competition between Li^+ and Mg^{2+} ions for Mg^{2+}-binding sites in biomolecules (12,33–36). Because Mg^{2+} is a cofactor for several enzymes, displacement of Mg^{2+} by Li^+ from biomolecules can affect several biological processes, and a combination of several effects, including metal ion binding to phospholipids, may be involved in the mode of action of Li^+ in the treatment of manic depression. Therefore, this study was devoted to probing Li^+/Mg^{2+} competition for RBC phospholipids and the RBC membrane by use of multinuclear NMR techniques.

In our NMR experiments, there are two layers: one organic and one aqueous. The organic layer contains essentially phospholipids, whereas the metal ions will reside in the aqueous layer. Because of the amphiphilic nature of phospholipid molecules, the tails will be fully embedded in the organic layer and the charged head groups will reside at the interface of the two layers. Therefore, the experimental design of our NMR experiments represents a good model system for understanding metal ion interactions to phospholipid head groups in biological membranes.

For human RBC membrane phospholipids, addition of up to 2.0 mM Mg^{2+} caused the ^{31}P NMR resonance shifts of PS to change by 0.5 ppm; this was the largest change that was observed (Fig. 1A). PS therefore interacted most strongly with Mg^{2+}, with marked broadening of the PS signal upon Mg^{2+} addition followed by quenching of the signal due to excessive broadening. Qualitatively, the signal that disappears first from the phospholipid spectral profile is considered to be interacting most strongly with the added cation. This obser-

TABLE 3
K_{app}, K_{Li}, and K_{Mg} for Unsealed and Cytoskeleton-Depleted RBC Membrane in Varying Concentrations of $MgCl_2$[a]

$[MgCl_2]$/(mM)	Unsealed RBC membrane K_{app} (M^{-1})	Cytoskeleton-depleted RBC membrane K_{app} (M^{-1})
0.0	202 ± 20	304 ± 30
0.05	190 ± 6	278 ± 21
0.10	167 ± 7	246 ± 11
0.15	134 ± 3	177 ± 15
0.20	113 ± 9	141 ± 17
K_{Li} (M^{-1})	221 ± 21	357 ± 24
K_{Mg} (M^{-1})	4397 ± 252	6996 ± 870

[a]These experiments were conducted in a 10-mm NMR probe. The values reported are the average values ± SD obtained from three separate trials. The r^2 values for all trials were greater than 0.90. The protein concentrations of the unsealed and the cytoskeleton-depleted RBC membranes were 6.9 ± 0.8 and 6.7 ± 0.9 mg/mL, respectively. The total phospholipid content was comparable for all samples. K_{app}, apparent binding constant; K_{Li}, lithium binding constant; K_{Mg}, magnesium binding constant.

vation was consistent with the K_{Mg} for PS, which was determined from a phospholipid extract. The relative order of K_{Mg} in the phospholipid extract samples calculated from the ^{31}P NMR δ values was PI ≫ PS > PE ≈ PEPLAS ≈ SM > PC.

Li^{+} titration of human RBC membrane phospholipids caused the ^{31}P NMR chemical shift of all phospholipids to move upfield by approximately 0.1 ppm, except for PC, when 17.6 mM LiCl was added (Fig. 1B). The changes in ^{31}P NMR δ values induced by an increase in Li^{+} concentration are very small when compared to the changes observed for Mg^{2+}. The relative order of Li^{+} binding constants in the phospholipid extract samples calculated from the ^{31}P NMR δ values was PS > PI > SM ≈ PE ≈ PEPLAS > PC. ^{7}Li NMR T_1 titrations also revealed that PS binds Li^{+} most strongly; SM and PI were found to have intermediate interaction. From the ^{31}P NMR δ data, it is interesting to note that in a phospholipid mixture, Mg^{2+} showed a preference for PI, whereas Li^{+} binds PS more strongly than PI.

The reversal of ordering of Li^{+} and Mg^{2+} binding to PS and PI is most likely related to a combination of two factors: differing coordination numbers and charge of the two metal ions. Because Li^{+} has a predominant coordination number of four, it binds to PI *via* one negatively charged oxygen of the phosphate group, whereas with PS, Li^{+} can bind in a bidentate manner to two charged oxygens of the phosphate head group and the carboxylate group of serine. Although the amino group of serine carries a +1 charge at the pH used in our studies, its repulsion by the low charge of Li^{+} is not very important. The PS-Mg^{2+} affinity constant is larger than that for the PS-Li^{+} affinity constant due to the higher charge of Mg^{2+}, thus making stronger interactions with the phosphate and carboxylic groups of PS (albeit also increasing the repulsion for its NH_4^{+} group).

Because Mg^{2+} has a coordination number of six (in contrast to four for Li^{+}), Mg^{2+} can bind simultaneously to phosphate and several hydroxyl groups of inositol. Formation of Mg^{2+} complexes of PI involving hydroxyl groups is more favored than in Li^{+} complexes of PI. The larger coordination number of Mg^{2+} allows binding of three hydroxyl groups of the inositol moiety pointing toward the same side of the ring, filling three vertices of its coordination polyhedron, whereas this ligand arrangement is not possible for Li^{+}. This type of proposed Mg^{2+} coordination to inositol has been observed before with Ca^{2+} chelates of inositol triphosphate (36,37). The fact that the ^{31}P shifts induced on PS and PI by Mg^{2+} binding are all upfield, whereas the ^{31}P shift induced on PI by Li^{+} binding is downfield, reflects the presence of chelate structures in the first two cases and their absence in the latter. Chelate formation drastically alters the torsion angles of the P–O bonds in the phosphodiester moieties of the phospholipids, which are one of the three factors that modify the chemical shielding of the ^{31}P nucleus (38).

It should be noted that Merchant and Glonek (11) saw a similar metal-binding trend in extracts containing various phospholipids present in the rat heart. In that study, it was determined that phosphatidic acid preferentially bound Ca^{2+}, whereas cardiolipin bound Mg^{2+} more strongly. Again, the reasoning behind this seems to be the difference in the coordination numbers of Mg^{2+} (six) and Ca^{2+} (greater than six).

To see whether there is competition between Li^{+} and Mg^{2+} for the phospholipid extract, we titrated the phospholipid extract sample with 0.50 mM Mg^{2+}. Addition of Mg^{2+} caused all of the resonances, except that of PC, to move upfield, with the maximum effect being the PS resonance with a 0.20-ppm shift (Figs. 2 and 3). When titrated with increasing amounts of LiCl, the resonance for the anionic phospholipid, PS, shifted downfield, whereas the resonances shifted slightly upfield. Therefore, a decrease in the δ change was observed. This effect is clearly seen in Figure 3; the slope of the δ difference for PS is positive, whereas for the other phospholipids present in the extract, the slope is negative. The signal of PS also sharpened; this, we believe, is due to Li^{+} displacing Mg^{2+} from the phosphate head group of PS.

^{6}Li NMR T_1 measurements were conducted with the phospholipid extracts both in the presence and absence of $MgCl_2$ (Fig. 4). The T_1 values were sensitive to LiCl addition to both samples. This is consistent with ^{31}P NMR results indicating that the phospholipids interact with Li^{+}. Moreover, the samples with Mg^{2+} gave higher T_1 values than did the samples without Mg^{2+} at the same Li^{+} concentration. This increase in T_1 values in the presence of Mg^{2+} is due to an increase in the free Li^{+} concentration, and this, we believe, is due to Mg^{2+} and Li^{+} occupying the same binding site. Mg^{2+} is presumably displacing Li^{+} from the phosphate head group of the phospholipids. Both the ^{31}P NMR δ measurements and the ^{6}Li T_1 measurements provide evidence for Li^{+}/Mg^{2+} competition.

The K_{Li} to the phospholipid extract without Mg^{2+} was calculated to be 45 ± 5 M^{-1} ($n = 2$, $r^2 > 0.90$) from the James-Noggle plot. This value is lower than the binding constant determined for the unsealed RBC membranes, which is close to 200 M^{-1} (3). These differences in Li^{+} binding constants may be attributed to the different media used—organic solvent mixture vs. water—in the two samples.

Although the phospholipids provide major binding sites for the metal ions, the removal of the lipids from the membrane skeleton could have decreased their affinity for the metal ions. This prompted us to characterize the role played by the cytoskeleton in metal ion binding and competition. To identify whether the cytoskeleton anchored on the inner leaflet of the lipid bilayer plays any significant role in metal ion binding and competition, we conducted ^{7}Li and ^{6}Li NMR studies with unsealed and cytoskeleton-depleted membranes. ^{7}Li NMR T_1 measurements were conducted for unsealed and cytoskeleton-depleted human RBC membranes titrated with increasing amounts of LiCl. It was previously determined from ^{7}Li NMR relaxation measurements that unsealed RBC membrane samples showed a biexponential relaxation of the ^{7}Li nucleus when the Li^{+} concentration was 20 mM (3). However, when the Li^{+} concentration exceeded 150 mM, Rong *et al.* (3) did not see evidence for the same biexponential decay. Removal of the cytoskeleton did not affect the sensitivity of the ^{7}Li T_1 values to the changes in LiCl concentra-

tion; this indicated that the removal of the cytoskeleton did not prevent Li^+ binding to the membranes. The Li^+ affinity binding constant to the cytoskeleton-depleted membrane was found to be 357 ± 24 M^{-1}, which is approximately twofold higher than the value determined for the unsealed membranes (221 ± 21 M^{-1}). It should also be noted that a similar trend was seen for ^6Li T_1 experiments; the binding constant was again larger for the cytoskeleton-depleted RBC membrane samples (259 ± 90 M^{-1}) compared to the unsealed RBC membrane samples (115 ± 33 M^{-1}). This increase in Li^+ affinity for the cytoskeleton-depleted membranes could be due to exposure of the anionic phospholipids. The inner leaflet of the RBC membrane contains a greater percentage of the negatively charged phospholipids (PI and PS) as compared to the outer leaflet, and removal of the spectrin–actin network could allow for easy interaction between Li^+ and the anionic phospholipids in the lipid bilayer. The competition between Li^+ and Mg^{2+} was found to be present even after the removal of the cytoskeleton. Hence, it was clear that the cytoskeleton had no major role in Li^+ binding or in Li^+/Mg^{2+} competition.

In summary, this study indicated that there is competition between Li^+ and Mg^{2+} for human RBC membranes and phospholipids. This study also showed that PS and PI bind Li^+ and Mg^{2+} most strongly, whereas the neutral phospholipid PC does not contribute significantly toward metal ion binding. These observations provide a possible explanation for the elevated K_{Li} obtained for the RBC membranes from manic-depressive patients undergoing Li^+ therapy, because their cell membranes contain a greater percentage of the anionic phospholipids (32).

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Fullerene Lipids: Synthesis of C_{60} Fullerene Derivatives Bearing a Long-Chain Saturated or Unsaturated Triester System

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ABSTRACT: Tris(hydroxymethyl)aminomethane was successfully esterified with saturated and unsaturated long-chain fatty acids. The resulting amino-triester intermediates were successively reacted with chloroacetyl chloride, sodium azide, and C_{60} fullerene. Spectral evidence showed that the aziridine ring is joined to the junction of [6,6]-fused rings of the fullerene. The structures of the various C_{60} fullerene derivatives bearing a long-chain saturated or unsaturated triester system were characterized by spectroscopic and spectrometric methods.

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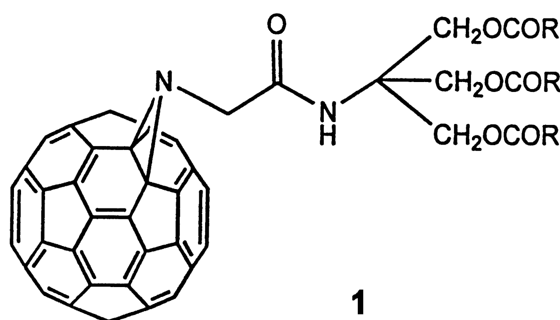
Studies of the reactivity and chemical derivatization of fullerenes have drawn keen and continuous attention after a method for bulk production of C_{60} fullerene was reported (1). A large range of functionalized fullerenes have been prepared for material and pharmaceutical applications (2–8). For instance, methanofullerene derivatives bearing the salts of carboxylic acids possess inhibitory activity against human immunodeficiency virus (HIV) protease (9,10). The biological activity of water-soluble fullerenes has also been explored, with results showing structural dependence of DNA cleavage, cytotoxicity, and enzyme inhibitory activities including HIV-protease inhibition (11). However, only two reports on C_{60} fullerene with lipophilic groups (containing long-chain ester functions) have been published (12,13). We have recently reported the synthesis of a series of 14 dialkyl 1,2-[6,6]-methano-[60]-fullerene dicarboxylate derivatives containing saturated and unsaturated alkyl groups (12). Murakami *et al.* (13) have described the synthesis of a C_{60} fullerene derivative containing three saturated fatty ester system attached *via* an aziridine bridge **1** (Scheme 1).

Compound **1** appears to be a very interesting fullerene lipid, as it can form self-organized multibilayer films despite the presence of a large spherical cage structure in the molecule. The degree of phase transitions between the crystalline phase and liquid crystalline phase of lipid bilayer membranes is very much dependent on the structure of the acyl chains. In view of this important factor, we propose to study the physical properties of a wider range of similar fullerene lipids by synthesizing analogs of compound **1** containing saturated acyl moieties of different chain lengths and analogs containing mono- and polyunsaturated acyl groups.



SCHEME 1

In this paper we describe the synthesis of seven fullerene lipids with structures similar to that of compound **1**, but containing either saturated (C_{12}, C_{14}, C_{16}, C_{18}, **1a–1d**, respectively) or unsaturated (oleic, linoleic, and stearolic acids, **1e–1g**, respectively) fatty acid moieties in the triester system (Schemes 2 and 3, respectively). The results of the study of the nuclear magnetic resonance (NMR) spectroscopic and other physical properties of these novel C_{60} fullerene derivatives are described.

MATERIALS AND METHODS

Melting points were determined by using a hot-stage apparatus and are uncorrected. Infrared (IR) spectra were recorded on a Bio-Rad FTS-165 FT IR spectrometer. Samples were made into KBr discs for solids or were run as neat films for liquids on KBr discs. Ultraviolet (UV) spectra of solutions in dichloromethane were recorded on a Hewlett-Packard Diode Array Spectrophotometer, model 8452A (Hewlett-Packard, Palo Alto, CA). NMR spectra were recorded on a Bruker Avance DPX_{300} (300 MHz) Fourier-transformed NMR spec-

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Abbreviations: APCI, atmospheric pressure chemical ionization; DCC, dicyclohexylcarbodiimide; DMAP, 4-dimethylaminopyridine; FTIR, Fourier transform infrared; IR, infrared; NMR, nuclear magnetic resonance; THF, tetrahydrofuran; TMS, tetramethylsilane; UV-VIS, ultraviolet-visible.

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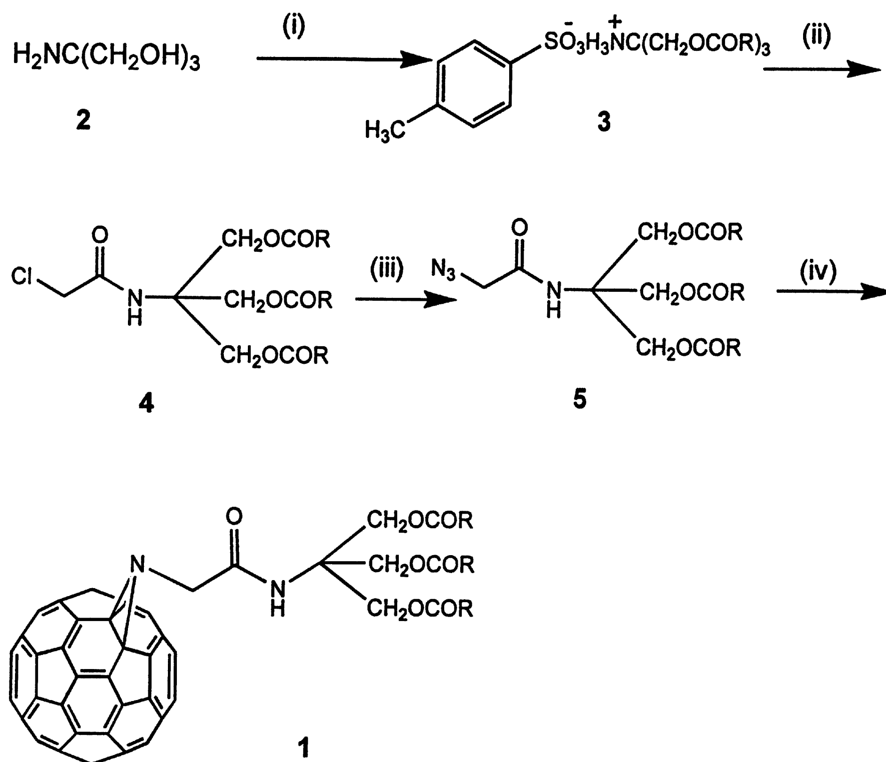


1 a R = $(CH_2)_{10}CH_3$
b R = $(CH_2)_{12}CH_3$
c R = $(CH_2)_{14}CH_3$
d R = $(CH_2)_{16}CH_3$

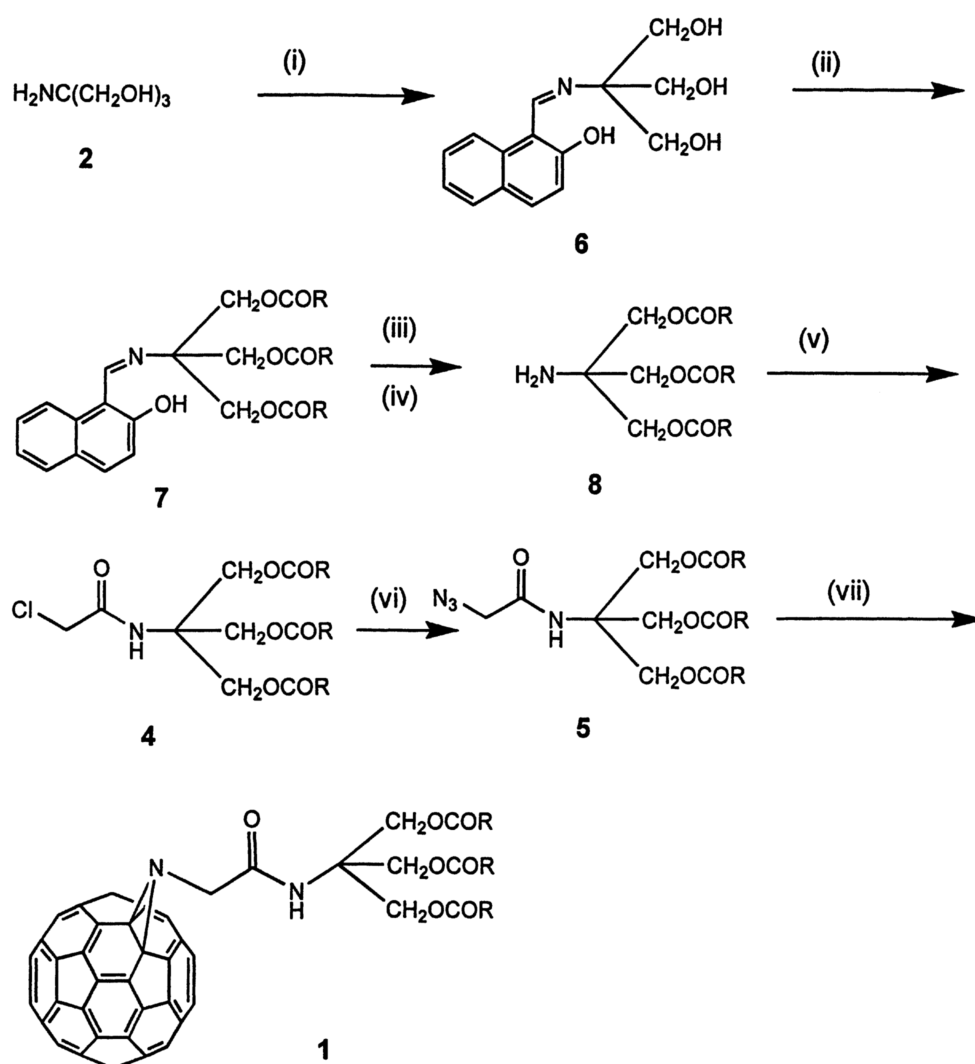
Reagents and conditions: (i) RCOOH, *p*-toluenesulfonic acid, Dean Stark water trap, reflux 20 h; (ii) chloroacetyl chloride, triethylamine, tetrahydrofuran, 24°C, 14 h; (iii) sodium azide, dimethylsulfoxide, 70°C, 5 h; (iv) C_{60} fullerene, chlorobenzene, reflux, 12 h.

SCHEME 2

trometer (Bruker, Fallanden, Switzerland) from solutions in deuteriochloroform ($CDCl_3$) with tetramethylsilane (TMS) as the internal reference standard. Chemicals shifts are given in δ-values in ppm downfield from TMS (δ_{TMS} = 0 ppm). Mass spectral analyses were carried out on Finnigan MAT-LCQ [atmospheric pressure chemical ionization (APCI)] (Finnigan Corp., San Jose, CA).

Oleic and linoleic acids were purchased from Aldrich Chemical Co. (Milwaukee, WI). Octadec-9-ynoic acid (stearolic acid) was prepared by bromination–dehydrobromination reaction of the corresponding oleic acid as described elsewhere (14). C_{60} fullerene was purchased from Lancaster Synthesis Ltd. (Eastgate, Lancashire, United Kingdom) or from Materials and Electrochemical Research Corp. (Tucson, AZ). Tris(hydroxymethyl)aminomethane was purchased from Sigma Chemical Co. (St. Louis, MO).

*General procedure for the preparation of C_{60} fullerene lipids containing saturated fatty acid moieties in the triester system (**1a–1d**) as exemplified by the synthesis of compound **1a** (Scheme 2).* A mixture of tris(hydroxymethyl)aminomethane **2** (1.6 g, 13.2 mmol), dodecanoic acid (9.5 g, 47 mmol), *p*-toluenesulfonic acid (4.7 g), and toluene (150 mL) was refluxed (fitted with a Dean-Stark trap) for 20 h. The reaction mixture was cooled, and the precipitate was isolated by filtration. The precipitate was recrystallized from a solution of acetonitrile and diethyl ether (4:1, vol/vol) to give the tridodecyl ester derivative **3** (9.2 g, 81.3%). The latter compound (2.44 g, 2.9 mmol) was mixed with triethylamine (363 mg, 3.6 mmol) in tetrahydrofuran (THF, 20 mL) at room temperature, followed by the addition of chloroacetyl chloride (542 mg, 4.8 mmol). The whole mixture was then stirred for 14 h. The solution was filtered, and the filtrate was evaporated under reduced pressure. The residue was recrystallized from methanol to give the chloroacetyl derivative **4** (1.2 g, 55%). Compound **4** (1.0 g, 1.48 mmol) was stirred with sodium azide (210 mg, 3.2 mmol) in dimethylsulfoxide (15 mL) at 70°C for 5 h. Water (50 mL)



1 e R = $(CH_2)_7CH{=}CH(CH_2)_7CH_3$ (*cis*)

1 f R = $(CH_2)_7CH{=}CHCH_2CH{=}CH(CH_2)_4CH_3$ (*cis*, *cis*)

1 g R = $(CH_2)_7C{\equiv}C(CH_2)_7CH_3$

Reagents and conditions: (i) 2-hydroxy-1-naphthaldehyde, methanol, reflux, 12 h; (ii) RCOOH, dichloromethane, dicyclohexylcarbodiimide, 4-dimethylaminopyridine, 24°C, 48 h; (iii) bromoacetic acid, tetrahydrofuran, 2°C, 2 h; (iv) *n*-butylamine, tetrahydrofuran, 2 min; (v) chloroacetyl chloride, triethylamine, tetrahydrofuran, 24°C, 12 h; (vi) sodium azide, dimethylsulfoxide, 70°C, 5 h; (vii) C_{60} fullerene, chlorobenzene, reflux, 12 h.

SCHEME 3

was added, and the reaction mixture was extracted with diethyl ether (4 × 50 mL). The ethereal extract was successively washed with water, brine, water (30 mL), dried over Na_2SO_4, and filtered. The filtrate was evaporated to give the corresponding azido derivative **5** (0.99 g, 89%). Compound **5** (315 mg, 0.42 mmol) in chlorobenzene (30 mL) was added dropwise to a refluxing solution of C_{60} fullerene (200 mg, 0.28 mmol) in chlorobenzene (150 mL) under an atmosphere of argon and was refluxed for a further 12 h. The reaction mixture was cooled, and the chlorobenzene was evaporated under re-

duced pressure to give a brown viscous residue. The residue was separated on a silica (30 g) column using toluene/*n*-hexane (1:1, vol/vol, 600 mL) to elute the unreacted C_{60} fullerene. The product was isolated by careful gradient elution using mixtures of diethyl ether/*n*-hexane (starting with 2:98, vol/vol, gradually increasing the amount of diethyl ether in the eluent until the final proportion reached 50:50, vol/vol; total volume of eluent used was 650 mL). Fractions of 10 mL each were collected during the chromatographic run, and the purity of the product was closely monitored by silica thin-layer chromatography. The requisite fullerene lipid **1a** (78.1 mg, 25% based on the amount of C_{60} fullerene consumed) was obtained as a brown solid.

Tris{[(dodecanoyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60]fullerene (**1a**): mp 51–54°C ; UV-VIS: λ_{max} (CH_2Cl_2, nm) 232, 258, 324; IR (KBr disc, cm^{-1}): 3372 (N-H, str.), 2924, 2852, 1744 (C=O, ester str.), 1692 (C=O, amide str.), 1524, 1465, 1378, 1151, 1113, 722, 527 (C_{60}, str.); ^1H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.88 (*s*, N*H*, 1H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, 2-*H* of acyl, 6H), 1.60–1.62 (*m*, 3-*H*, 6H), 1.24–1.42 (*m*, C*H*$_2$, 48H), 0.88 (*t*, *J* = 6.6, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.26 (C=O, ester), 168.13 (C=O, amide), 145.30, 144.94, 144.80, 144.60, 144.43, 143.97, 143.84, 143.54, 143.22, 142.96, 142.15, 141.52, 141.05 (C_{60}-*sp*^{2} C), 83.36 (C_{60}-*sp*^{3} C), 62.60 (*C*H$_2$OCOR), 58.27 [N*C*(CH_2OCOR)$_3$], 54.06 (C_{60}-N*C*H$_2$CO), 34.14 (C-2), 31.93 (C-10), 29.67, 29.38, 29.34, 29.22 (*C*H$_2$), 24.91 (C-3), 22.71 (C-11), 14.16 (C-12); mass spectral analyses (APCI): *m/z* 1443.4 (M$^+$), 1443.753 (calc.).

Tris{[(tetradecanoyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60] fullerene (**1b**): mp 53–54°C ; UV-VIS: λ_{max} (CH_2Cl_2) 232, 258, 324; IR (KBr disc, cm^{-1}): 3372 (N-H, str.), 2956, 2923, 2852, 1745 (C=O, ester str.), 1692 (C=O, amide str.), 1522, 1465, 1378, 1156, 1115, 722, 527 (C_{60}, str.); ^1H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.88 (*s*, N*H*, 1H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, 2-*H* of acyl, 6H), 1.60–1.69 (*m*, 3-*H*, 6H), 1.24–1.43 (*m*, C*H*$_2$, 60H), 0.87 (*t*, *J* = 6.6, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.25 (C=O, ester), 168.12 (C=O, amide), 145.29, 144.93, 144.79, 144.65, 144.47, 143.82, 143.41, 143.21, 142.95, 142.13, 141.03 (C_{60}-*sp*^{2} C), 83.37 (C_{60}-*sp*^{3} C), 62.56 (*C*H$_2$OCOR), 58.26 [N*C*(CH_2OCOR)$_3$], 54.04 (C_{60}-N*C*H$_2$CO), 34.14 (C-2), 31.94 (C-12), 29.71, 29.68, 29.57, 29.39, 29.34, 29.22 (*C*H$_2$), 24.90 (C-3), 22.71 (C-13), 14.16 (C-14); mass spectral analyses (APCI): *m/z* 1528.3 (M$^+$), 1527.915 (calc.).

Tris{[(hexadecanoyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2',3':1,2][60] fullerene (**1c**): mp 52–53°C [literature (Ref. 13): 50–51°C]; UV-VIS: λ_{max} (CH_2Cl_2) 232, 258, 324; IR (KBr disc, cm^{-1}): 3386 (N-H, str.), 2924, 2853, 1745 (C=O, ester str.), 1693 (C=O, amide str.), 1525, 1465, 1379, 1152, 1113, 721, 527 (C_{60} str.); ^1H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.88 (*s*, N*H*, 1H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.34 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, 2-*H*, 6H), 1.60-1.62 (*m*, 3-*H*, 6H), 1.20-1.30 (*m*, C*H*$_2$, 72H), 0.88 (*t*, *J* = 6.5, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.25 (C=O, ester), 168.13 (C=O, amide), 145.30, 144.94, 144.81, 144.66, 144.49, 143.83, 143.54, 143.23, 142.97, 142.17, 141.06 (C_{60}-*sp*^{2} C), 83.41 (C_{60}-*sp*^{3} C), 62.63 (*C*H$_2$OCOR), 58.29 [N*C*(CH_2OCOR)$_3$], 54.08 (C_{60}-N*C*H$_2$CO), 34.16 (C-2), 31.98 (C-14), 29.77, 29.73, 29.61, 29.43, 29.38, 29.25 (*C*H$_2$), 24.94 (C-3), 22.75 (C-15), 14.21 (*C*-16); mass spectral analyses (APCI): *m/z* 1611.7 (M$^+$), 1612.269 (calc).

Tris{[(octadecanoyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60] fullerene: (**1d**) mp 52–53°C ; UV-VIS: λ_{max} (CH_2Cl_2, nm) 234, 258, 324; IR (KBr disc, cm^{-1}): 3473 (N-H str), 2953, 2918, 2850, 1744 (C=O, ester str.), 1688 (C=O, amide str.), 1524, 1467, 1381, 1166, 1115.4, 739, 527 (C_{60} str.); ^1H NMR ($CDCl_3$, δ_H, J/Hz) 7.88 (*s*, N*H*, 1H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, C*H*$_2$COO, 6H), 1.60–1.62 (*m*, C*H*$_2$CH$_2$COO, 6H), 1.20–1.30 (*m*, C*H*$_2$, 84H), 0.88 (*t*, *J* = 6.6, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.23 (C=O, ester), 168.12 (C=O, amide), 145.28, 144.92, 144.78, 144.64, 143.82, 143.20, 142.94, 142.12, 141.04 (C_{60}-*sp*^{2} C), 83.37 (C_{60}-*sp*^{3} C), 62.59 (*C*H$_2$OCOR), 58.26 [N*C*(CH_2OCOR)$_3$], 54.04 (C_{60}-N*C*H$_2$CO), 34.13 (C-2), 31.94 (C-16), 29.74, 29.70, 29.58, 29.39, 29.35, 29.22 (*C*H$_2$), 24.90 (C-3), 22.71 (C-17), 14.16 (C-18); mass spectral analyses (APCI): *m/z* 1695.7 (M$^+$), 1696.239 (calc.)

*General procedure for the preparation of C_{60} fullerene derivatives containing unsaturated fatty acid moieties in the triester system (**1e–1g**) as exemplified by the synthesis of compound **1e** (Scheme 3).* A mixture of tris(hydroxymethyl)aminomethane **2** (3.0 g, 25 mmol), 2-hydroxy-1-naphthaldehyde (5.9 g, 25 mmol), and methanol (40 mL) was refluxed for 12 h. The solvent was evaporated under reduced pressure. The brown solid residue was washed with diethyl ether (2 × 20 mL) to give crude *N*-(2-hydroxynaphthyl)tris(hydroxymethyl)iminomethane **6** (5.3 g, 19.3 mmol, 77%) as a brownish yellow powder. The latter compound was mixed with oleic acid (5.0 g, 17.7 mmol), dicyclohexylcarbodiimide (5.0g, 24 mmol), 4-dimethylaminopyridine (90 mg, 0.7 mmol), and pyridine (20 mL), and the mixture was stirred vigorously at room temperature for 2 d. The reaction mixture was filtered. Water (30 mL) was added to the filtrate. The mixture was extracted with dichloromethane (2 × 30 mL). The organic extract was successively washed with dilute HCl (2 M, 30 mL) and water (20 mL), and dried over anhydrous Na_2SO_4. The solvent was evaporated under reduced pressure. The residue was separated on a silica (200 g) column by gradient elution using mixtures of diethyl ether/*n*-hexane to yield the triester **7** (1.42 g, 36%) as a fluorescent yellow oil.

A solution of the triester **7** (1.0 g, 0.94 mmol) in THF (20 mL) was cooled to 0°C in an ice bath. A precooled solution of bromoacetic acid (3.0 g) in THF (10 mL) was added dropwise to the triester solution, and the temperature of the reaction mixture was maintained at 0–2°C for 2 h. During this period the fluorescent yellow solution turned to a pale nonfluorescing yellow color. An additional amount of THF (20 mL) was added, and the reaction temperature was allowed to reach 20°C. *n*-Butylamine (2 mL) was then added when the reaction turned fluorescent yellow in color. Water (30 mL) was

added, and the reaction mixture was extracted with *n*-hexane (3×50 mL). The organic extract was washed with water (3×30 mL) and dried over anhydrous Na_2SO_4. The filtrate was evaporated under reduced pressure, and the residue was separated by flash silica (100 g) column chromatography (gradient elution with a mixture of diethyl ether/*n*-hexane) to give the amino-triester **8** (0.43 g, 50%) as a pale yellow oil.

Compound **8** (0.43 g, 0.47 mmol) and triethylamine (77 mg, 0.76 mmol) were dissolved in THF (20 mL). Chloroacetyl chloride (142 mg, 1.26 mmol) was slowly added. The reaction mixture was stirred for 12 h at room temperature and filtered. The filtrate was evaporated under reduced pressure, and the residue was separated by silica (70 g) column chromatography (gradient elution with a mixture of diethyl ether/*n*-hexane) to give the chloro-amido derivative **4** (0.39 g, 84%) as a viscous oil.

The chloro-amido derivative **4** (0.36 g, 0.36 mmol) was heated at 55°C with sodium azide (0.47 g, 0.73 mmol) in dimethyl sulfoxide (20 mL) for 12 h. A reddish orange oil was found to float on top of the reaction mixture. Aqueous sodium chloride solution (10%, 50 mL) was added, and the reaction mixture was extracted with diethyl ether (3×30 mL). The organic extract was washed with aqueous sodium chloride solution (10%, 2×20 mL) and dried over anhydrous sodium sulfate. The filtrate was evaporated under reduced pressure to give the corresponding azido derivative **5** (0.36 g, 98%) as a reddish orange oil.

A solution of the azido derivative **5** (0.36 g, 0.36 mmol) in chlorobenzene (50 mL) was added through a condenser to a refluxing solution of C_{60} fullerene (0.25 g, 0.35 mmol) in chlorobenzene (250 mL) under an argon atmosphere. The reaction mixture was refluxed for a further 12 h. The color of the reaction mixture turned from purple to dark brown. The solvent was evaporated under reduced pressure. The resulting viscous brown residue was dissolved in diethyl ether (100 mL) and filtered. The filtrate was evaporated, and the residue was separated by silica (30 g) chromatography using mixture of toluene/*n*-hexane, 1:1 vol/vol (100 mL), followed by gradient elution with mixtures of diethyl ether/*n*-hexane (starting with 2:98, vol/vol, gradually increasing the amount of diethyl ether in the eluent until the final proportion reached 50:50, vol/vol: total volume of eluent used was 650 mL). Fractions of 10 mL each were collected during the chromatographic run, and the purity of the product was closely monitored by silica thin-layer chromatography. The requisite fullerene lipid **1e** (72 mg, 24% based on the amount of C_{60} fullerene consumed) was obtained as a brown liquid.

Tris{[(oleyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60]fullerene **(1e):** UV-VIS: λ_{max} (CH_2Cl_2, nm) 232, 258, 328; IR (neat, cm^{-1}) 3349 (N-H str.), 3010 (C-H, olefin, str.), 2924, 2853, 1747 (C=O, ester str.), 1697 (C=O, amide str.), 1540, 1521, 1430, 1092, 821, 730, 526 (C_{60} str.), 417; ^{1}H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.88 (*s*, N*H,* 1H), 5.31–5.35 (*m*, C*H*=C*H*, 6H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, 2-*H* of acyl, 6H), 1.88–2.01 (*m*, 8-*H*, 11-*H*, 12H), 1.60–1.65 (*m*, 3-*H*, 6H), 1.21–1.29 (*m*, C*H*$_2$, 60H), 0.88 (*t*, *J* = 6.7, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.22 (C=O, ester), 168.11 (C=O, amide), 145.29, 144.93, 144.80, 144.65, 143.84, 143.22, 142.96, 142.16, 141.48, 141.04 (C_{60}-*sp*^{2} C), 130.03, 129.69 (*C*H=*C*H of acyl), 83.38 (C_{60}-*sp*^{3} C), 62.59 (*C*H$_2$OCOR), 58.27 [N*C*(CH$_2$OCOR)$_3$], 54.06 (C_{60}-N*C*H$_2$CO), 34.12 (C-2 of acyl), 31.92 (C-16), 29.79, 29.75, 29.55, 29.25, 29.21 (*C*H$_2$), 27.26, 27.22 (C-8, C-11), 24.89 (C-3), 22.70 (C-17), 14.15 (C-18); mass spectral analyses (APCI): *m/z* 1689.8 (M$^+$), 1690.191 (calc.).

Tris{[(linoleyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60]fullerene (**1f**): UV-VIS: λ_{max} (CH_2Cl_2, nm) 234, 256, 326; IR (neat, cm^{-1}): 3398 (N-H str.), 3009 (C-H olefin, str.), 2927, 2854, 1748 (C=O, ester str.), 1683 (C=O, amide str.), 1540, 1507.3, 1141, 1092, 1043, 724, 526 (C_{60}, str.), 418; ^{1}H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.88 (*s*, N*H,* 1H), 5.27–5.42 (*m*, C*H*=C*H,* 12H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.76 (*t*, *J* = 5.8, 11-*H*, 6H), 2.34 (*t*, *J* = 7.6, 2-*H* of acyl, 6H), 2.01–2.05 (*m*, 8-*H*, 13-*H*, 12H), 1.60–1.65 (*m*, 3-*H*, 6H), 1.30–1.47 (*m*, C*H*$_2$, 42H), 0.89 (*t*, *J* = 6.8, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.18 (C=O, ester), 168.10 (C=O, amide), 145.28, 144.92, 144.78, 144.64, 143.82, 143.21, 142.95, 142.14, 141.03 (C_{60}-*sp*^{2} C), 130.21, 122.97, 128.08, 127.90 (*C*H=*C*H of linoleyl), 83.37 (C_{60}-*sp*^{3} C), 62.57 (*C*H$_2$OCOR), 58.27 [N*C*(CH$_2$OCOR)$_3$], 54.04 (C_{60}-N*C*H$_2$CO), 34.09 (C-2), 31.52 (C-16), 29.64, 29.34, 29.23, 29.19 (*C*H$_2$), 27.21 (C-8, C-14), 25.65 (C-11), 24.86 (C-3), 22.59 (C-17), 14.11 (C-18); mass spectral analyses (APCI): *m/z* 1683.5 (M$^+$), 1684.143 (calc.).

Tris{[(stearolyl)oxy]methyl}iminomethane-*N,N*-acetyl-aziridino-[2′,3′:1,2][60]fullerene (**1g**): UV-VIS: λ_{max} (CH_2Cl_2, nm) 232, 256, 324; IR (neat, cm^{-1}): 3393 (N-H str.), 2928, 2854, 1747 (C=O, ester str.), 1697 (C=O, amide str.), 1522, 1458, 1157, 1095, 1035, 723, 526 (C_{60}, str.), 418; ^{1}H NMR ($CDCl_3$, δ_H, *J*/Hz) 7.87 (*s*, N*H*, 1H), 4.61 (*s*, C*H*$_2$OCOR, 6H), 4.35 (*s*, C_{60}-NC*H*$_2$CO, 2H), 2.34 (*t*, *J* = 7.6, 2-*H* of acyl, 6H), 2.12 (*t*, *J* = 5.9, 8-*H*, 11-*H*, 12H), 1.65–1.60 (*m*, 3-*H*, 6H), 1.46–1.27 (*m*, C*H*$_2$, 60H), 0.88 (*t*, *J* = 6.6, C*H*$_3$, 9H); ^{13}C NMR ($CDCl_3$, δ_C) 173.18 (C=O, ester), 168.12 (C=O, amide), 145.28, 144.93, 144.80, 144.65, 144.44, 143.83, 143.22, 142.96, 142.15, 141.55, 141.04 (C_{60}-*sp*^{2} C), 83.37 (C_{60}-*sp*^{3} C), 80.38, 80.05 (*C*≡*C*), 62.56 (*C*H$_2$OCOR), 58.3 [N*C*(CH$_2$OCOR)$_3$], 54.04 (C_{60}-N*C*H$_2$CO), 34.07 (C-2), 31.86 (C-16), 29.73, 29.23, 29.19, 29.14, 29.10, 28.91, 28.85, 28.73 (*C*H$_2$), 24.9 (C-3), 22.68 (C-18), 18.80 (C-8, C-11), 14.14 (C-18); mass spectral analyses (APCI): *m/z* 1684.5 (M$^+$), 1684.143 (calc.).

DISCUSSION

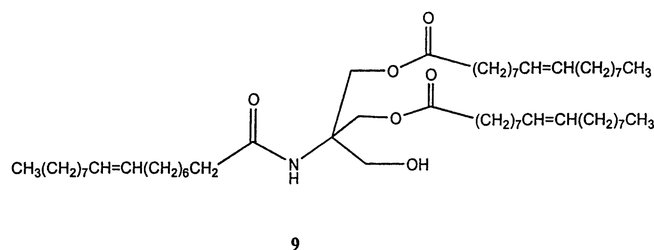
The formation of aziridinofullerene by reacting C_{60} fullerene with an azide is one of the promising protocols of fullerene functionalization (15). Murakami *et al.* (13) have reported a C_{60} fullerene lipid molecule **1** containing an interesting triester system, which was attached to C_{60} fullerene *via* an

aziridino bridge. These workers have produced only one C_{60} fullerene lipid where the triester system was made up of saturated C_{16} (palmitic) acyl chains. They have studied the phase transition properties of this molecule. Details of the synthesis methods used were not included in the publication. As fullerene chemistry is still in its infancy, we propose to study the synthesis of a series of homologs (containing C_{12} , C_{14}, C_{16}, C_{18} fatty acid acyl chains) of the C_{60} fullerene lipid **1** and to produce three similar C_{60} fullerene lipids but containing unsaturated fatty acid moieties in the triester (oleic, linoleic, and stearolic) system.

We found that preparation of the four C_{60} fullerene lipids (compounds **1a–d,** containing C_{12}, C_{14}, C_{16}, C_{18} fatty acid acyl chains) could be readily achieved once the precise methodology was established (Scheme 2). The esterification of tris(hydroxymethyl)-aminomethane **2** with saturated fatty acids in the presence of *p*-toluenesulfonic acid proceeded smoothly in high yields. The sulfonate salt of the amino-triester **3** reacted with chloroacetyl chloride to give the chloroamido derivative **4.** The latter, on treatment with sodium azide, furnished the key azido intermediate **5**. Reaction of C_{60} fullerene with the azido intermediate **5** furnished the requisite products **1a, 1b, 1c** and **1d** in 28, 46, 45, and 27%, respectively (based on the amount of C_{60} fullerene reacted). The most difficult part was encountered during silica column chromatographic separation of the fullerenoid products at the final stage. It was necessary to use a very careful gradient elution system (with small and increasing amounts of diethyl ether in *n*-hexane as the eluting solvent). The entire process was closely monitored by thin-layer chromatography.

When unsaturated fatty acids (oleic, linoleic, and octadec-9-ynoic acid) were reacted with tris(hydroxymethyl)aminomethane **2** in the presence of *p*-toluenesulfonic acid, a complex mixture of products was obtained in each case. *p*-Toluenesulfonic acid was also found to attack the double or acetylenic bond in the fatty acid. The reaction products could not be separated or purified by silica column chromatography for detailed analysis. The mixture of products showed that both the unsaturated centers of the fatty acids and the amino group of the reactant **2** were attacked by *p*-toluenesulfonic acid. Attempts to conduct the esterification process in the presence of dicyclohexylcarbodiimide (DCC) and 4-dimethylaminopyridine (DMAP) as described by Hassner and Alexanian (16) or by using a lipase (Novozyme 435, C*andida antarctica)* as the catalyst for the esterification were likewise unsuccessful.

It was therefore decided to block the amino function by reaction of this group with 2-hydro-1-naphthaldehyde (17). The blocked amino intermediate **6** was readily esterified with oleic acid in the presence of dicyclohexylcarbodiimide and 4-dimethylaminopyridine in pyridine to yield the corresponding ester **7** in good yields. Problems arose when attempts were made to deblock the protected amino group of compound **7** by the standard method (18) (*viz*., with 2 M HCl, CF_3COOH at room temperature). The ester groups were completely or partially hydrolyzed to yield fatty acids. We then decided to perform the deprotection procedure with bromoacetic acid, instead of HCl/CF_3COOH. However, when this reaction was carried out at room temperature, an undesired product **9** was isolated in >50% yield (Scheme 4). The structure of compound **9** was elucidated by a combination of spectroscopic analyses. Absorption bands in the IR spectrum showed the presence of a hydroxy group (3687 cm^{-1}), amide N-H (3602 cm^{-1}) group, and C=O stretching vibrations for the ester (1736 cm^{-1}) and amide (1665 cm^{-1}). The shifts of the N-H proton of the amide and O-H (D_2O-exchangeable) appeared at δ_H 5.99 (*s*) and 4.75 (*br.*, *s*), respectively. The presence of a hydroxy group was confirmed by the signal at δ_H 3.76 (*s*, CH_2OH, 2H). The shifts of the protons of the methylene group located between the quaternary carbon atom and the two oleyl acyl chains appeared as two distinct doublets at 4.43 (*d*, J = 11.5, CH_2OCOR, 2H) and 4.16 (*d*, J = 11.5, CH_2OCOR, 2H).



SCHEME 4

There were also two signals (2:1 intensity) for the 2-*H* shifts of the oleyl chains, which could be assigned to the ester acyl chains 2.34 (*t*, J = 7.5, CH_2COO, 4H) and to those of the amide acyl chain 2.19 (*t*, J = 7.5, CH_2CONH-, 2H). The shifts of the olefinic protons of the oleyl chains appeared at 5.20–5.40 (*m*, CH=CH, 6H). The following carbon shifts provided further proof of the structure of compound **9**: ^{13}C NMR ($CDCl_3$, δ_C) 174.60 (C=O, amide), 173.75 (C=O, ester), 130.06, 129.68 (CH=CH), 63.81 (-CH$_2$OCO-), 63.00 (-CH$_2$OH), 60.42 [NC(CH_2OCOR)$_2$$CH_2OH$], 37.29 ($CH_2$CONH), 34.12 ($CH_2$COOR), 31.92 (C-16 of acyl), 29.78, 29.75, 29.72, 29.55, 29.34, 29.29, 29.20, 29.13 (CH_2), 27.24, 27.18 (allylic carbon), 25.68 (CH$_2$CH$_2$CONH), 24.89 (CH$_2$CH$_2$COOR), 22.70 (C-17), 14.14 (C-18).

We rationalized that during this deprotection process, one of the three acyl groups in compound **7** migrated to the amino group to form an amido function (compound **9**). No anticipated free amino-triester **8** was isolated. Some of the starting material (compound **7)** was recovered.

After many trials to find a suitable deprotection procedure for compound **7**, we ultimately discovered a successful method for this purpose. Deprotection (to the extent of 50%) was made possible when the reaction was carried out with bromoacetic acid at 2°C for 2 h. Excess *n*-butylamine was immediately added to neutralize the reaction in order to prevent acyl migration. Despite the low temperature conditions, followed by neutralization of the reaction medium (with *n*-butylamine), the undesired product **9** was still obtained at about 20%. However, this undesired side product was readily sepa-

rated by silica column chromatography from the requisite amino-triester **8**. The isolated amino-triester **8** was then reacted with chloroacetyl chloride without delay. The reactions with sodium azide and subsequently with C_{60} fullerene were successfully accomplished.

By esterifying the protected amino intermediate **6** with oleic acid, linoleic acid, or octadec-9-ynoic acid, compounds **1e**, **1f**, and **1g** were obtained in 24, 37, and 23% yield (based on the amount of C_{60} fullerene reacted), respectively, at the last step of the reaction sequence. The four saturated C_{60} fullerene lipids (**1a–1d**) are brown solids with melting points in the range of 51–54°C. The three unsaturated C_{60} fullerene derivatives (**1e–1g**) are very viscous brown oils. Both sets of fullerene lipids are readily soluble in diethyl ether, dichloromethane or chloroform.

The proton NMR spectra of all C_{60} fullerene lipids **1** showed the characteristic shift of the N-H proton at δ_H 7.88. The signal for the shift of the methylene protons located between the aziridine ring and the amido function appeared at δ_H 4.35 as a singlet. The shifts of the methylene protons of the initial triol (δ_H 4.61, *s*) were readily differentiated from the 2-*H* (δ_H 2.34, *t*, J = 7.6 Hz) methylene of the long-chain acyl chain. The olefinic protons of the triesters containing oleic acid or linoleic acid appeared in the δ_H 5.3–5.0 region of the spectrum.

The ^{13}C NMR spectra showed 9–12 signals (δ_C 140–146) for the sp^2 carbon nuclei of the fullerene cage. The appearance of a signal (2C equivalent) at δ_C 83.4 is characteristic of the shifts of sp^3 carbon nuclei of the fullerene cage at which the aziridine ring is attached. This specific signal also indicates that the aziridine ring is joined to the junction of [6,6]-fused rings of the fullerene (13).

The shift of the carbonyl carbon for the ester and the amide were δ_C 173 and 168 respectively. The methylene carbon of the initial triol appeared at δ_C 54.1, whereas the quaternary carbon nucleus was shifted to δ_C 58.3. The signals corresponding to saturated and unsaturated (olefinic and acetylenic) fatty acyl chains were readily assigned by referring to results described for the carbon NMR properties of long-chain saturated and unsaturated fatty acids or esters (19). IR spectra of both saturated and unsaturated products showed vibrations for the N-H stretching (3370–3472 cm^{-1}), C=O stretching (1744–1748 cm^{-1}), and $(CH_2)_n$ rocking (about 720 cm^{-1}) (20). The characteristic C_{60} fullerene stretching vibrations appeared in 526–527 cm^{-1}.

The mass spectrometric analysis of the fullerene lipids (**1a–1g**) gave the corresponding M + 1 peak by atmospheric pressure chemical ionization (APCI) technique with the base peak appearing at 720. When these compounds were analyzed by either electron ionization or by fast atom bombardment technique, only the base peak was found at m/z = 720.

We conclude from this study that the synthesis of fullerenoid lipids containing saturated or unsaturated fatty acid moieties in the ester system can be achieved. From the NMR spectroscopic study it is evident that the aziridine ring is joined to the junction of [6,6]-fused rings of the fullerene.

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Separation and Determination of Glycolipids from Edible Plant Sources by High-Performance Liquid Chromatography and Evaporative Light-Scattering Detection

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ABSTRACT: Glycolipids from edible plant sources were accurately quantified by silica-based, normal-phase high-performance liquid chromatography using an evaporative light-scattering detector. Five major glycolipid classes (acylated steryl glucoside, steryl glucoside, ceramide monohexoside, monogalactosyldiacylglycerol, and digalactosyldiacylglycerol) were separated and determined with a binary gradient system consisting of chloroform and methanol/water (95:5, vol/vol) without any interference from other lipid classes and pigments. The described method was applied to 48 edible plants available in Japan including cereals, legumes, vegetables, and fruits. Examined plant species contained glycolipids in wide concentration ranges, such as 5–645 mg/100 g tissue.

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Glycolipids in higher plants mainly consist of steryl glucosides (SG), sphingoglycolipids, and glyceroglycolipids. These glycolipids are widely distributed in edible plants such as cereals, legumes, vegetables, and fruits (1,2). In previous studies (3–10), plant glycolipids have been quantified gravimetrically or colorimetrically after separating and isolating them with column chromatography or with thin-layer chromatography (TLC); however, these techniques are time-consuming and labor intensive.

High-performance liquid chromatography (HPLC) is one of the most popular tools for the analysis of lipid classes. Since gradient solvent systems have been employed for the separation of glycolipids, quantitative analysis with HPLC has been recognized to be difficult even with post-column detection by ultraviolet (UV), refractive index (RI), and fluorescence detectors. Recently, an evaporative light-scattering detector (ELSD) for HPLC, which is insensitive to the mobile-phase solvents and thus allows direct quantification, has been applied to lipid class analyses (11–20). Christie (11,12) and Lutzke and Braughler (13) have quantified lipid classes in rat liver, heart, plasma, and brain by HPLC–ELSD with a ternary gradient elution system. The HPLC–ELSD analyses of phospholipids in cooked beef (14), soybean lecithin (15), rat liver and heart (16), and human brain (17) have also been carried out.

For the HPLC–ELSD analysis of plant glycolipids, a complex ternary solvent gradient system (19) and several pretreatments for sample lipid fractionation before HPLC (20) have been needed. HPLC–ELSD has never been applied to the analysis of ceramide monohexoside (CMH) which is commonly distributed in plants (21–24).

The aim of this study was to develop the optimal HPLC–ELSD conditions for the separation and quantification of glycolipids in edible plants by using a simpler binary gradient system without any pretreatment of the Folch's lipid extract.

MATERIALS AND METHODS

Materials. Monogalactosyldiacylglycerol (MGDG) and digalactosyldiacylglycerol (DGDG) from wheat flour, and phosphatidylglycerol (PG) sodium salt from egg lecithin were purchased from Sigma Chemical Co. (St. Louis, MO). SG and acylated steryl glucoside (ASG) from soybeans were purchased from Funakoshi Co. (Tokyo, Japan). Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) from porcine liver, phosphatidic acid (PA) from egg PC, and phosphatidylserine (PS) from bovine brain were purchased from Funakoshi Co.

To isolate wheat flour CMH and remove glycerolipids, the total lipids extracted with chloroform/methanol (2:1, vol/vol) were saponified with 0.4 M KOH in methanol at 38°C for 2 h (23). The alkali-stable lipid fraction was recovered and applied to silica TLC (silica gel 60 F_{254}, 20 × 20 cm; Merck, Darmstadt, Germany) developed in chloroform/methanol/water (65:16:2, by vol) for the purification of CMH. Under UV light irradiation, the visualized lipid band corresponding to CMH was collected and extracted with chloroform/methanol (2:1, vol/vol). The wheat flour CMH isolated was analyzed by electrospray ionization mass spectrometry (ESI–MS) using a Mariner ESI/time of flight (ESI/TOF) mass

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Abbrevations: ASG, acylated steryl glucoside; CMH, ceramide monohexoside; DGDG, digalactosyldiacylglycerol; ELSD, evaporative light-scattering detector; ESI, electrospray ionization; HPLC, high-performance liquid chromatography; LC–MS, liquid chromatography–mass spectrometry; MGDG, monogalactosyldiacylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PS, phosphatidylserine; RI, refractive index; SG, steryl glucoside; TGDG, trigalactosyldiacylglycerol; TLC, thin-layer chromatography; TOF, time of flight; UV, ultraviolet.

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spectrometer (PE Biosystems, Milford, MA). In the mass spectrum of CMH, $[M + H]^+$ ions demonstrating molecular weight were found at *m/z* 716 and 772 corresponding to the principal species having ceramide moieties, in which 8-sphingenine was combined with hydroxy fatty acids of C_{16} or C_{20} (24). Thus, the wheat flour CMH served as a standard in HPLC.

All other reagents and chemicals used were extra-pure grade. All edible plants or products were purchased from a local supermarket in Sendai, Japan.

Lipid extraction. Five grams of the edible part of fresh plant samples was cut into pieces and heated for 1 min in a microwave oven (model RE-26-H5P, 500 W; Sharp Co., Osaka, Japan) to inactivate hydrolytic enzymes. Then the sample plants were further homogenized in a Waring blender with 20 mL of chloroform/methanol (2:1, vol/vol), and the total lipids (the Folch's lipid extract) were prepared as followed by the method of Folch *et al.* (25). The total lipids were redissolved in an aliquot of chloroform and were subjected to HPLC–ELSD for the analysis of glycolipids. The glycolipid concentrations were expressed as the mean of a paired analysis of the same sample.

HPLC–ELSD conditions. The HPLC system consisted of a sample injector (100 µL, Rheodyne model 7125; Rohnert Park, CA), two JASCO 980-PU HPLC pumps (Japan Spectroscopic Co., Tokyo, Japan), and a JASCO 860-CO column oven (40°C). The ELSD detector at post-column was an SEDEX model 55 (Sedere, Virty sur Seine, France), kept at an evaporation temperature of 60°C and at 2.0 bar pressure (2.7 L/min) for nebulization gas (nitrogen). The photomultiplier sensitivity was adjusted to gain six. The peak area was calculated with a Chromatocorder 21 (System Instruments, Tokyo, Japan). The HPLC column was a LiChrospher Si 60 (5 µm, 125 × 4 mm i.d., Merck).

The mobile phase consisted of chloroform and methanol/water (95:5, vol/vol) (Table 1), and the flow rate was 1.0 mL/min. It was necessary to reequilibrate the column with the starting solvent mixture for 10 min prior to a subsequent injection.

Liquid chromatography–mass spectrometry (LC–MS) analysis. LC–MS analysis was performed using a Mariner ESI/TOF mass spectrometer with a NANOSPACE HPLC apparatus (Shiseido, Tokyo, Japan) using the same conditions as described above, except that the methanol/water included 5 mM ammonium acetate as the mobile phase. The ESI mode (positive) was used for detection.

TABLE 1
Elution Program for a Binary Gradient System[a]

Time (min)	A (%)	B (%)
0	99	1
15	75	25
20	10	90
25	10	90
30	99	1

[a]Solvents: (A) chloroform; (B) methanol/water (95:5, vol/vol).

RESULTS

HPLC–ELSD of glycolipids. The glycolipids (ASG, MGDG, SG, CMH, and DGDG) were completely separated from each other with the new HPLC–ELSD conditions (Fig. 1). The elution order was ASG, MGDG, SG, CMH, and DGDG. The detection limits at a signal-to-noise ratio of 3 were 0.2 µg for SG, MGDG, and DGDG, and 0.5 µg for ASG and CMH.

The calibration curves were run with concentrations from 5 to 40 µg of glycolipids. The calibration curves were expressed as the equation $y = ax^b$, and the regression correlation coefficients were above 0.998 (Fig. 2). Detection sensitivity was highest for SG, with peak areas about three times greater than those of ASG and CMH. For three determinations at different concentrations of glycolipids, the standard deviation values were within 4% of the mean.

HPLC–ELSD of tomato and pumpkin extract. Figure 3 shows HPLC–ELSD chromatograms of Folch's extract of tomato. At least 10 peaks were detected for the tomato extract (Fig. 3A). ASG, MGDG, SG, CMH, and DGDG were eluted between 5 and 13 min following injection. The neutral lipids and pigments were eluted within the first 3 min. Phospholipids such as PE, PA, PI, and PC were eluted at retention times between 15 and 25 min. PI was coeluted with PA. The peak components detected by HPLC–ELSD of tomato extract were fur-

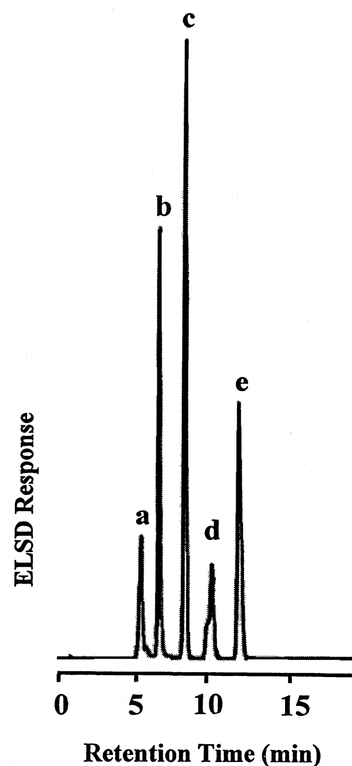

FIG. 1. High-performance liquid chromatograph–evaporative light-scattering detector (HPLC–ELSD) chromatogram of standard glycolipids. A mixture of 20 µg each of standard glycolipids was injected. Peaks: (a) acylated steryl glucoside (ASG); (b) monogalactosyldiacylglycerol (MGDG); (c) steryl glucoside (SG); (d) ceramide monohexoside (CMH); and (e) digalactosyldiacylglycerol (DGDG).

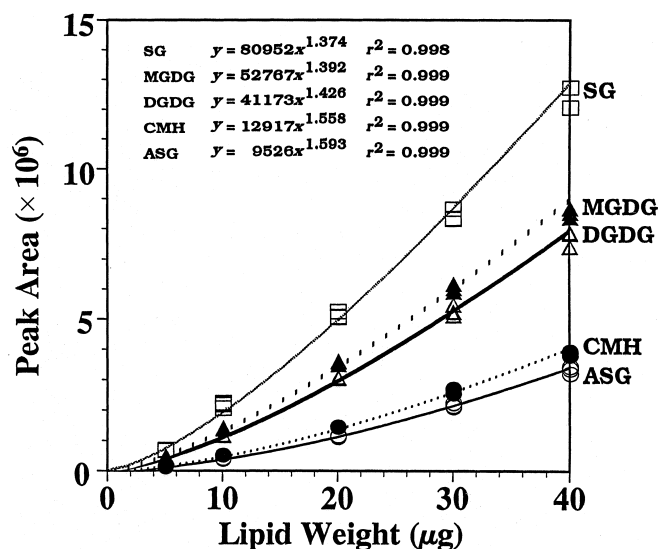

FIG. 2. Calibration curves of major plant glycolipids in HPLC–ELSD. Three determinations for each sample were given at different concentrations. ○, ASG; ●, CMH; ▲, MGDG; △, DGDG; and □, SG. See Figure 1 for abbreviations.

ther identified by LC–MS. In the mass spectra, the SG and ASG showed mainly molecular ions $[M + NH_4]^+$ at *m/z* 594 and 832 identical to the β-sitosteryl glucoside and the palmitate of β-sitosteryl glucoside, respectively (Fig. 4). The molecular ion peaks corresponding to the different constituent sterols (campesterol and stigmasterol) were also found. The MGDG and DGDG gave $[M + NH_4]^+$ at *m/z* 792 and 954, respectively, corresponding to the predominant species including 18:3 (α-linolenic acid)/18:3. In the mass spectrum of CMH, $[M + H]^+$ ion was found at *m/z* 714 originating from the species having 4,8-sphingadienine and C_{16} hydroxy fatty acid

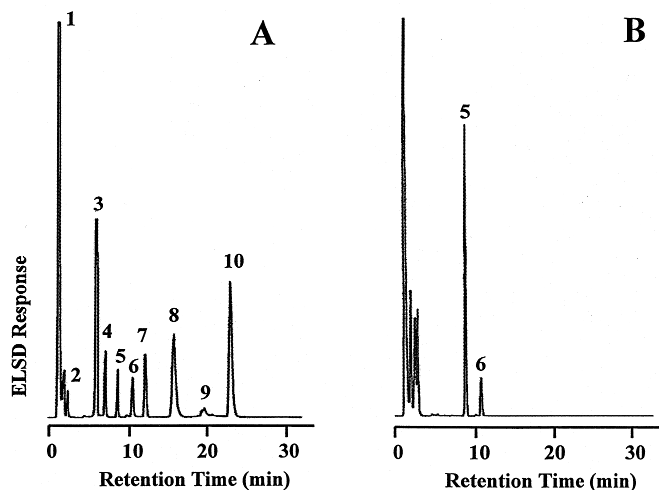

FIG. 3. HPLC–ELSD chromatograms of tomato extract. (A) Tomato extract (140 μg of lipid extract): (B) tomato extract after saponification for 2 h (injected an equivalent of A). Peaks: (1) neutral lipids + pigments; (2) free fatty acids; (3) ASG; (4) MGDG; (5) SG; (6) CMH; (7) DGDG; (8) phosphatidylethanolamine (PE); (9) phosphatidic acid + phosphatidylinositol (PA + PI); and (10) phosphatidylcholine (PC). See Figure 1 for abbreviations.

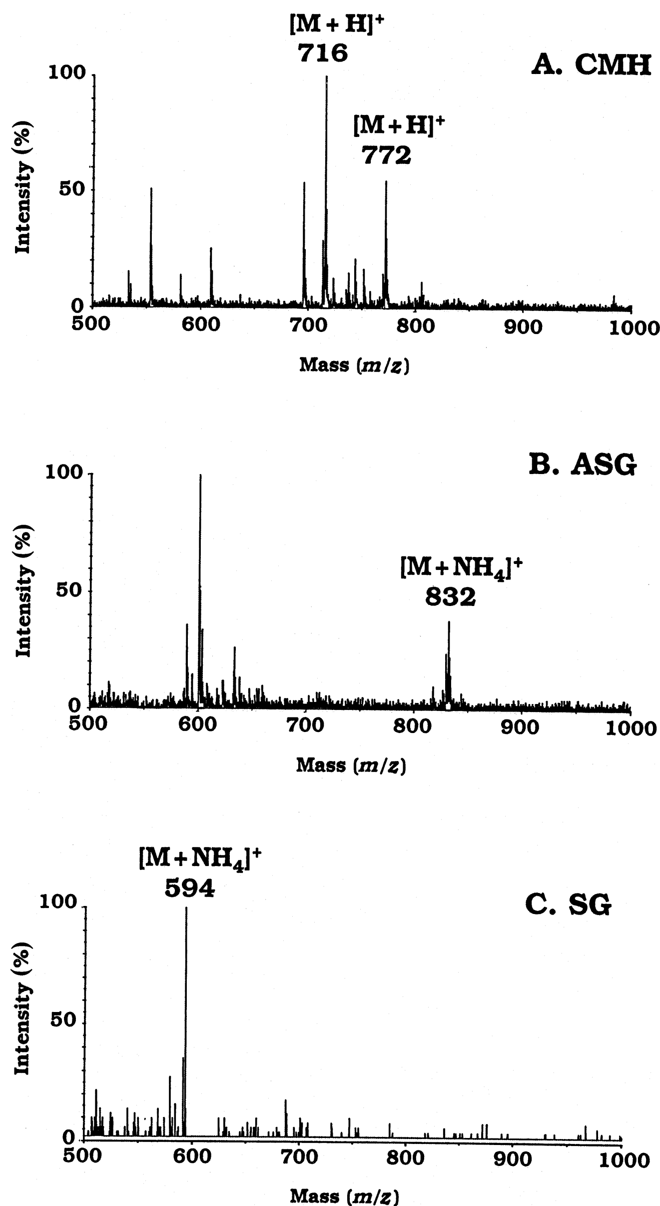

FIG. 4. Electrospray ionization–mass spectrometry (ESI–MS) spectra of CMH and SG. (A) Wheat flour CMH isolated as standard; (B) ASG of tomato extract; (C) SG of tomato extract. See Figure 1 for abbreviations.

(26). After mild alkaline treatment of plant lipid extract, only two peaks ascribed to SG and CMH were recovered (Fig. 3B).

For the pumpkin extract (Fig. 5), more than 10 peaks were detected and identified by co-chromatography with standard lipids. A peak component at retention time of 16.1 min was tentatively identified as trigalactosyldiacylglycerol (TGDG) because its molar ratio in fatty acid/sugar was 2.00:2.84. An LC–MS assay was further conducted to confirm the structure of TGDG. The mass spectrum of TGDG peak component showed $[M + NH_4]^+$ peak at *m/z* 1120 corresponding to the predominant molecular ion including 18:2/18:2 acyl residues (Fig. 6). The TGDG was also found in Folch's extract prepared from potato (data not shown).

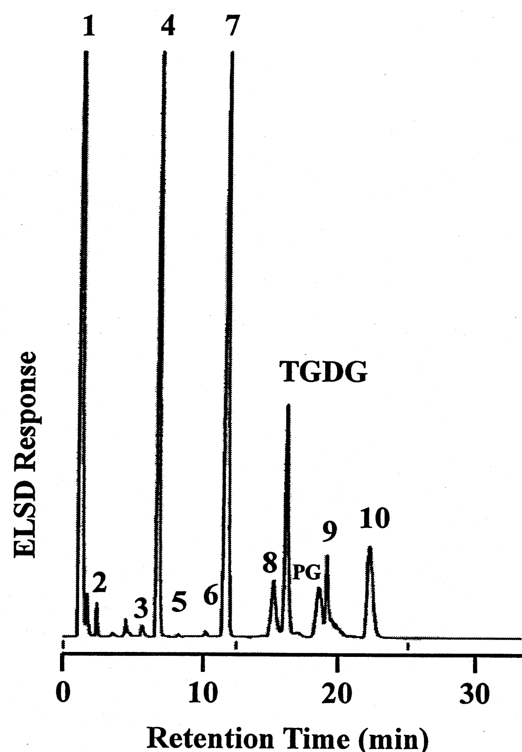

FIG. 5. HPLC–ELSD chromatogram of pumpkin extract. The Folch's extract (230 μg of lipid extract) from fresh pumpkin was injected into the HPLC–ELSD. Peak numbering and abbreviations are the same as given in Figure 3. TGDG, trigalactosyldiacylglycerol; PG, phosphatidylglycerol.

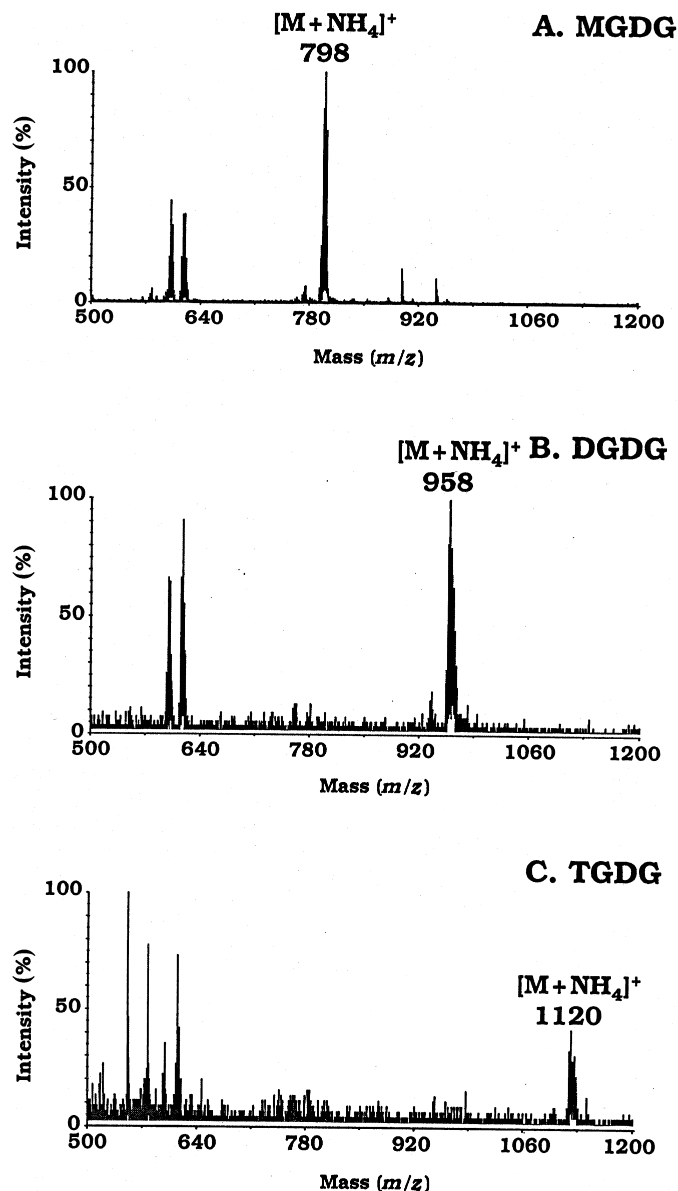

FIG. 6. ESI–MS spectra of glyceroglycolipids of pumpkin extract. See Figures 1, 4, and 5 for abbreviations.

Glycolipid composition of edible plants. Table 2 summarizes the glycolipid composition as tentatively determined by HPLC–ELSD method. The glycolipid profile was profoundly different among the plants examined, i.e., from 5.3 mg/100 g for Japanese radish to 644.9 mg/100 g for perilla leaf. Total glycolipid content was high in cereals (barley, corn, wheat flour), legumes (azuki bean, black soybean, pea), and leafy vegetables (leek, parsley, perilla, spinach). Barley, corn, and wheat flour were rich in DGDG; leek, parsley, perilla, spinach, and pumpkin were rich in MGDG and DGDG; azuki bean, black soybean, and perilla also contained remarkably high concentrations of ASG. Many fruits showed lower glycolipid concentrations, but were relatively high in ASG. The CMH also was uniformly distributed in those plants.

DISCUSSION

This study demonstrated the new HPLC–ELSD procedures for the separation and quantitative analyses of plant glycolipids using a binary solvent system. Since the plant glycolipids could be detected and quantified in a single HPLC run without any interference from other nonglycolipids, the procedure has advantages because it does not require subfractionation of glycolipids from Folch's lipid extract by TLC or solid-phase extraction before HPLC analysis. The present study revealed that the standard curves are quite different for ASG, MGDG, SG, CMH, and DGDG and are not linear relationships. Such variation as observed for the ELSD response may be partly ascribed to a dissimilarity in lipid specific particle sizes causing different light scattering (27).

Previous studies have reported that the ELSD response of neutral lipids (18), phospholipids (18,20,28), and glycolipids (20) is not proportional to their concentrations. Therefore, a separate calibration curve for each glycolipid was needed for accurate quantitative analysis with ELSD. Although dependent on lipid content, approximately 0.3 g of plant fresh weight was sufficient to determine glycolipid concentration by the present HPLC–ELSD conditions.

One strength of the present chloroform/methanol/water HPLC method is that it achieves better separation of the glycolipids than previously reported for hexane/2-propanol/

TABLE 2
Major Glycolipids of Edible Plant Tissues as Determined by HPLC–ELSD[a]

	ASG	SG	CMH	MGDG	DGDG	Total	S : C : G[b]
				(mg/100 g)[c]			
Cereals							
Barley	39.5	6.6	14.4	34.7	179.7	274.9	17 : 5 : 78
Corn	15.4	3.4	11.5	46.9	78.0	155.2	12 : 7 : 81
Rice (milled)	23.6	1.9	2.5	0.7	0.6	29.4	87 : 9 : 4
Wheat flour	25.6	5.4	21.0	40.1	134.2	226.3	14 : 9 : 77
Legumes							
Azuki bean	125.1	9.1	30.3	2.8	43.1	210.4	64 : 14 : 22
Black soybean	99.1	27.0	22.2	6.6	30.9	185.8	68 : 12 : 20
Pea	35.8	6.8	17.3	2.2	69.8	131.9	32 : 13 : 55
Snap bean (kidney bean)	6.8	4.6	15.8	23.0	21.1	71.3	16 : 22 : 62
Soybean	38.0	11.6	8.0	ND	5.0	62.6	79 : 13 : 8
Leaf vegetables							
Cabbage	4.5	3.2	10.0	10.5	8.3	36.5	21 : 27 : 51
Chinese cabbage	3.1	1.5	6.8	3.9	2.6	17.9	26 : 38 : 36
Leek	14.5	1.7	6.4	77.8	58.8	159.1	10 : 4 : 86
Lettuce	14.6	2.7	5.1	13.5	14.8	50.8	34 : 10 : 56
Parsley	23.0	3.8	4.4	183.8	115.7	330.8	8 : 1 : 91
Perilla	63.6	15.7	40.9	285.1	239.6	644.9	12 : 6 : 81
Spinach	25.3	12.3	13.9	85.0	56.3	192.9	20 : 7 : 73
Stem vegetables							
Asparagus	15.3	5.7	14.5	26.2	22.5	84.3	25 : 17 : 58
Broccoli	11.4	10.8	27.9	37.7	24.2	112.0	20 : 25 : 55
Cauliflower	15.5	6.3	18.2	10.8	14.1	64.9	34 : 28 : 38
Celery	5.9	1.1	2.0	2.6	2.9	14.6	48 : 14 : 38
Green onion	11.4	3.6	5.1	2.0	9.2	31.2	48 : 16 : 36
Onion	2.5	1.5	3.4	0.3	2.2	9.9	41 : 34 : 25
Tubers and root vegetables							
Aroid	16.9	2.2	15.1	22.8	26.4	83.4	23 : 18 : 59
Burdock	9.2	2.8	7.3	1.8	1.8	22.9	53 : 32 : 16
Carrot	7.2	2.2	1.4	4.0	6.6	21.4	44 : 6 : 50
Chinese yam	3.4	0.8	6.2	4.6	7.1	22.2	19 : 28 : 53
Garlic	17.2	3.4	5.9	2.2	3.2	31.9	65 : 19 : 17
Japanese radish	0.2	0.5	3.4	0.6	0.5	5.3	14 : 64 : 22
Lotus root	18.2	2.1	7.9	6.8	7.5	42.6	48 : 18 : 34
Potato	5.2	0.7	3.1	1.9	4.7	15.6	38 : 20 : 42
Sweet potato	15.1	5.6	14.1	9.7	22.6	67.0	31 : 21 : 48
Turnip	2.3	2.3	5.8	2.0	1.5	13.8	33 : 42 : 25
Fruit vegetables							
Cucumber	10.6	5.0	15.9	13.8	11.7	56.9	27 : 28 : 45
Eggplant	16.8	3.5	14.3	5.7	7.1	47.3	43 : 30 : 27
Pimento	9.5	2.3	14.8	14.7	14.5	55.7	21 : 26 : 52
Pumpkin	9.0	1.1	4.6	61.7	68.6	145.0	7 : 3 : 90
Tomato	8.6	1.2	3.4	2.1	2.3	17.6	56 : 19 : 25
Watermelon	3.0	1.0	1.4	1.2	3.3	9.9	41 : 14 : 46
Fruits							
Apple	1.4	3.2	7.1	0.7	1.2	13.6	34 : 52 : 14
Banana	1.0	1.4	1.2	0.8	3.8	8.2	29 : 15 : 56
Grape	3.2	3.1	7.9	1.0	0.9	16.2	39 : 49 : 12
Grapefruit	15.2	5.6	8.9	1.2	2.4	33.5	62 : 27 : 11
Japanese persimmon	3.2	2.6	3.8	1.3	3.5	14.3	40 : 26 : 33
Kiwifruit	7.9	3.8	6.2	6.5	7.9	32.3	36 : 19 : 45
Lemon	35.2	6.2	7.3	0.6	0.7	50.0	83 : 15 : 3
Satsuma orange	12.7	3.8	4.9	5.4	1.0	27.7	60 : 17 : 23
Pear	3.2	4.7	6.8	1.7	8.5	24.9	32 : 27 : 41
Strawberry	5.3	1.9	3.4	1.1	1.0	12.7	56 : 27 : 17
Total average	17.9	4.6	10.1	22.8	28.2	83.1	27 : 12 : 61

[a]Values are the mean of a paired analysis of the same tissue sample. ND, not detected.
[b]S:C:G = (ASG + SG)/CMH/(MGDG + DGDG) (%). ASG, acylated steryl glucoside; SG, steryl glucoside; CMH, ceramide monohexoside; MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.
[c]Fresh weight basis (barley, rice, wheat flour, azuki bean, black bean, pea, soybean were obtained by dry matter). HPLC–ELSD, high-performance liquid chromatography–evaporative light-scattering detection.

water methods (19,20). The mobile phase based on chloroform/methanol/water is popular for glycolipid separation in open-column silica gel chromatography (29). The ELSD detection made the gradient elution possible even with chloroform as mobile phase, which has low UV transparency, because chloroform emerging from HPLC column is immediately evaporated under a stream of nebulization gas.

Under the present chromatographic conditions, the retention times of human Gaucher's spleen glucocerebroside (9.4 and 11.2 min) and of bovine brain galactocerebroside (9.0 and 9.9 min) were clearly different from those of wheat flour CMH (10.1 and 10.4 min). The sphingoid bases (4-sphingenine, *d*18:1 and sphinganine, *d*18:0) of sphingolipids in mammals differ from those of plant CMH including 8-sphingenine (*d*18:1), 4,8-sphingadienine (*d*18:2) and 4-hydroxy-8-sphingenine (*t*18:1) (22). With mild alkaline treatment of plant lipid extracts, only two peaks ascribed to SG and CMH remained (Fig. 3B), since only O-acyl linkage is hydrolyzed, but N-acyl linkage is not (30). Thus, saponification was helpful to identify SG and CMH.

Morrison *et al*. (7) have reported that glycolipids in a high-grade spring wheat flour consist of 72 mg ASG, 94 mg MGDG, and 230 mg DGDG per 100 g dry weight. From our present data, wheat flour glycolipid concentrations were 30 mg ASG, 47 mg MGDG, and 158 mg DGDG per 100 g dry weight. Since the concentration of glycolipids in plants can vary tremendously depending on the cultivar, growth condition, stage of development, and harvested days, the data as given in Table 2 do not imply universal glycolipid contents. Furthermore, the natural diversity between members of the same species needs recognition. Thus, the data given in Table 2 represent typical glycolipid contents of commercial plant foodstuffs available in Japan from summer to winter. However, the present data compare well with those of previous studies (3–10) in light of probable differences in cultivar, locality, and harvest time of plants. The HPLC–ELSD condition as described here seems useful for the quantitation of plant glycolipids.

As shown in Table 3, glycolipids were not degraded and no artifacts were yielded as compared with the conventional hot 2-propanol during the present inactive procedure by microwave oven (31). The data given in Table 3 differ from those of Table 2 because of variations in locality, growth conditions, and harvest time.

TABLE 3
A Comparison of Recovery of Spinach Glycolipids When Hydrolytic Enzymes Are Inactivated by Conventional Hot 2-Propanol and Microwave Oven[a]

	Hot 2-propanol	Microwave oven
	(mg/100 g fresh spinach)	
ASG	19.5 ± 1.0	20.5 ± 1.6
SG	5.2 ± 0.6	4.8 ± 0.2
CMH	10.1 ± 1.2	10.6 ± 0.3
MGDG	112.4 ± 1.4	113.5 ± 0.6
DGDG	73.7 ± 2.1	78.3 ± 2.9

[a]Values are the mean ± SD of three experiments of the same sample. Spinach was harvested at Hokkaido in September 1999. Microwave oven from Sharp Co. (Osaka, Japan). See Table 2 for abbreviations

Dark green plants with abundant chloroplasts, such as leek, parsley, perilla and spinach leaves, had more glyceroglycolipids than light green plants (Table 2). It has been reported that in photosynthetic tissues, the amount of MGDG commonly exceeds DGDG, while in nonphotosynthetic tissues, DGDG is predominant (1,2). The occurrence of TGDG has been reported previously (1,32–34). In the present study, TGDG was detected in the pumpkin lipid extract by HPLC–ELSD and identified by LC–MS (Figs. 5 and 6). TGDG was also found in Folch's extract prepared from potato.

Although a limited number of samples were collected from local markets, the glycolipid composition of different species is given in Table 2. The biological significance of the ratio of sterol lipids/sphingoglycolipids/glyceroglycolipids is extremely interesting and merits further attention because of their nutritional function in humans.

Edible plant glycolipids have been expected to play a role in human nutrition as a nutrient, but little is known about them in intestinal digestion and absorption in mammals (35–37). Since linolenic acid (18:3n-3) accounts for more than 90% of the total fatty acids of MGDG in most plant leaves (2), plant glycolipids would be expected to be an important source for such n-3 essential fatty acid. To clarify the nutritional roles of plant glycolipids, estimating the daily intake in human body may be necessary. Based on the present data as given in Table 2 and the average daily food consumption per person (38), the average daily intake of glycolipids is estimated to be 140 mg ASG, 65 mg SG, 50 mg CMH, 90 mg MGDG, and 220 mg DGDG in a human.

In conclusion, the new HPLC–ELSD condition as described here is convenient and reliable for separation and determination of the plant glycolipids.

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REVIEW

Lipids in Human Milk

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ABSTRACT: I have reviewed recent (March 1995–December 1997) papers on human milk lipids including many on fatty acid (FA) composition. The effects of maternal diets on the profiles are apparent. However, more data on the composition of milk lipids are needed. It is noteworthy that so few papers on milk FA composition have reported analyses using high-resolution gas–liquid chromatography columns. Two of these were on milk from women in North America. The diets in North America are varied and the number of analyses few. We do not have a reliable data base showing the ranges of biologically important acids. Except for the gangliosides, few new data on the other lipids appeared during this period.

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In this paper, I review and evaluate papers on human milk lipids that were published from March 1995 through December 1997. Some relevant earlier papers are included. It is the latest in a series of my reviews on the subject, which began in 1978. The last was published in 1996 (1).

THE NATURE OF MILK

Milk is a very complex fluid, containing carbohydrates and salts in true solution, caseins in colloidal dispersion, cells and cellular debris, and lipids mostly in emulsified globules.

The lipids are triacylglycerols (TG, 98%), phospholipids (PL, 0.8%), cholesterol (C, 0.5%), and many others. The lipids (3–5%) occur as globules emulsified in the aqueous phase (87%) of milk. Nonpolar lipids, e.g., TG, cholesteryl esters, and retinyl esters, are found in the core of the globules (1,2). The globules are covered with bipolar materials—PL proteins, mucopolysaccharides, C, enzymes—organized into a loose layer called the milk lipid globule membrane (MLGM) (2). The MLGM acts as an emulsion stabilizer and represents the membrane of the secretory mammary gland cell. The globules range in size from 1 to 10 μM, with most of the globules less than 1 μM, but those around 4 μM accounting for the most weight. The globules present a large surface area (500 cm^2/mL) to lipolytic enzymes and other adhesive components.

Effects of milk lipids. Based on my earlier reviews, I have listed in Table 1 effects of milk lipids, both beneficial and detrimental. Further discussion will be found throughout the review. The unperturbed system delivers energy, nutrients, protective components, and metabolic messages to the infant. The emulsion is thermodynamically unstable, maintaining its original compartmentation for the few minutes of nursing. Thereafter, some constituents may change compartments, but the original value of milk for the infants apparently remains. However, off-flavors may develop to the extent that the infant will not consume stored milk.

Fatty acids (FA) in milk lipids (90% of TG as esters) are the only components that can be altered to any extent by maternal dietary manipulation. This observation suggests that the FA composition of milk may not be usable as a standard for the preparation of formulas because the range in contents is broad. However, profiles of milk FA related to the optimal growth and development of the infant could be used. As of December 1997, more analyses of milk FA by the best methods available were needed.

SAMPLING FOR AND DETERMINATION OF LIPIDS

Sampling. It is difficult to sample milk and obtain an aliquot that contains the true amount of lipid. The heterogeneously sized globules, which have a density of 0.9, rise at different rates and must be shaken at ≥38°C, to achieve a random distribution. The globules must be liquid. The globules also clump and these must be dispersed. In addition to the problems caused by the physical nature of the globules, the lactation process causes more difficulties, e.g., the lipid content rises as a nursing proceeds. Detailed sampling protocols are available and must be followed to obtain a representative sample (3–5). However, not discussed in References 3–5 are the cyclic changes in lipid content that occur in milks from malnourished women on low-fat, high-carbohydrate diets, relative to ingestion of meals. The lipid content changes diurnally, reaching maxima about 8 h after a meal. Several samples that bracket meal time should be taken and averaged to ascertain the true lipid content.

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Abbreviations: BSSL, bile salt-stimulated lipase; C, cholesterol; CE, cholesteryl ester; CLA, conjugated linoleic acid; DHA, docosahexaenoic acid; DG: diglyceride, diacylglycerol; FA, fatty acid; FAME, fatty acid methyl ester; FFA, free fatty acid; GLC, gas–liquid chromatography; HODE, hydroxyoctadecadienoic; HPLC, high-performance liquid chromatography; LBSA, lipid-bound sialic acid; LCPUFA, long-chain polyunsaturated fatty acids; LDL, low density lipoproteins; MG, monoglyceride, monoacylglycerol; MLGM, milk lipid globule membrane; MP, melting point; MMP, mean melting point; MS, mass spectrometry; PL, phospholipid; PUFA, polyunsaturated fatty acid; TG: triglyceride, triacylglycerol; TLC, thin-layer chromatography.

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TABLE 1
The Effects of Lipids in Human Milk[a]

1.	Source of 50–60% of the calories, about 70 kcal/dL, in human milk. Not usually responsive to diet.
2.	Fatty acids combined into triacylglycerols to maintain a bulk melting point below 38°C.
3.	Provide about 15 mg of cholesterol/dL. May predispose the infant to efficiently metabolize dietary cholesterol as an adult. Precursor of steroid hormones and other derivatives in humans. Not affected by changes in maternal diet.
4.	Contain the essential polyunsaturated fatty acids 18:2n-6 and 18:3n-3 and their products, 20:4n-6, 20:5n-3, and 22:6n-3. Required in maternal diet.
5.	The 20:4n-6, 20:5n-3, and 22:6n-3 in proper balance may be required for maturation and optimal function of the visual process and brain and nervous system.
6.	Contain 8:0–14:0, which if absorbed in the stomach, are transported to the liver and oxidized in decreasing amounts as the molecular weight increases. Quantities in milk dependent on amount of carbohydrate in diet.
7.	If present, the conjugated fatty acid, *c*9, *t*11-18:2, or rumenic acid, may exert anticarcinogenic, antiatherosclerotic, and other beneficial effects. Responds to ruminant products, particularly dairy products.
8.	*Trans* unsaturates believed by some to adversely affect infants growth. Some positional isomers of *c*-18:1 may ameliorate atherosclerosis.
9.	When produced by lipolysis in the stomach and small intestine, 12:0 and 18:2 and their monoacylglycerols have potent cidal effects against some microorganisms. Soluble and dispersible salts of fatty acids may act against microorganisms.
10.	Milk gangliosides inactivate cholera and other enterotoxins.
11.	Contain eicosanoids and their precursors, which act as first and second messengers. Precursors respond to diet.
12.	Lipolysis of triacylglycerols by gastric lipase, regioselective for *sn*-3/*sn*-1,3/1; produces *sn*-1,2-diacylglycerols, which can be second messengers in stomach and small intestine.
13.	Milk lipid globule membrane binds, protects, and releases bioactive compounds as needed. Graded doses of lipid provided. Membrane stabilizes globules in an oil/water emulsion.
14.	Increases in lipid content and numbers of globules during a nursing may help develop appetite and its control in the infant by approaching satiety and by the tactile effect of the globules.
15.	Lipid content increases as lactation progresses to help provide for growth and development of infant.
16.	Carrier of fat-soluble vitamins.
17.	Can transport undesirable compounds such as dioxins.
18.	When milk is stored in the frozen state, it can be the source of soapy flavor due to free 12:0 and to oxidized flavors (cardboardy) due to polyunsaturated fatty acids.
19.	Carotenoids, tocopherols, and conjugated 18:2 may be antioxidants.

[a]Derived from References 1–9. Specific references are given in the text.

To obtain the measured intake of milk and its contents by the infant, the amount of milk consumed must be determined. The phrase "measured intake" was used by Quinsey *et al*. (6) and aptly defines our goal: What does the infant receive in milk? The methods as described by Neville (7) are: (i) weighing of infant or mother before and after nursing, (ii) dilution of stable isotopes, and (iii) topographical computer imaging of the breast. Weighing is the least expensive and most readily applicable method. In brief, to determine the measured intake of milk lipids by a nursing infant, the investigator must (i) obtain a representative sample of milk, (ii) record descriptive data about the mother, the infant, and the event, and (iii) acquire the amount of milk consumed by the baby. With regard to point (i), it should be remembered that unless external manipulation is applied, there is no time during nursing or storage thereafter that the lipid globules in milk will be randomly dispersed to provide a sample containing the true amount of lipid.

Factors affecting total lipid content. These are listed in Table 2 with an indication of the nature of the change where known. Investigators must design their research and identify their subjects to control the relevant factors. Events that alter volume, i.e., time postpartum, mastitis, etc., must be considered.

The lipid content of milks from mildly malnourished women who were consuming a low-fat, high-carbohydrate diet oscillated diurnally. The maxima were reached in many but not all women about 8 h after a meal (8). The apparent causes of the decrease in lipid content were a rise in the synthesis rate of 8:0–14:0 and a drop in dietary 18:1 (see Fatty Acid section, which follows)

Determination. The methods most widely used are the Creamatocrit and solvent extraction followed by gravimetry (1,5,9). In the Creamatocrit procedure, a capillary tube containing milk is centrifuged and the length of the fat column is measured and calculated as a percentage of the total length of the milk column (10). This figure is converted to percentage fat using standard curves obtained by one of the other methods of determining fat, usually solvent extraction. The method is useful for fieldwork or where large numbers of samples must be analyzed. The Folch extraction, originally done with 2:1 chloroform/methanol, is and has been employed (11). Since chloroform is toxic and possibly carcinogenic, we and others substituted dichloromethane. A useful dry column extraction with these solvents is available (12). Jensen *et al*. (5,13) discussed the basics of and problems associated with solvent extraction of milk lipids.

TABLE 2
Factors Associated with Changes in the Total Lipid Content of Human Milk[a]

Factor	Effect
Duration of a nursing or feed	Increases
Age postpartum, stage of lactation	Increases
Diurnal rhythm	Variable; related to time of samples and maternal meals
Between breasts	Occurs
Gestational age at birth; preterm vs. term	Occurs
Diet regionality	May occur
Nutritional status	Malnourished women, decrease
High carbohydrate, low fat diet	May decrease
Infections, metabolic disorders	Usually decreases
Medication	
Mothers menstrual cycle or pregnancy	
Parity	Decreases
Season	Related to diet and regionality
Age	
Individuality	Adiposity increases
Miscellaneous	

[a]Adapted from Jensen (1).

Other procedures for quantitation are enzymatic, turbidimetry, colorimetry, gas–liquid chromatography (GLC), and infrared spectrophotometry (1,3,9).

Data on changes in the total lipid, protein, carbohydrate, and energy contents in 2,554 Danish milk samples from 224 donors to a milk bank, not previously shown in my reviews, are presented in Table 3 (14). The contents were measured by infrared spectrophotometry. Few papers are available with results based upon such a large number of samples. The authors observed that the lipid content (i) decreased during the first 4 mon, followed by an almost linear increase; (ii) increased slightly with increasing body mass index of the mother; (iii) was related in a U-shaped fashion to the infant's birth weight; <3.1 kg, 3.72%; 3.5, 3.21 after 4 mo., and ≥4.0, 4.04; and (iv) was higher in mothers who delivered large amounts of milk. We have accepted as fact the belief that volume and lipid content are inversely related. Volume and lipid content may respond to the infant's requirements.

LIPIDS IN MILK

Data on the general classes of lipids in humans are shown in Table 4. These data have been corrected for the products of lipolysis, that is, diacylglycerols (DG), monoacylglycerols (MG), and free fatty acids (FFA). Traces of these compounds will be found unless new milk is extracted immediately. The amounts will increase as storage lengthens at all temperatures except −70°C. The lipases in milk are inactive at this temperature.

TRIACYLGLYCEROLS

About 98% of the total lipid in milk is TG, and 90% of this is FA. Thus, esterified FA are 88% of the total lipid. Because of this and because it is much easier to analyze milk lipids for FA than for TG with more data on FA, we tend to ignore TG. This is a mistake because milk FA are secreted, consumed, and hydrolyzed as TG in globules.

The organization of FA into TG creates another factor to consider in the metabolism of TG—their structure. Structure can be defined as the identity of the FA in each of the *sn*-1, -2, and -3 positions of TG and the arrangement of these into individual TG. The positional identities are determined by stereospecific analyses and the individual TG by a variety of procedures (17). Structure of TG is important because it controls both the liquidity of the lipid globules and the identity of the products formed by the sequential lipolysis of the TG in the stomach and small intestine (18). The resultant FFA and MG are absorbed.

Milk lipids must be liquid so as to form microdroplets in the secreting cells; subsequently these fuse into droplets, which are then secreted as globules (19). The globules remain liquid as they encounter the lipases in the infant's stomach and small intestine. The large total surface of the globules

TABLE 3
Percentile Distribution of Protein, Fat, Carbohydrate, and Energy Contents of Milk from Danish Mothers[a]

	Percentiles								
	Avg.	2.5	10	25	50	75	90	97.5	Number of samples
Protein (g/L)	9.0	6.3	6.9	7.6	8.6	9.7	11.4	14.3	2553
Fat (g/L)	39.0	18.4	23.8	29.4	36.1	43.4	54.6	89.0	2554
Carbohydrate (g/L)	71.9	64.2	68.4	70.6	72.4	73.8	75.2	76.5	2554
Energy (kcal/L)	696	500	557	606	668	737	840	1155	2553

[a]Adapted from Michaelsen *et al.* (14). Components determined by infrared spectrophotometry, energy by calculation. Milk samples collected at a milk bank from 224 donors.

TABLE 4
Lipid Class Composition of Human Milk During Lactation[a]

Lipid class	Percentage of total lipids at lactation day 3	7	21	42	84	Immediate extraction
Total lipid % in milk	2.04 ± 1.32[b]	2.89 ± 0.31	3.45 ± 0.37	3.19 ± 0.43	4.87 ± 0.62	3.54
Phospholipid	1.1	0.8	0.8	0.6	0.6	0.81
Monoacylglycerol						ND[c]
Free fatty acids						0.08
Cholesterol (%)	1.3	0.7	0.5	0.5	0.4	0.34
(mg/d)[c]	34.5	20.2	17.3	17.3	19.5	—
1,2-Diacylglycerol						0.01
1,3-Diacylglycerol						ND
Triacylglycerol	97.6	98.5	98.7	98.9	99.0	98.76
Cholesteryl esters[d] (mg/dL)	5	1	1	1	1	1
n	39	41	25	18	8	6

[a]Adapted from Bitman *et al.* (15).
[b]Mean ± SEM, wt%.
[c]Total cholesterol content ranges from 10 to 20 mg/dL after 21 d postpartum in most milks.
[d]From Bitman *et al.* (16). ND, none detected.

provides a supersubstrate for the lipases. Holman *et al*. (20) developed a measure of liquidity or fluidity of membranes. They calculated the mean melting point (MMP) of the FA in plasma phospholipid (PL). We calculated MMP for some milk fats, finding a range of 27 to 32°C. A temperature of 27°C has been reported for milk lipids (21). Obviously, it would be much easier to determine melting point (MP) of extracted lipid in a capillary tube. This might show a relation between MP and changes in the FA composition. The MP is the average of all TG MP in the mixture. Changes in the FA composition of TG have a profound effect on MP, e.g., 16:0-16:0-16:0 has a MP of 66°C; 16:0-18:1-18:0, 38°C; and 18:1-18:1-16:0, 24°C (17).

Structure. Human milk generally contains seven major FA in amounts >1%. If these are randomly distributed among the three hydroxyls of glycerol, 343 (or 7 to the third power) TG would result (17). If a new FA is introduced to the maternal diet, then there would be more types of TG, i.e., 8^3 or 512. If the amount of an acid already present is increased, the quantities of the TG containing the acid are increased. Although the distribution of FA in milk TG is not random, the potential number of TG is probably in the thousands because about 200 FA have been detected. The total theoretically possible is 200^3, or 8,000,000! The TG structure is organ-specific because of the greater than random amounts of 16:0, 60–70 mol%, in the *sn*-2 position. The random quantity would be 33.3 mol%. Very little has been reported on TG structure since my last review (1).

The identities of the FA in *sn*-1, -2, and -3 positions have been determined by stereospecific analyses. Some data from French milks are given in Martin *et al.* (22). The structure of TG in colostrum was very similar to that of mature milk. Other data are presented in References 23–29. The distributions are unique with much of the 16:0 at *sn*-2 and other acids located unequally. The location of 16:0 at the *sn*-2 position improves the absorption of fat and calcium in the small intes-

TABLE 5
Amounts Exeeding 1% of Triglyceride (TG) in Pooled Human Milk with Proven Fatty Acid Profiles[a]

% of total (mol/100 mol TG)	Type of TG	% of total Type of TG (mol/100 mol TG)	Type of TG
11.8	16:0-18:1-18:1	2.1	16:0-18:1*t*-18:1
10.0	16:0-18:1-18:2	2.1	18:1-18:1-18:1
4.4	16:0-16:0-18:1	1.9	18:1-18:2-18:2
3.3	18:1-18:1-18:2	1.9	16:0-16:1-18:1
3.3	14:0-16:0-18:1	1.7	16:0-18:0-18:2
3.2	16:0-18:2-18:2	1.5	14:0-16:0-18:2
3.1	12:0-16:0-18:1	1.4	12:0-18:1-18:1
3.1	16:0-18:0-18:1	1.3	12:0-16:0-18:2
2.8	14:0-18:1-18:1	1.2	12:0-18:1-18:2
2.4	16:0-16:0-18:2	1.1	12:0-14:0-18:1
2.3	14:0-18:1-18:2	1.0	18:0-18:1-18:2

[a]Adapted from Winter *et al.* (28). Amounts are rounded to one decimal point. The most abundant TG of 170 are reported. Samples were obtained from Dutch mothers on a typical Western diet. The fatty acid sequences listed may not correspond with the actual structures.

tine. Carnielli *et al*. (24) fed formulas containing 13, 39, and 66% 16:0 esterified at the 2-position to infants. Absorption of fat was 90.4, 93.0, and 97.6% in the 13, 39, and 66% formulas. Calcium excretion was lowest in the 66% preparation.

Winter *et al*. (28) utilized high-performance liquid chromatography (HPLC) separation of milk TG with a silver ion column and light-scattering detector. Further fractionation and analyses resulted in a determination of 170 TG. Those present in quantities of 1% or more are given in Table 5. The amounts were nonrandom. According to Winter *et al*., the relatively nonpolar 16:0 and 18:0 appeared to be balanced by association with 18:1 and 18:2 or 8:0–12:0, thus maintaining the liquidity of the globule TG. The 8:0–12:0 FA were present in 19.0 mol% of the TG. Of these, 7.2 mol% were likely to be associated in enantiomeric structures, with an 8:0–12:0 at *sn*-3, 16:0 or 14:0 at *sn*-2, and 18:1 or 18:2 at *sn*-1.

To evaluate milk TG as a substrate for the lipase system described below, we need not determine individual TG. Identification of the acids in the *sn*-1, -2, and -3 positions will show the FA available to the lipases with different selectivities. Liquidity can be easily assessed by determining the MP of the extracted lipid in a capillary tube. Whether MP is related to TG structure in milk remains to be seen. A positive relationship has been observed in other fats (17).

Digestion of TG. Digestion of milk TG occurs in two phases, gastric and intestinal (17,18,30,31). Gastric lipase initiates lipolysis in the stomach, hydrolyzing 40–60% of dietary TG. The enzyme is regioselective for primary esters stereoselective for *sn* 3/1; 4/1, and it preferentially hydrolyzes shorter acids from a TG such as 18:1-18:1-10:0. A relatively large amount of *sn*-1,2 DG and shorter FA are produced. The DG could act as second messengers, and the acids are absorbed through the stomach wall in decreasing amounts as the molecular weight increases. They are then transferred *via* the portal vein to the liver. Gastric lipolysis is needed to initiate intestinal lipolysis because the globules resist pancreatic and bile salt-stimulated lipases (BSSL). Armand *et al*. (32) fed milk and formula to premature infants. They found that different FA profiles of globule sizes did not affect the level of gastric lipase. The TG within the milk lipid globules appeared to be more accessible than those in formula. Lauric acid (12:0) and 18:1 and their MG are potent microbicides at the high concentrations attained during digestion, helping control the growth of some microorganisms in the stomach and small intestine (33).

In the intestinal phase, the globules partially hydrolyzed by gastric lipase are further digested by the pancreatic-colipase system and by milk BSSL (18,31). Pancreatic lipase is highly regiospecific for *sn*-1+3 esters of TG. BSSL hydrolyzes esters from all positions, i.e., it has no regiospecificity. The lipase is elaborated in the mammary gland of humans, a few other primates, and some carnivores. The enzyme contributes to absorption of milk FA in the infant when pancreatic lipase and bile salt concentrations are suboptimal (32). With a nonregiospecific lipase present, there is a possibility that all of the milk acylglycerol FA are hydrolyzed. However, Innis *et al*. (34) recently found that the *sn*-2 16:0 was preserved in plasma TG of breast-fed infants (23.3% of the acids), but not in babies fed formula (7.4%). These results are convincing evidence that some 16:0 is absorbed as the 2-MG. The three lipases hydrolyze long-chain polyunsaturated fatty acids (LCPUFA) from the TG (35).

PL AND COMPLEX LIPIDS

The average amount of total PL is 0.6% of mature milk lipid, with a range of 0.39 to 0.97% (1). This is about 25 mg/dL of mature milk containing 4% fat [see also Glew *et al*. (36)]. The sources of variation are the decrease in PL content as lactation progresses (37) and the methods used. A method for the analysis of organic PL in milk lipids that does not require acid digestion is available (38). References 38–41 include data on types and amounts of milk PL. The PL originate on the secreting cell and envelop the globule as it is extruded. Most of the PL are located in the milk lipid globule membrane. In Reference 42, analyses of 2,000 Japanese milk samples showed seasonal changes in milk PL. Postpartum decreases in milk PL are given in Reference 43.

Classes. Recent data on kinds and quantities of PL are shown in Table 6. The separations carried out by Harzer *et al*. (39), Bitman *et al*. (37), and Glew *et al*. (36) were done with thin-layer chromatography (TLC) and those of Hundreiser

TABLE 6
Phospholipids (% of total, means ± SD) in Human Milk

	Glew *et al.*[a]		Holmes-McNary *et al.*[b]
Phospholipid	MM (mol%)	SM (mol%)	(mol%)
Phosphatidyl choline	27.7 ± 0.9	22.1 ± 1.1	82 ± 6
Phosphatidyl ethanolamine	15.2 ± 1.9	12.8 ± 1.3	
Phosphatidylserine + -inositol	5.9 ± 1.0	5.6 ± 2.5	
Sphingomyelin	51.3 ± 1.6	59.5 ± 4.0	24 ± 9
Choline			116 ± 22
Glycerophosphocholine			362 ± 70
Phosphocholine			370 ± 36

[a]Adapted from Glew *et al.* (36). Nigerian donors; MM, moderately malnourished, n = 10; SM, severely malnourished, n = 3. Phospholipids separated by thin-layer chromatography (TLC).
[b]Adapted from Holmes-McNary *et al.* (44). U.S. donors, hospital milk banks, mature samples. Separation by high-performance liquid chromatography (HPLC).

and Clark (40), van Beusekom *et al*. (41), and Holmes-McNary *et al.* (44) with HPLC. The breast-fed infant derives most of its choline, a conditional required nutrient, from the compounds containing choline (44), as listed in Table 6. Glyceryl ethers have also been identified in human milk (45,46).

Complex lipids other than PL. Milk contains sphingomyelins, acidic glycosphingolipids or gangliosides, and neutral glycosylceramides (1,13). The amounts of those components are: sphingomyelins, 100–200 µM (47–49); neutral glycosylceramides, 1–20 mg/L (49–52); and gangliosides, 1–20 mg/L (51–53). Sphingomyelin is usually classified and reported as a PL, but it is also a sphingolipid. It is the phosphorylcholine ester of an *N*-acyl-sphingosine or ceramide. Neutral glycolipids are composed of a ceramide bound to one or more glycosyl units. Monoglycosylceramides are termed cerebrosides. Sulfatides are galactosyl-3-sulfate esters of cerebrosides and are acidic. Information about these lipids in human milk is available in References 1, 13, and 53–57. Gangliosides are glycosphingolipids that contain sialic acid (*N*-actylneuraminic acid, NANA) as part of the carbohydrate group and are acidic. The nomenclature of Svennerholm (58) will be used in the remainder of this section for the gangliosides. Briefly, G is ganglioside; M, D, and T are mono-, di-, and trisialo groups, respectively, and the number is arbitrarily assigned on the basis of chromatographic migration. These compounds are amphiphilic (bipolar), have several important biological effects, and are concentrated in the milk lipid globule membrane.

The FA attached to sphingosine in milk by a peptide linkage are 18:1 (61.8%) with most of the remainder being 20:0, 22:0, 24:0, and 24:1 (13). The monohexosyl ceramides contained long-chain hydroxylated and nonhydroxylated FA, mostly 24:0.

Takamizawa *et al*. (52) noted that GD3 was high early in lactation, decreasing as lactation progressed and GM3 increased. The ganglioside GM1 binds the enterotoxins of *Vibrio cholerae, Escherichia coli,* and *Campylobacter jejuni* (51,57). Keenan and Patton (59) postulated that gangliosides promote fusion of microdroplets of lipid into globules in the mammary gland secreting cell. Newburg *et al.* (56) found that the globo- series of glycolipids bound shigatoxin and the shiga-like toxin receptor, Gb3. Sulfatide and sulfated lactosyl ceramides have been identified in milk (54). The same compounds may be relevant to the protection of the breast-fed infant from several pathogens including HIV.

New information on gangliosides was reported by Rueda *et al*. (53,60,61). In addition to GM3 and GD3, they found six others, possibly GD1a, GD1b, GT1b, and GQ1b. Two were tentatively identified as polysialogangliosides. They observed that GD3 was relatively the most abundant ganglioside during the first 3 wk of lactation (53) and was higher in preterm than in term colostrum (60). Conversely, GM3 was present in greater amounts 1 mon postpartum and in term as compared to preterm colostrum. They noted that GD3 is usually found in developing tissues. Rueda *et al.* (61) found no significant changes in content and distribution of gangliosides in human milk from Spanish and Panamanian mothers. Profound differences would not be expected since gangliosides are part of the MLGM and originate from the secreting cell. The ganglioside content, expressed as µg lipid-bound sialic acid (LBSA) per g of lipid, decreased as lactation progressed. The decrease was significant in Panamanian milks but not in samples from Spain. These data were obtained by TLC separation, utilization of the bands, and densitometry and are given as µg of LBSA.

Readers who are interested in the relation between the complex lipids and brain development should read the book by Jumpsen and Clandinin (62). It contains useful and enlightening information.

Sterols. Very few papers on milk sterols have appeared during the review period. Earlier research established that the sterol content in mature human milk ranges from 10 to 20 mg/dL with C as the major component (1,13). Glew *et al*. (36) found 25 mg/dL with TLC and GLC. Huisman *et al*. (63) detected 12 to 16.6 mg/dL using GLC and did not report any other sterols. They also present data on mono- and disaccharides and sugar alcohols. Japanese data on C contents relative to time postpartum appeared in References 42 and 43. Most of the C is located in the MLGM, and the amount is not affected by diet or by maternal plasma levels, but is correlated with fat content (1,2). About 15% occurs as the ester. C has been determined by colorimetry (*o*-phthalaldehyde), enzymatic treatment, GLC, and HPLC. We use and recommend the *o*-phthalaldehyde method of Bachman and Wilcox (64) for routine measurement of C. GLC must be employed for analysis of sterols. Kallio *et al*. (65) analyzed milk for C, its precursors, and other sterols by GLC–mass spectrometry (MS) (Table 7). They concluded that in the mammary gland, C is synthesized from lanosterol *via* preservation of the side-chain double bond. However, the amount of C synthesized in the gland is unknown. Note that C accounted for 88.2% and desmosterol 8.4% of the total sterols at 2 mon. In contrast to earlier reports, the amounts of phytosterols were negligible. Small amounts of other sterols have been reported (65–69).

C content increases along with fat content during a nursing (1,13). Diurnal rhythms have been detected, but this may

TABLE 7
Amounts (µg/dL) of Cholesterol and Its Precursors and Triacylglycerol in Human Milk at 2, 6, and 9 mon of Lactation[a]

	Months of lactation		
	2 (*n* = 88)	6 (*n* = 28)	9 (*n* = 6)
Squalene	386	493	452
Lanosterol	94	98	115
Dimethylsterol	45	62	62
Methostenol	48	72	88
Lathosterol	43	89	112
Desmosterol	1,509	1,351	1,140
Cholesterol	15,800	18,000	18,900
Triacylglycerol (g/dL)	2.96	3.89	4.25

[a]Adapted from Kallio *et al.* (65).

be dependent on the interval of sampling (70). The infants in this study consumed 94 mg C/d. Wong *et al*. (71) reported an intake of 116 mg/d. A range of consumption can be calculated as 75 to 150 mg/d based on milk contents of 10 to 20 mg/dL and a daily consumption of 750 mL. The C content of milk decreases [incorrectly stated as increases in (1)] as lactation advances: for example, (i) days postpartum; (ii) mg C/dL of milk; and (iii) lipid content (%); 0 to 4, 36.0, 1.0; 5 to 9, 19.7, 2.7; and 10 to 30, 19.0, 4.3 (72; see also 42,43). Boersma *et al*. (72) suggested that this continual decline in mg C/dL while fat levels were also increasing, was associated with the increase in globule diameter and thinner membranes that occur as lactation lengthens. No data have been published yet on globule numbers and diameters and C content. With a given amount of fat, the total globule area decreases as the average globule diameter increases.

Reiser *et al*. (73) suggested that the "high" levels of C in human milk could contribute to homeostasis of the sterol in the adult who was breast-fed as an infant. Although the C content of milk is not high (15 mg/dL), the consumption by the infant as compared to the adult is elevated. A 4-kg infant can have a daily intake of 25 mg/kg body weight (100 mg C/4 kg). A 70-kg adult should consume no more than 300 mg of C daily or 4.3 mg/kg body weight. The infant's intake is 5.8 times that of the adult.

In this summary, the effects of milk and formulas fed to infants on C metabolism were compared. Exceptions are noted. Concentrations of mRNA for low density lipoprotein (LDL) receptors were increased in baboons (74). The fractional synthesis rate of C was decreased (71), while concentrations of LDL and total serum C increased (73–76). C excretion was increased and endogenous synthesis decreased (76). Weaning from milk increased the concentrations of serum lipoprotein (a), which is associated with higher risk of coronary artery disease (77). Cruz *et al*. (78) noted that isoflavones (phytoestrogens) in soy-based formulas were absorbed, excreted, and negatively associated with the synthesis of C, but not serum C. The soy isoflavones daidzein and genistein were quantified in human milk (79). The contents were raised when roasted soybeans were fed to the donors.

FA AND RELATED COMPOUNDS

I have discussed how to analyze FA with GLC in previous reviews (1,9,13,80) and will not repeat all of it here. I will present the contents of relevant papers and summarize my suggestions for analysis of milk FA. The important message is that there are few papers containing data on milk FA in which GLC analyses were done with high-resolution columns. Nevertheless, some reliable data have been published. A method for determination of total TG content of milk by GLC of FA has been described (81). An added internal standard is the basis for the calculation of the weight in each GLC peak. Kohn *et al*. (82) examined several methods for the transesterification of milk TG, PL, and CE to fatty acid methyl esters (FAME). They used extracted milk fat that contained 10% FFA, prepared FAME, and analyzed these by GLC. They recommended the boron trifluoride method for milk and the sodium methoxide method for formulas, but not milk. Their milk sample contained FFA, which cannot be methylated by sodium methoxide. Instead, calcium salts (i.e., soaps) formed, which cannot be methylated. This is well known and has been mentioned (80). Acid and base catalysts are required (discussed below). Unless stored, human milk samples contain very low concentrations of FFA. Kohn *et al*. (82) did not test the use of 10% sulfuric acid (83) or the direct, no extraction method of Lepage and Roy (84). These, the boron trifluoride (85), and the HCl-catalyzed (86) methylations have all been employed for analysis of human milk. Assessing the conversion of TG to FAME would have been much easier with TLC. The disappearance of TG and appearance of FAME can be easily monitored. We have done this with all the methods above and observed complete conversion to FAME.

Kramer *et al*. (87) found that sodium methoxide-catalyzed methylation was the best compromise for bovine milk FA followed by hydrochloric acid or boron trifluoride. This procedure caused less isomerization of conjugated dienes, but sphingomyelin was ignored. However, there is very little sphingomyelin in milk PL. The failure of sodium methoxide to methylate FFA can be eliminated as described above, and the recommended methods can be used to methylate the FA in human milk lipids.

Analysis of milk FA by HPLC was done by Takayama (88). The method, as reported, was not superior to GLC analysis. For example, 14:0 and 22:6n-3 coeluted.

FA compositions are usually reported as relative weight percentages based on the peak areas from the GLC chart. Although rarely given, weights of FA/dL or gravimetric reports are necessary so that the actual FA intake by the infant can be calculated and the real effects of intervention seen. The amounts of FA can be ascertained gravimetrically or by use of an internal standard (81).

Another method for presentation of total fat and FA contents on food labels is being required in the United States by the U.S. Food and Drug Administration and the Department of Agriculture (89). Fat must be expressed as the sum of TG equivalents. For example, if the weight of 16:0 taken from the GLC chart is 1 g, then it is reported as 1.04946 g of tripalmitin. The conversion factor of 1.04946 is derived as follows:

$$\frac{\text{mol wt of tripalmitin}}{3\ (\text{mol wt of 16:0}} = \frac{807}{3(256)} = 1.04946 \qquad [1]$$

This calculation is to be done for each acid, or the factors published in Reference 89 can be used for total fat. The factors range from 1.074 for 10:0 to 1.037 for 22:0. Most are close to 1.05, which can be used. If FAME (16:0 ME, 3 × 270 = 810) are inserted into the calculation, a factor for conversion of ME to TG of 0.99+ results. Although not stated in Reference 89, this means that the weights of FAME and triglyceride fatty acid are equal and that the FAME units summed equal the

sample weight with allowance for dilutions. Conversely, the sample weight multiplied by the relative percentage of a FAME equals the weight of the FAME. The glyceride fragments of CH_3O- and CH_2O- and the methyl portion CH_3O esterified to FA have almost the same molecular weights, 30 and 31. The point is that the weights of FA calculated from the areas on a GLC chart in relation to an internal standard will not equal the weight of the extracted lipid because the glyceride fragment is not included. The difference can be corrected by using the conversion factors, usually 1.05 (Eq. 1). Milk FA enter the infant as TG, but we ignore the glycerol. It can be oxidized, enter a biosynthetic sequence, or be absorbed as a 2-MG. It is not lost and should be reported as a portion of the TG.

My instructions for determination of milk FA are these: (i) Obtain a representative sample of milk with documentation (3–5). (ii) Extract the sample using an approved method and weigh the total lipid (3). If not used immediately, store in hexane at −70°C. (iii) Convert the TG to FAME using sodium methoxide (85) for fresh samples and for samples with minimal lipolysis or else use a procedure that employs base and acid catalysts when lipolysis is suspected in samples stored at −20°C for two or more weeks (90). Add an internal standard for calculation of sample total lipid weight (81,89). (iv) Analyze the FAME by GLC using a wall-coated open tubular column with a polar coating of SP-2340, SP-2560 (Supelco, Bellefonte, PA), or equivalent, at least 30-m long, preferably 100 m. The column must be capable of resolving polyunsaturated fatty acids (PUFA) and *cis* and *trans* isomers of 18:1. (v) Identify every FA to the extent possible. Report the amounts of unknown FA. (vi) Report the total lipid content, the relative weight percentage of every FA that has an integration value, the weight (mg or g) of each FA/dL milk, and the TGFA in weight/dL. Do not eliminate FA because they are less than some arbitrary value. Use both acid- and base-catalyzed transesterification for frozen samples kept longer than 3 wk (90).

Factors affecting composition. Many of the factors affecting lipid content, listed in Table 2, do not alter the FA profile of milk, although the absolute quantities can be changed. Exceptions are time postpartum, gestational age, parity, diseases, individuality, and diet (1,8,80). Diet has the greatest effect. The effects of geographic region and season are undoubtedly due to diet. I will present information from recent papers and some earlier publications that were not quoted in my recent reviews. First, I shall include information about the regulation of TG synthesis in the mammary gland from a review by Neville and Picciano (91).

Neville and Picciano (91) discuss evidence that FFA inhibit acetyl CoA carboxylase in a feedback regulatory loop. This hypothesis explains why synthesis of 8:0–14:0 in the mammary gland is decreased on high-fat and fasting diets. Lipoprotein lipase produces FFA on the endothelial surface of the mammary gland cells on the high-fat diet. Adipose tissue lipase releases FFA in the fasting regimen. These enter the circulation and are transported to the gland and other tissues.

Conversely, it is well known that a high-carbohydrate, low-fat diet stimulates synthesis of 8:0–14:0 in the mammary gland. The total 8:0–14:0 rises from about 10 to 20% or greater. The quantities of FFA, from the diet or from adipose tissue, needed to inhibit acetyl Co-A carboxylase are not available. Glucose is the precursor of the mammary gland 8:0-14:0 FA.

TABLE 8
Saturated Fatty Acids (wt%) in Human Milk from Japanese Donors at Various Times Postpartum[a] and in Hungarian Milk[b], for Comparison of GLC Columns

	Japanese donors, days postpartum (number of samples)[a]								
Fatty acid	3–5 (110)	6–10 (181)	11–15 (177)	16–30 (351)	31–60 (562)	61–120 (314)	121–240 (280)	241–482 (149)	Hungary[b] (250)
6:0	0.07	0.10	0.12	0.10	0.08	0.08	0.10	0.05	—
10:0	0.81	1.29	1.62	1.45	1.33	1.25	1.34	1.09	—
12:0	4.50	6.00	6.95	5.72	3.20	5.21	5.95	5.80	2.1
13:0	0.04	0.04	0.03	0.02	0.02	0.02	0.02	0.03	—
14:0	7.26	7.67	7.64	6.58	6.15	6.42	7.28	8.54	4.9
i-15:0[c]	0.06	0.17	0.06	0.06	0.07	0.06	0.07	0.06	—
ai-15:0[c]	0.08	0.09	0.08	0.08	0.08	0.08	0.08	0.07	—
15:0	0.32	0.33	0.29	0.29	0.29	0.28	0.28	0.27	—
i-16:0	0.06	0.06	0.06	0.06	0.06	0.05	0.06	0.06	—
16:0	23.92	22.90	21.46	21.42	21.36	21.38	20.74	21.88	26.20
i-17:0	0.13	0.13	0.12	0.13	0.14	0.13	0.13	0.12	—
ai-17:0	0.15	0.15	0.15	0.16	0.16	0.15	0.15	0.14	—
17:0	0.34	0.35	0.32	0.33	0.33	0.32	0.32	0.32	—
18:0	5.49	5.67	5.77	6.04	6.13	6.53	6.33	6.47	9.80
20:0	0.21	0.19	0.20	0.02	0.21	0.21	0.20	0.21	—
22:0	0.08	0.07	0.06	0.06	0.07	0.07	0.06	0.06	—
24:0	0.12	0.07	0.05	0.04	0.04	0.04	0.04	0.03	—

[a]Adapted from Idota *et al.* (92). Neither paper in last review (1); 30-m capillary column, DB wax coating.
[b]Adapted from Gere *et al.* (93); packed column. Samples from 1, 3, and 6 mon combined.
[c]i = iso, ai = anteiso. GLC, gas-liquid chromatographic.

TABLE 9
Saturated Fatty Acids (wt%) in Human Milk from Japanese Donors at Various Times Postpartum[a] and in Hungarian Milk[b], for Comparison of GLC Columns

	Japanese donors, days postpartum (number of samples)								
Fatty acid	3–5 (110)	6–10 (181)	11–15 (177)	16–30 (351)	31–60 (562)	61–120 (314)	121–240 (280)	241–482 (149)	Hungary[b] (250)
14:1	0.15	0.18	0.18	0.18	0.17	0.16	0.17	0.13	—
16:1n-7	0.49	0.46	0.41	1.41	0.41	0.41	0.40	0.38	—
16:1n-9	2.55	2.64	2.50	2.69	2.62	2.43	2.38	2.13	—
16:1	0.13	0.12	0.11	0.11	0.09	0.07	0.07	0.09	—
17:1	0.19	0.21	0.19	0.20	0.20	0.19	0.20	0.18	2.6
18:1n-9	28.43	27.83	27.64	29.44	29.97	29.64	29.33	27.93	43.4[c]
18:1n-7	2.96	2.84	2.56	2.64	2.63	2.60	2.46	2.50	—
20:1n-11	0.23	0.21	0.19	0.22	0.23	0.24	0.20	0.24	—
20:1n-9	0.76	0.62	0.55	0.58	0.58	0.57	0.52	0.54	—
20:1n-7	0.09	0.07	0.06	0.05	0.05	0.05	0.04	0.04	—
22:1n-11	0.08	0.08	0.08	0.10	0.11	0.12	0.10	0.13	—
22:1n-9	0.23	0.17	0.15	0.14	0.15	0.14	0.13	0.14	—
24:1n-9	0.22	0.13	0.09	0.07	0.06	0.06	0.05	0.05	—

[a]Adapted from Idota *et al.* (92). Neither paper in last review (1); 30-m capillary column, DB wax coating.
[b]Adapted from Gere *et al.* (93); packed column. Samples from 1, 3, and 6 mon combined.
[c]18:1n-9 + 18:1n-7. See Table 8 for abbreviation.

The data from papers that I should have included in my last review are presented in Tables 8–13. These papers contain (i) data from regions where information is deficient, i.e., Japan, Hungary (Tables 8–10); (ii) data on changes in FA composition as lactation progresses (Tables 8–10); and (iii) a comparison of omnivorous and macrobiotic diet (Table 11). These studies give us information on the effects of regionally unique diets and adherence to them, which would be impossible to obtain in controlled experiments where costs and numbers of experiments would be prohibitive. Ethical considerations would prevent the initiation of research that could, even remotely, be harmful. An excellent example is the data in Table 11. The consumers of macrobiotic diets avoid foods of animal origin, except fish. Their fat consumption is 20% of energy intake (94). Interestingly, the increase in 8:0–14:0 usually observed when the donor is on a high-carbohydrate diet did not occur. The study was confounded since dietary cross-over between both groups occurred. The macrobiotic milk contained less vitamin B_{12} than the regular milks. Tables 12 and 13 contain data on milks from women in Nigeria, Japan, and Israel.

TABLE 10
Polyunsaturated Fatty Acids (wt%) in Milk from Japanese Donors at Various Times Postpartum[a] and in Hungarian Mothers[b], for Comparison of GLC Columns

	Japanese donors, days postpartum (number of samples)[a]								
Fatty acid	3–5 (110)	6–10 (181)	13–15 (177)	16–30 (351)	31–60 (562)	61–120 (314)	120–240 (280)	240–482 (149)	Hungary[b] (250)
18:2n-6	11.87	12.04	12.88	13.33	13.96	14.25	14.18	13.99	10.1
18:3n-6	0.06	0.07	0.09	0.10	0.11	0.11	0.10	0.08	0.9[c]
18:3	0.11	0.11	0.12	0.14	0.15	0.14	0.14	0.14	
18:3n-3	1.14	1.20	1.37	1.44	1.53	1.48	1.47	1.42	
18:2	0.22	0.24	0.22	0.24	0.23	0.21	0.22	0.21	
20:2	0.09	0.09	0.07	0.07	0.07	0.07	0.07	0.06	
20:2n-6	0.71	0.51	0.41	0.35	0.33	0.30	0.27	0.27	
20:3n-6	0.53	0.44	0.39	0.35	0.32	0.28	0.24	0.21	
20:4n-6	0.59	0.54	0.47	0.42	0.40	0.39	0.37	0.36	
20:3n-3	0.13	0.10	0.08	0.07	0.06	0.06	0.05	0.05	
20:5n-3	0.20	0.22	0.23	0.23	0.24	0.24	0.23	0.22	
22:2	0.13	0.09	0.06	0.05	0.04	0.03	0.02	0.02	
22:4n-6	0.27	0.17	0.11	0.09	0.08	0.07	0.07	0.07	
22:5n-3	0.50	0.37	0.32	0.30	0.30	0.29	0.27	0.28	
22:6n-3	1.33	1.24	1.09	1.02	1.00	1.00	0.95	1.04	
n-6 PUFA	14.03	13.77	14.35	14.64	15.20	15.40	15.23	14.98	
n-3 PUFA	3.48	3.28	3.24	3.21	3.26	3.18	3.07	3.10	

[a]Adapted from Idota *et al.* (92). Neither paper in last review (1); 30-m capillary column, DB wax coating.
[b]Adapted from Gere *et al.* (93); packed column. Samples from 1, 3, and 6 mon combined.
[c]Includes 18:3n-3. PUFA, polyunsaturated fatty acid; see Table 8 for other abbreviation.

TABLE 11
Fatty Acid Composition (wt%) of Milk from Dutch Women on Omnivorous and Macrobiotic Diets[a]

Fatty acid	Omnivorous (± SD)	Macrobiotic (± SD)	*P* value
n	10	27	
Lipid (%)	2.40 ± 1.02	2.34 ± 0.83	NS[b]
8:0	0.08 ± 0.04	0.12 ± 0.04	0.020
10:0	0.81 ± 0.29	0.98 ± 0.32	NS
12:0	5.14 ± 2.67	4.84 ± 1.83	NS
14:0	8.00 ± 2.58	7.12 ± 4.07	NS
15:0	0.22 ± 0.11	0.42 ± 0.10	<0.001
16:0	17.85 ± 2.17	21.78 ± 2.21	<0.001
17:0	0.21 ± 0.08	0.33 ± 0.12	0.002
18:0	5.32 ± 1.45	7.94 ± 1.13	<0.001
20:0	0.22 ± 0.08	0.33 ± 0.08	0.002
14:1	0.11 ± 0.06	0.22 ± 0.08	<0.001
16:1	1.44 ± 0.64	1.67 ± 0.42	NS
18:1	32.25 ± 5.25	33.62 ± 2.51	NS
20:1	0.42 ± 0.12	0.57 ± 0.15	0.007
18:2	22.42 ± 4.72	16.46 ± 5.16	<0.001
20:2	0.45 ± 0.15	0.33 ± 0.08	0.030
18:3	1.10 ± 0.47	0.88 ± 0.08	NS
Not identified	3.84 ± 5.10	3.91 ± 1.27	NS

[a]Adapted from Dagnelie *et al.* (94). Not in last review (1). Twenty-five-meter capillary column, coated with CP Sil8 CB (Chrompack, Middelburg, The Netherlands).
[b]NS, not significant, $P > 0.05$.

TABLE 13
Polyunsaturated Fatty Acids in Milks from Nigerian, Japanese, and Israeli Mothers

Fatty acid	Nigerian[a] Mean ± SD (mol%)	Japanese[a] Mean ± SD (mol%)	Israeli[b] Mean ± SD (mol%)
n	20	53	26
Lipid %	—	—	3.89 ± 0.18
n-6 series	15.57 ± 0.17	15.38 ± 1.11	20.02 ± 3.65
18:2			
18:3	0.07 ± 0.01	0.12 ± 0.00	—
20:2	ND	0.17 ± 0.01	—
20:3	0.40 ± 0.01[c]	0.20 ± 0.09	—
20:4	0.56 ± 0.02[c]	0.36 ± 0.01	0.58 ± 0.12
22:4	0.22 ± 0.00	0.07 ± 0.01	—
n-3 series			
18:3	0.93 ± 0.02[c]	1.82 ± 0.06[c]	1.57 ± 0.44
20:5	0.09 ± 0.01[c]	0.20 ± 0.01	—
22:5	0.17 ± 0.10	0.13 ± 0.09	—
22:6	0.34 ± 0.01[d]	0.53 ± 0.10	0.37 ± 0.17
22:3	ND	0.02 ± 0.00	—
Other LCPUFA			0.40 ± 0.17

[a]Ogunleye *et al.* (95). Not in last review (1); Twenty-five-meter capillary column.
[b]Budowski *et al.* (96). Not in last review (1). Capillary column coated with Supelcowax 10.
[c]Significantly different from Japanese values at $P < 0.05$.
[d]Significantly different from Japanese values at $P < 0.01$. LCPUFA, long-chain polyunsaturated fatty acids.

Most of the findings of Chen *et al.* (97) in Tables 14–16 were reviewed before (1). They are included here because they contain almost the only information on FA in milks from North American women in which a GLC column of high resolution was used. Researchers in Europe are using these columns (98). Chen *et al.* (97) found a relatively high *trans* FA content of 7.19 ± 3.03% (range 0.10 to 17.15%) not reported in earlier papers. The distribution of 18:1 positional isomers (Table 16) was similar to that in partially hydrogenated soybean and canola oils. I will discuss *trans* FA at length shortly. Chen *et al.* found one previously unidentified FA; 20:2Δ8*c*,14*c*.

TABLE 12
Saturated and Monounsaturated Fatty Acids in Milk from Nigerian, Japanese, and Israeli Mothers

Fatty acid	Nigerian[a] Mean ± SD (mol%) (*n* = 20)	Japanese[a] Mean ± SD (mol%) (*n* = 53)	Israeli[b] Mean ± SD (wt%) (*n* = 26)
			3.89% lipid
Saturates			
12:0	10.20 ± 0.26[c]	7.82 ± 1.99)	
14:0	9.05 ± 0.11	8.69 ± 3.67)	15.15 ± 9.5
16:0	22.14 ± 0.10[c]	23.19 ± 3.06	20.14 ± 2.42
17:0	0.31 ± 0.01	0.30 ± 0.12	NR
18:0	6.77 ± 0.04	6.34 ± 1.83	5.53 ± 1.09
20:0	0.17 ± 0.01	0.19 ± 0.09)	
22:0	0.06 ± 0.02	0.07 ± 0.09)	0.24 ± 0.08
24:0	0.05±0.01	0.05±0.01	NR
Monounsaturates			
16:1n-7	0.74 ± 0.22[c]	2.58 ± 0.76	2.63 ± 0.78
17:1	0.09 ± 0.00[d]	0.22 ± 0.14	NR
18:1n-9	28.38 ± 0.17	29.10 ± 1.14)	30.07 ± 2.93
18:1n-7	2.61 ± 0.13[d]	1.78 ± 1.08)	
20:ln-11	0.45 ± 0.22	0.13 ± 0.04)	
20:1n-9	0.04 ± 0.01	0.42 ± 0.20)	0.47 ± 0.13
22:1	NR	NR	0.10 ± 0.10
24:1n-9	0.06 ± 0.03	0.06 ± 0.01	NR

[a]Ogunleye *et al.* (95). Not in last review (1). Twenty-five-meter-capillary column. NR, not reported.
[b]Budowski *et al.* (96). Not in last review. Capillary column coated with Supelcowax 10.
[c]Significantly different from Japanese values at $P < 0.05$.
[d]Significantly different from Japanese values at $P < 0.01$.

Chardigny *et al.* (98) reported, along with Chen *et al.* (97), the most comprehensive analyses of milk FA to date (December 1997) in Tables 17–19. They employed techniques not utilized in most other analyses. They noted cyclic monomers for the first time and identified six (Table 19). These monomers made up 0.04% of total FA. The compounds are produced by thermal degradation of 18:3n-3 during vegetable oil deodorization–heat treatments. That these compounds have been detected in human milk is not surprising. Any compound that is fat soluble, is in the maternal diet, and passes through the metabolic gates should appear in milk lipids. Chardigny *et al.* (98) identified the same positional isomers of 18:1*t*, 6-16, as Chen *et al.* (97), but the total amount was only 1.91%. Most of these were the 11-isomer, vaccenic acid, derived from dairy products. In the milk sample analyzed by Chardigny *et al.*, 50% of the total 18:3n-3 were *trans* isomers. Four times a week, the women ate French fries cooked in rapeseed oil, which contains relatively large amounts of *trans* FA.

Data on Nigerian milks can be seen in Table 20. Information on milk from malnourished women was derived from analyses by a packed GLC column, but the quantities were given as μmol/mL. While this mode is scientifically accept-

TABLE 14
Fatty Acids (wt%) in Canadian Milks[a]

Fatty acids	Mean ± SD	Range	Fatty acids	Mean ± SD	Range
Saturates			*cis* monounsaturates		
10:0	1.39 ± 0.59	0.46–4.42	14:1–9*c*	0.28 ± 0.08	0.06–0.66
11:0	0.01 ± 0.02	Tr–0.08	16:1–9*c*	2.27 ± 0.56	1.11–3.88
12:0	5.68 ± 2.01	2.32–11.77	16:1–7*c*	0.41 ± 0.33	0.20–0.70
13:0	0.03 ± 0.03	Tr–0.14	17:1–10*c*	0.21 ± 0.06	Tr–0.44
14:0	6.10 ± 1.73	2.26–11.68	18:1–9*c*	30.65 ± 2.66	23.55–40.64
15:0	0.37 ± 0.12	0.12–0.67	18:1–11*c*	1.91 ± 0.17	1.26–2.33
16:0	18.30 ± 2.25	12.90–24.06	Sum other 18:1*c*	2.31 ± 0.23	1.44–3.97
17:0	0.32 ± 0.08	0.03–0.44	20:1–11*c*	0.39 ± 0.13	0.13–0.65
18:0	6.15 ± 0.97	3.49–9.85	20:1–13*c*	0.14 ± 0.09	Tr–0.42
20:0	0.15 ± 0.09	Tr–0.36	22:1–13*c*	0.02 ± 0.03	Tr–0.11
14:0 Br	0.14 ± 0.06	Tr–0.26	Total	38.59 ± 2.78	
16:0 Br	0.14 ± 0.06	0.04–0.45			
Total	38.50 ± 2.94				

[a]Adapted from Chen *et al.* (97). One hundred-meter capillary column, SP - 2560 coating. Samples taken 3 to 4 wk postpartum. n = 198, lipid content, 3.16 ± 0.94%. Prior separation with $AgNO_3$–TLC. Other fatty acids (FA) included FA < 10:0, minor branched (Br) FA, 18:2 conjugated FA, and unknowns. Tr, trace; for other abbreviation see Table 6.

TABLE 15
PUFA and *Trans* FA (wt%) in Canadian Milks[a]

Fatty acids	Mean + SD	Range	Fatty acids	Mean + SD	Range
n-6 PUFA			n-3 PUFA		
18:2	10.47 ± 2.62	5.79–21.35	18:3	1.16 ± 0.37	0.58–1.90
18:3	0.08 ± 0.06	Tr–0.21	20:4	0.06 ± 0.06	Tr–0.26
20:2	0.17 ± 0.37	Tr–0.35	20:5	0.05 ± 0.05	Tr–0.25
20:3	0.26 ± 0.09	Tr–0.46	22:5	0.08 ± 0.06	Tr–0.45
20:4	0.35 ± 0.11	0.05–0.69	22:6	0.14 ± 0.10	Tr–0.53
22:4	0.04 ± 0.05	Tr–0.18	Total	1.49 ± 0.44	
22:5	0.10 ± 0.02	Tr–0.16	Unusual *cis*-PUFA		
Total	11.39 ± 3.18		18:2~9*c*/15*c*	0.07 ± 0.05	Tr–0.15
Trans FA			20:2~8*c*,14*c*	0.19 ± 0.08	Tr–0.49
14:1-7	0.09 ± 0.05	Tr–0.47	20:3~5*c*,11*c*,14*c*	0.02 ± 0.02	Tr–0.12
16:1-9	0.18 ± 0.08	Tr–0.42	20:3~5*c*,8*c*,11*c*	0.05 ± 0.06	Tr–0.34
Total 18:1	4.65 ± 1.99	0.10–15.42	Total	0.33	
18:2~9*t*,12*t*	0.05 ± 0.08	Tr–0.28			
18:2~9*c*,13*t*/8*t*,12*c*	0.36 ± 0.14	Tr–0.76	Others	2.24 ± 0.09	
18:2~9*c*,12*t*	0.29 ± 0.15	Tr–0.78			
18:2~9*t*,12*c*	0.24 ± 0.12	Tr–0.59			
Total 18:3*t*	0.11 ± 0.08	Tr–0.34			
Total	7.19 ± 3.03				

[a]Adapted from Chen *et al.* (97). One hundred-meter capillary column, SP-2560 coating. Samples taken 3 to 4 wk postpartum. n = 198, lipid content, 3.16 ± 0.94%. Prior separation with $AgNO_3$–TLC. Other FA detected include FA < 10:0, minor Br FA, 18:2 conjugated FA, and unknowns. For abbreviations see Tables 6 and 14.

able, the nutritionist and others must calculate the actual weights of each quantity per deciliter and know the volume of milk consumed so as to determine the absolute amounts of FA in the milk the baby received. The TG in Table 20 were lipolyzed about 7% then separated by TLC. The FA composition of postlipolysis TG is about the same as the original because the bile salt-stimulated lipase of human milk is not regioselective (30,31) except that *sn*-2 16:0 is not hydrolyzed. This FA profile may be atypical. The enzyme loses its bile salt dependency and remains active at −20°C, and the TG content is reduced. The Nigerian milks were high in 12:0 and 14:0, the typical response to a high-carbohydrate diet deficient in

TABLE 16
Positional Isomers (wt%) of *Cis* and *Trans* 18:1[a]

Position	*cis*	*trans*	Position	*cis*	*trans*
7	Tr	Tr	12	2.22	12.00
8	1.20	3.00	13	1.22	10.00
9	88.20	15.00	14	0.70	6.00
10	1.70	22.00	15	Tr	4.00
11	4.80	24.00	16	Tr	2.00
			% of total lipid	36.88	5.87

[a]Adapted from Chen *et al.* (97). For abbreviation see Table 14.

TABLE 17
Saturated FA (wt%) in Milks from French Mothers[a]

FA	Range	Means	SEM	FA	Range	Means	SEM
4:0	0.01–0.06	0.03	0.01	Iso-14:0	0.01–0.06	0.03	0.01
5:0	0.01–0.11	0.02	0.01	15:0	0.23–0.62	0.42	0.04
6:0	0.01–0.19	0.07	0.02	Iso-15:0	0.01–0.14	0.08	0.01
7:0	0.01–0.09	0.04	0.01	16:0	14.80–24.83	21.62	0.97
8:0	0.06–0.32	0.19	0.03	Iso-16:0	0.04–0.25	0.12	0.02
9:0	0.01–0.04	0.01	0.01	17:0	0.21–0.54	0.36	0.03
10:0	0.29–2.21	1.23	0.19	18:0	4.79–10.65	7.64	0.50
11:0	0.01–0.05	0.02	0.00	20:0	0.12–0.34	0.22	0.02
12:0	2.01–9.59	5.15	0.74	22:0	0.00–0.20	0.06	0.02
13:0	0.01–0.07	0.04	0.01	Total	39.44–46.68	44.32	0.71
14:0	4.77–8.86	6.90	0.45				

[a]Adapted from Chardigny *et al.* (98). Fifty- and 30-m capillary GLC columns with polar coatings, HPLC, and $AgNO_3$–TLC employed. Samples taken from 0–90 d postpartum, $n = 10$. See Tables 6, 8, and 14 for abbreviations.

18:3n-3, the precursor of 22:5-6. Palm oil was the major dietary fat. This oil contains about 40% each of 16:0 and 18:1 and virtually no 12:0, 14:0, or 18:3n-3 (99).

FA profiles of milks from Sudanese and Chinese mothers are listed in Tables 21–23. I have presented the grouped results of Laryea *et al*. from Sudan (101). They analyzed their data for the possible effects of maternal age, time postpartum, and parity. The quantities of 22:6n-3 in Sudanese milk (Table 23) were low, 0.07 ± 0.03 wt%. Laryea *et al*. (Table 22) detected 0.61 wt% total *trans* FA, mostly the 18:1 isomer. The major dietary fat was unhydrogenated cottonseed oil. The data in Tables 21–23 prove that regional diets are diversified and the FA profiles of milk from those regions typify the diets, i.e., high carbohydrate–low fat, and the sources of particular fatty acids (102). The 22:6n-3 contents of the milk from women in the marine region of China were 2.78% (Table 23), which is extraordinarily high. These women had access to seafood. The regional diets are given in Table 24.

More FA profiles are presented in Tables 25 and 26, i.e., Spanish (103); Dutch, days postpartum (63), and Dutch,

TABLE 18
Unsaturated FA (wt%) in Milks from French Mothers[a]

FA	Range Min	Range Max	Mean	SEM	FA	Range Min	Range Max	Mean	SEM
cis Monoenes					n-6 Polyunsaturates				
10:1	0.01	0.14	0.03	0.01	18:2	8.92	22.82	14.67	1.38
14:1[a]	0.15	0.59	0.35	0.04	18:2 *ct*	0.05	0.38	0.15	0.03
16:1	1.31	3.10	2.15	0.17	18:2 *tc*	0.07	0.26	0.12	0.02
18:1 *c*[b]	24.41	35.77	32.15	0.92	18:3	0.05	0.16	0.11	0.01
18:1 *t*[c]	1.20	3.01	1.91	0.20	20:2	0.15	1.17	0.52	0.11
20:1	0.41	1.26	0.78	0.09	20:3	0.18	0.72	0.39	0.05
22:1	0.00	0.23	0.12	0.03	20:4	0.25	0.87	0.50	0.06
Total *cis*	26.82	40.01	35.58	1.06	22:4	0.00	0.60	0.17	0.07
22:5	0.00	0.14	0.03	0.02					
					Total *cis*	10.26	25.10	16.39	1.48
n-3 Polyunsaturates									
18:3	0.25	1.64	0.70	0.11					
18:3 *tcc*	0.00	0.22	0.03	0.02					
18:3 *ctc*	0.00	0.07	0.00	0.00					
18:3 *cct*	0.01	0.26	0.04	0.02					
18:3 *tct*	0.00	0.20	0.02	0.02					
20:3	0.00	0.08	0.04	0.00					
20:4	0.00	0.44	0.08	0.04					
20:5	0.00	0.08	0.02	0.01					
22:5	0.07	0.43	0.16	0.04					
22:6	0.00	0.82	0.32	0.08					
Total *cis*	0.84	2.03	1.32	0.12					

[a]Adapted from Chardigny *et al.* (98). Fifty- and 30-m capillary GLC columns with polar coatings, HPLC, and $AgNO_3$–TLC employed. Samples taken from 0 to 90 d postpartum, $n = 10$. For abbreviations see Tables 6 and 14.
[b]Contains some ante-iso 15:0.
[c]Including all positional isomers.

TABLE 19
Relative Proportions of Hydrogenated Cyclic FA Monomers in Human Milk[a]

Identity	Proportion[b] (%)
Methyl 9-(2-butylcyclopentyl) nonanoate *trans*	20
Methyl 10-(2-propylcyclopentyl) decanoate *trans*	22
Methyl 9-(2-butylcyclopentyl) nonanoate *cis*	9
Methyl 9-(propylcyclohexyl) nonanoate *trans*	16
Methyl 10-(2-propylcyclopentyl) decanoate *cis*	17
Methyl 9-(propylcyclohexyl) nonanoate *cis*	16

[a]Adapted from Chardigny *et al.* (98) based on gas chromatographic analysis of methyl esters. Amount 0.04% of total FA. Listed in order of elution on GLC chart.
[b]Proportions calculated by author based on peak height, are approximate. For abbreviations see Tables 6 and 8.

preterm (104). I have pooled the Spanish data. De la Presa-Owens *et al.* (103) showed profiles from two regions, Navarre and Catalonia. The compositions are similar to those in milk from other studies of European women herein and in Reference 1. The purpose of the research by Huisman *et al.* (63) was to compare the composition of Dutch milks to 10 types of formulas. Beijers and Schaafsma (104) analyzed milk for FA in response to the recognized importance of 20:4n-6 and 22:6n-3 in the nutrition of preterm infants. They observed (data not shown in Table 26) that the 22:6n-3 and 20:4n-6 contents of very preterm milks (<31 wk) decreased as lactation progressed. In preterm milks (31 to 36 wk), 22:6n-3 did not change and 20:4n-6 decreased. They suggested that 0.34%, the mean amount of 22:6n-3 from their study, be regarded as the norm. The expected increases in quantities of 8:0, 10:0, 12:0, and 14:0, the only FA synthesized by the mammary gland as lactation progresses, were observed. Useful information on 24-h volumes of milk produced by these mothers is shown in this paper (104) and in Table 27.

TABLE 20
FA in Milks from Nigerian Mothers

	Ibadan (mol%), 1995[a]		
FA	MM[b] (*n* = 13)	SM[b] (*n* = 3)	Udo 1991[c] (wt%)
10:0	2.10 ± 2.00	ND	0.54
12:0	31.40 ± 25.60	25.60 ± 6.10	8.34
14:0	9.00 ± 1.50	7.50 ± 3.90	9.57
16:0	20.10 ± 2.20	26.80 ± 7.80	23.35
16:1n-7	3.10 ± 2.20	1.30 ± 0.20	1.18
18:0	3.40 ± 0.40	4.30 ± 1.00	10.15
18:1n-9	20.80 ± 1.70	25.40 ± 5.70	18.52
18:2n-6	8.20 ± 0.60	8.10 ± 2.40	11.06
18:3n-3	ND	ND	0.09
20:0	NR		0.42
20:1n-9	0.90 ± 0.20	0.60 ± 0.10	0.34
22:0	0.10 ± 0.03	ND	0.41
20:4n-6	0.60 ± 0.40	ND	0.82
20:5n-3	TR	ND	0.48
22:5n-3	TR	ND	0.39
22:6n-3	0.30 ± 0.10	0.10 ± 0.07	0.93

[a]Adapted from Glew *et al.* (36). Packed column. ND, not detected; NR, not reported. Ibadan and Udo are villages.
[b]MM moderately malnourished, body mass index (BMI), 20.2; SM, severely malnourished; BMI, 16.4.
[c]Adapted from Koletzko *et al.* (100), capillary GLC column. Included for comparison. For abbreviations see Tables 8, 14, and 17.

The changes in FA profiles of term and preterm milks were investigated by Genzel-Boroviczeny *et al.* (105), who employed a high-resolution GLC column (Table 28). Their purpose, similar to that of Beijers and Schaafsma (104), was to determine the contents of LCPUFA in preterm milk as a guide for the design of infant formulas. Significant increases were seen in 10:0, 12:0, 18:2n-6, and 18:3n-3 from day 5 to day 10. Total n-6 LCPUFA, 22:6n-3, and total n-3 LCPUFA decreased significantly from day 5 to day 10. Amounts of 18:2n-

TABLE 21
Saturated FA (wt%) in Milks from Sudanese and Chinese Mothers

	Sudan[a,b]		China[b,c] (mean ± SD)				
FA	Mean ± SD (*n* = 77)	Range	Marine (*n* = 45)	Urban 1 (*n* = 39)	Urban 2 (*n* = 22)	Rural (*n* = 16)	Pastoral (*n* = 24)
8:0	NR	NR	0.12 ± 0.07	0.12 ± 0.06	0.11 ± 0.06	0.11 ± 0.06	0.13 ± 0.05
10:0	0.59 ± 0.43	0.00–1.73	1.38 ± 0.33[b]	1.20 ± 0.39[a,b]	1.08 ± 0.48[a]	1.19 ± 0.37[a,b]	1.06 ± 0.32[b]
11:0	0.01 ± 0.01	0.00–0.04	NR	NR	NR		
12:0	6.66 ± 2.27	1.59–12.16	6.25 ± 2.00	5.31 ± 2.08	5.27 ± 2.31	4.92 ± 1.47	5.20 ± 1.87
13:0	0.04 ± 0.02	0.02–0.14	NR	NR	NR	NR	NR
14:0	10.13 ± 3.11	4.27–17.72	6.52 ± 2.84	5.34 ± 2.24	5.99 ± 2.91	5.25 ± 1.64	6.01 ± 3.35
15:0	NR	NR	NR	NR	NR	NR	NR
16:0	22.10 ± 2.69	16.05–29.77	18.97 ± 2.93	18.85 ± 2.80	18.93 ± 3.90	18.86 ± 3.24	17.32 ± 2.32
17:0	0.50 ± 0.12	0.28–0.84	0.40 ± 0.14[b]	0.29 ± 0.11[a]	0.29 ± 0.14[a]	0.28 ± 0.14[a]	0.48 ± 0.21
18:0	5.21 ± 1.05	3.04–8.13	5.00 ± 1.39[a]	5.57 ± 1.15[a,b]	5.86 ± 2.65[a,b]	5.83 ± 1.01[a,b]	6.71 ± 2.65[ab]
20:0	0.14 ± 0.21	0.00–0.67	0.41 ± 0.14[b]	0.34 ± 0.16[a,b]	0.26 ± 0.17	0.32 ± 0.18[a,b]	0.41 ± 0.16[a,b]
21:0	0.11 ± 0.07	0.00–0.29	NR	NR	NR	NR	NR
22:0	0.07 ± 0.03	0.03–0.17	NR	NR	NR	NR	NR
24:0	0.06 ± 0.02	0.00–0.14	NR	NR	NR	NR	NR
Total	45.61 ± 4.78	36.16–55.54	39.05 ± 5.33	37.02 ± 7.57	38.69 ± 6.51	36.76 ± 5.24	37.32 ± 4.74

[a]Adapted from Laryea *et al.* (101), 50-m capillary GLC column coated with CP Sil 88. Subjects well-nourished on indigenous diet. NR = not reported; for other abbreviations, see Tables 8 and 14.
[b]Within a row of data, values with different roman letters superscript are significantly different, $P < 0.05$.
[c]Adapted from Chulei *et al.* (102). Two 2.1-m packed columns with diethyleneglycol succinate (DEGS) coating. Dietary intakes are in Table 32.

TABLE 22
Monounsaturated FA (wt%) in Milks from Sudanese and Chinese Mothers

	Sudan[a,b]		China[b,c] (mean ± SD)				
FA	Mean ± SD (*n* = 77)	Range	Marine (*n* = 45)	Urban 1 (*n* = 39)	Urban 2 (*n* = 22)	Rural (*n* = 16)	Pastoral (*n* = 24)
14:1 *t*	0.08 ± SD	0.03–0.29					
14:1 *c*	0.41 ± 0.13	0.18–0.87					
16:1 *t*	0.1 ± 0.04	0.04– 0.24					
16:1 *c*	2.47 ± 0.77	1.04–4.49	3.42 ± 1.51	3.42 ± 1.73	2.94 ± 1.85	3.50 ± 2.23	4.39 ± 1.92
17:1 *c*	0.29 ± 0.05	0.16–0.54					
18:1n-9*t*	0.43 ± 0.2	0.14–1.09					
18:1n-9	29.05 ± 3.74	21.49–39.21	25.10 ± 4.36[b]	29.13 ± 4.06	27.56 ± 4.15	30.64 ± 4.21	24.93 ± 4.12
20:1n-9	0.37 ± 0.12	0.18–0.42					
22:1n-9	0.07 ± 0.05	0.00–0.32					
24:1n-9	0.04± 0.03	0.00–0.17					
Total	32.81± 4.1	25.09–43.01	28.52 ± 4.79[a]	32.55 ± 4.54[b]	30.50 ± 5.32[a,b]	34.14 ± 4.74[b]	20.32 ± 4.81

[a]Adapted from Laryea *et al.* (101), 50-m capillary GLC column coated with CP Sil 88. Subjects well-nourished on indigenous diet.
[b]Within a row of data, values with different superscript roman letters are significantly different, $P < 0.05$.
[c]Adapted from Chulei *et al.* (102). Two 2.1-m packed columns with DEGS coating. Dietary intakes are in Table 32. For abbreviations see Tables 8, 14, and 21.

6, 18:3n-3, and LCPUFA did not differ in term and preterm milks at any time. There was significantly more 10:0, 12:0, and 14:0 in preterm as compared to term milk on days 5, 10, and 20. The authors concluded that milk from mothers of preterm infants is not better suited to provide their infants with all the required LCPUFA than other sources during the first few weeks postpartum. The slightly greater contents of 10:0–14:0 might enhance fat and calcium absorption in the preterm infant. These differences in FA profiles are probably due to immaturity of the mammary gland and not to a process resulting from evolution to provide necessary nutrients for the preterm infant. A premature birth is an abnormal event.

Desci *et al*. (106) analyzed milks obtained from Hungarian mothers at 3 to 9 d postpartum. The results (not presented) are similar to those in Table 28.

The effects of time postpartum on the PUFA contents in milks from French mothers (107) and from Australian women (108) are presented in Table 29 (see also Reference 109). Information on the effects of n-3 PUFA-enriched eggs (110) or 22:6n-3 (111) in the maternal diet on milk FA profiles is

TABLE 23
PUFA (wt%) in Milk from Sudanese and Chinese Mothers

	Sudan[a,b]		China[b,c] (mean ± SD)				
FA	Mean ± SD (*n* = 77)	Range	Marine (*n* = 45)	Urban 1 (*n* = 39)	Urban 2 (*n* = 22)	Rural (*n* = 16)	Pastoral (*n* = 24)
18:2 *ct*	0.05 ± 0.01	0.02–0.09					
18:2 *tt*	0.00 ± 0.01	0.00–0.07					
18:2 *tt*	0.03 ± 0.02	0.00–0.08					
18:2 *cc*	18.28 ± 4.11	9.31–32.7	18.48 ± 4.71	20.57 ± 3.94	19.73 ± 5.42	18.43 ± 5.84	20.43 ± 4.59
18:3n-6	0.17 ± 0.06	0.06–0.38					
20:2	0.39 ± 0.07	6.27–0.56					
20:3	0.44 ± 0.11	0.23–0.75					
20:4	0.50 ± 0.10	0.28–0.86	1.17 ± 0.33[b]	0.89 ± 0.44[a]	1.01 ± 0.50[a,b]	0.80 ± 0.14[a]	1.22 ± 0.32[b]
22:2	0.08 ± 0.03	0.04–0.23					
22:4	0.15 ± 0.04	0.07–0.29					
22:5	0.07± 0.03	0					
Total n-6	20.08 ± 4.20	10.67–34.02	19.65	21.46	20.74	19.23	21.65
n-3 series							
18:3	0.67 ± 0.54	0.20–2.7	3.03 ± 0.80[b]	2.97 ± 0.69[b]	2.95 ± 1.03[b]	2.71 ± 0.60[b]	2.08 ± 0.49[a]
20:3	0.02 ± 0.02	0–0.14					
20:5	0.01 ± 0.02	0–0.13	1.01 ± 0.50[b]	0.50 ± 0.29[a]	0.66 ± 0.43[a]	0.35 ± 0.18[d]	0.66 ± 0.27[a]
22:5	0.11 ± 0.04	0–0.33					
22:6	0.07 ± 0.05	0–0.28	2.78 ± 1.20[b]	0.88 ± 0.34	0.82 ± 0.35[a]	0.68 ± 0.29[a]	0.14 ± 0.29[d]
Total n-3	0.89 ± 0.6	0.25–3.13	6.82	4.55	4.43	3.74	3.18

[a]Adapted from Laryea *et al.* (101), 50-m capillary GLC column coated with CP Sil 88. Subjects well-nourished on indigenous diet.
[b]Within a row of data, values with different superscript roman letters are significantly different, $P < 0.05$.
[c]Adapted from Chulei *et al.* (102). Two 2.1-m packed columns with DEGS coating. Dietary intakes are in Table 32. For abbreviations see Tables 4, 13, 14 and 21.

TABLE 24
Dietary Intakes of Mothers in Several Regions of China[a]

	Marine (*n* = 10)	Urban I and II (*n* = 15)	Rural (*n* = 5)	Pastoral (*n* = 5)
Energy, MJ/d	10.71 ± 1.37	11.03 ± 1.65	11.39 ± 1.23	10.99 ± 1.94
Protein, g/d (%KJ)	83 ± 14 (13%)	71 ± 9 (11%)	72 ± 7 (11%)	80 ± 16 (12%)
Fat, g/d (%KJ)	76 ± 7 (27%)[a]	105 ± 17 (36%)[b]	106 ± 15 (35%)[b]	78 ± 8 (27%)[a]
Carbohydrate, g/d (%KJ)	383 ± 33 (60%)	349 ± 55 (53%)	375 ± 33 (55%)	399 ± 42 (61%)
Cholesterol, mg/d	434	441	442	326
Food, g/d (edible portion)				
Rice and wheat flour	497 ± 75	446 ± 82	503 ± 87	425 ± 62
Millet	none[a]	none[a]	none[a]	80[b]
Pork	33 ± 22[a]	97 ± 30[b]	95 ± 44[b]	26 ± 5[a]
Beef and mutton	none[a]	4 ± 4[a]	none[a]	34 ± 9[b]
Eggs	75 ± 18	75 ± 26	80 ± 15	48 ± 21
Milk[a] cows'	32 ± 28[a]	94 ± 60[b]	none[a]	512 ± 101[c]
Fish and shellfish	136 ± 29[c]	49 ± 27[b]	11 ± 6[a]	none[a]
Lard	3 ± 3[a]	none[a]	20 ± 8[b]	4 ± 3[a]
Mutton fat	none[a]	none[a]	none[a]	6 ± 2[b]
Soybean oil (% KJ)	34 ± 14 (12%)[c]	25 ± 8 (9%)[b]	5 ± 3 (2%)[a]	8 ± 3 (3%)[a]

[a]Adapted from Chulei *et al.* (102).
[b]Except for cholesterol, values are means ± SD.
[c]Milk products expressed as whole fat fluid. Within a row of data, values with different superscript roman letters are significantly different, $P < 0.05$.

shown in Table 30. The subjects on eggs consumed two per day. Each enriched egg contained 690 mg of n-3 FA and 165 mg of n-3 LCPUFA. The amounts of plasma C and TG were not changed by ingestion of the eggs. The n-3 enriched eggs could be used as an alternative source of 18:3n-3 in the maternal diet. In the study by Makrides *et al*. (111), six capsules containing 500 mg of a special oil with 200 mg of 22:6n-3 (42.9%) were taken per day by the subjects. The effects on milk FA are also presented in Table 30. These authors employed appropriate capillary columns, but did not report *trans* FA. The FA contents were typical of milks from mothers on Western diets, although Makrides *et al*. observed that the 22:6n-3 contents, 0.21%, were lower than the 0.32% they gave in a 1981 paper (109). Although this could be related to differences in columns [packed (109) vs. capillary], Makrides *et al*. (111) suggested that changes in the maternal diets were responsible. They noted, "It does demonstrate the sensitivity of breast milk FA to dietary change and highlights the need

TABLE 25
Saturated FA (wt%) in Term Milk from Spanish and in Term and Preterm Milk from Dutch Mothers

	Spanish[a]		Dutch term[b] (mean ± SD), days postpartum			Dutch preterm[c] (mean ± SD)	
FA	Mean	Range	14	42	89	VPT	PT
n	40		99	99	25	50	12
Lipid (%)	—	—	2.94 ± 0.59	2.86 ± 0.61	2.57 ± 0.55	3.50 ± 0.60	3.3 ± 0.53
6:0	—	—	0.28 ± 0.05	0.32 ± 0.64	0.38 ± 0.06	—	—
8:0	0.13	0.03–0.22	0.67 ± 0.10	0.66 ± 0.10	0.66 ± 0.09	0.12 ± 0.06	0.14 ± 0.09
10:0	1.32	0.5–2.11	2.87 ± 0.54	2.67 ± 0.54	2.58 ± 0.47	1.59 ± 0.28	1.46 ± 0.35
11:0	—	—	—	—	—	—	—
12:0	5.9	2.59–11.08	8.73 ± 2.51	8.16 ± 2.60	7.75 ± 1.79	8.14 ± 2.20	6.44 ± 1.71
13:0	1.13	0.06–0.56	—	—	—	—	—
14:0	6.25	3.09–11.41	8.38 ± 1.85	8.01 ± 1.95	7.68 ± 1.66	9.69 ± 2.40	7.42 ± 1.05
15:0	0.30	0.15–0.72	—	—	—	—	—
16:0	19.45	15.05–27.5	23.10 ± 2.12	23.04 ± 2.19	22.74 ± 2.77	22.01 ± 3.02	21.95 ± 2.74
17:0	0.36	0.21–0.81	—	—	—	—	—
18:0	6.85	4.84–10.04	7.21 ± 0.87	7.25 ± 0.92	7.27 ± 0.79	7.55 ± 1.65	8.28 ± 1.51
20:0	0.23	0.17–0.37	—	—	—	0.24 ± 0.07	0.27 ± 0.08
21:0	—	NR	—	—	—	—	—
22:0	—	NR	—	—	—	—	—
24:0	—	NR	—	—	—	—	—
Total	41.09	32.12–50.87	51.64 ± 4.25	50.50 ± 4.90	49.46 ± 4.85		

[a]Adapted from de la Presa-Owens *et al.* (103). Thirty-meter capillary GLC column coated with SP-2330. Samples taken 20–30 d postpartum.
[b]Adapted from Huisman *et al.* (63). 24-h samples at days 14, 42, and 89 postpartum. Capillary GLC columns.
[c]Adapted from Beijers and Schaafsma (104). Capillary GLC column coated with CP-wax 52 CB Chrompak. VPT, very preterm, 28.1 wk; PT, preterm, 32.2 wk. Samples taken at 26 d postpartum. For abbreviation see Tables 8, 14, and 21.

TABLE 26
Unsaturated FA (wt%) in Term Milks from Spanish and in Term and Preterm Milks from Dutch Mothers

	Spanish[a]		Dutch term[b] (mean ± SD), days postpartum			Dutch preterm[c] (mean ± SD)	
FA	Mean	Range	14	42	89	VPT	PT
n	40		99	99	25	50	12
Lipid (%)	-	-	2.94 ± 0.59	2.86 ± 0.61	2.57 ± 0.55	3.50 ± 0.60	3.3 ± 0.53
14:1n-5	0.21	0.09–0.72		Monounsaturated		—	—
15:1n-5	0.12	0–0.81		FA		—	—
16:1n-7	2.07	1.16–3.75		not		—	—
17:1n-7	0.30	0.18–1.0		reported		—	—
18:1n-9,7	38.39	26.29–55.25				25.70 ± 3.07	25.58 ± 2.87
20:1n-9	0.51	0.27–1.09				0.55 ± 0.14	0.64 ± 0.18
Total	41.97	28.29–58.33	32.69 ± 3.38	33.19 ± 8.33	33.42 ± 3.46	26.25	26.24
n-6 series							
18:2	12.02	6.57–27.58	12.81 ± 3.54	13.62 ± 4.24	14.42 ± 4.87	11.72 ± 3.38	11.80 ± 3.84
18:3	—	—	0.09 ± 0.03	0.11 ± 6.03	0.11± 0.04	0.07 ± 0.04	0.06 ± 0.02
20:2	0.41	0.17–0.9	0.35 ± 0.08	0.29 ± 0.07	0.26± 0.05	0.44 ± 0.13	0.40 ± 0.11
20:3	0.5	6.22–1.05	0.38 ± 0.09	0.34 ± 0.06	0.30 ± 0.06	0.33 ± 0.12	0.27 ± 0.08
20:4	0.5	0.22–0.79	0.41 ± 0.07	0.34 ± 0.06	0.34 ± 0.05	0.31 ± 0.11	0.37 ± 0.12
22:4	0.17	0.08–0.56	0.08 ± 0.02	0.06 ± 0.01	0.06 ± 0.01	0.08 ± 0.03	0.09 ± 0.05
22:5	—	—	0.04 ± 0.01	0.03 ± 0.01	0.02 ± 0.01	—	—
22:6	—	—	—	—	—	0.24 ± 0.13	0.34 ± 0.15
Total	13.73	8.09–29.54	14.15 ± 3.64	14.79 ± 4.34	15.50 ± 4.97	13.31 ± 3.56	13.16 ± 4.02
n-3 series							
18:3	0.78	0.58–1.2	1.07± 0.28	1.11± 0.35=	1.20 ± 0.40	0.81 ± 0.29	0.96 ± 0.43
20:5	0.14	0.06–0.42	0.05 ± 0.02	0.06 ± 0.04	0.07 ± 0.01	0.07 ± 0.03	0.07 ± 0.03
22:5	0.12	0.07–0.71	0.12 ± 0.02	0.133 ± 0.03	1.13 ± 0.03	—	—
22:6	0.34	0.15–1.02	0.23 ± 0.06	0.19 ± 0.11	0.18 ± 0.12	0.24 ± 0.13	0.34 ± 0.15
Total	1.39	1.10–2.54	1.47 ± 0.29	1.48 ± 0.38	1.58 ± 0.44	1.13 ± 0.38	1.37 ± 0.44

[a]Adapted from de la Presa-Owens *et al.* (103). 30-m capillary GLC column coated with SP-2330. Samples taken 20–30 d postpartum.
[b]Adapted from Huisman *et al.* (63). 24-h samples at days 14, 42, and 89 postpartum. Capillary GLC columns.
[c]Adapted from Beijers and Schaafsma (104). Capillary GLC column coated with CP-wax 52 CB Chrompak. For abbreviations see Tables 8, 14, and 25.

for studies on appropriate diets for lactating women." Milk levels of 22:6n-3 were increased from 0.2 to 1.7% by supplementation. Maternal plasma and erythrocyte contents also went up. Maternal vitamin A and E levels, indicators of antioxidants status, were not affected.

Other pertinent references are discussed briefly below for purposes of completeness. Similar information on most of these points is available in papers discussed previously.

Al-Othman *et al*. (112) analyzed the milks from Saudi mothers. They utilized a packed GLC column and did not detect 20:4n-6, 20:5n-3, and 22:6n-3. The FA profiles were not unique. The FA compositions reported by Luukkainen *et al*. (113) on milks donated by Finnish subjects resemble those from other women consuming Western diets. They also presented data on regional formulas. Jorgensen *et al*. (114) compared the FA profiles of Danish formulas to human milk. Again the data were typical of milks from mothers ingesting Western foods. Jorgensen *et al*. did find 2.5% *trans* FA. The *trans* FA included 18:1, *tt* 18:2, *ct* 18:2, and *tc* 18:2. Although not stated, most of the *trans* FA was probably 18:1 (97,98). The conjugated isomer, *c*9,*t*11-18:2, may have been present. Once again, very few investigators have sought, identified, and reported *trans* FA. Some researchers have employed GLC columns which resolve these FA, but did not identify them. Sera *et al.* (115) analyzed the FA in milks from Italian women obtained twice a day from postpartum days 1 through 28. There were no differences between the A.M. and P.M. samples. The amounts of LCPUFA declined as lactation progressed.

Villalpando *et al*. (116) presented evidence which possibly explains the abnormally low lipid contents and variations in FA profiles often seen in milks from malnourished women. They noted that in milks from individuals, diurnal changes occurred in relative wt% and real amounts of total lipids and some FA. Unsaturated FA, 16:1, 18:1, and 18:2, identified as of dietary origin, increased after each meal. These peaks were associated with troughs in the wt% of 8:0–14:0; these acids

TABLE 27
Weighed 24-h Expressions of VPT and PT Breast Milk from Dutch Mothers[a]

Lactation period	VPT milk (g)	PT milk (g)
Colostrum		
Mean volume	154.0	330.0
SD	161.5	215.9
Range	16.0–770.0	74.0–734.0
Transitory milk		
Mean volume	289.0	263.0
SD	179.4	186.4
Range	45.0–739.0	47.0–648.0
Mature milk		
Mean volume	397.0	458.0
SD	245.1	235.5
Range	41.0–880.0	28.0–760.0

[a]Adapted from Beijers and Schaafsma (104). VPT, very preterm, <31 wk, PT, preterm, ≥31 to 36 wk of gestation.

TABLE 28
FA Composition of Milk Lipids (wt%) as Median (interquartile/range) for Term and Preterm Milk from Day 5 to Day 30[a]

	Day 5		Day 10		Day 20		Day 30	
	Term = 32	Preterm = 19	Term = 38	Preterm = 19	Term = 38	Preterm = 17	Term = 38	Preterm = 17
10:0	0.50	0.46	0.95[a]	1.06[a]	1.05	1.15	1.01	1.08
	(0.10)	(0.09)	(0.22)	(0.13)	(0.26)	(0.22)	(0.30)	(9.24)
12:0	3.42	4.17	5.18[a]	6.19[a,e]	5.39	7.17[d]	5.21	6.07
	(0.91)	(0.81)	(1.31)	(1.88)	(1.19)	(1.17)	(1.50)	(1.64)
14:0	5.86	7.65	6.49	9.00[e]	8.93	8.97[c]	6.90	7.93
	(1.25)	(1.50)	(1.47)	(1.67)	(1.21)	(1.70)	(2.05)	(2.02)
16:0	24.97	24.79	22.56[b]	22.62	23.18	22.58	22.47	22.84
	(2.36)	(1.24)	(2.00)	(2.11)	(2.09)	(1.90)	(2.31)	(1.26)
18:0	7.07	6.34	7.42	7.54[e]	7.74	7.85	7.40	7.65
	(0.73)	(1.59)	(0.90)	(0.94)	(0.75)	(0.90)	(0.82)	(0.72)
Total Sat	43.65	44.16	43.05	47.41[c,e]	46.28	48.09[e]	44.30	46.78
	(3.55)	(3.20)	(3.67)	(3.60)	(3.83)	(3.50)	(5.00)	(2.46)
18:1n-9	32.10	33.83	32.10	30.81[c]	31.97	29.81	31.50	31.38
	(1.87)	(2.30)	(2.61)	(2.36)	(1.73)	(2.98)	(2.49)	(3.95)
Total *trans*	0.96	1.01	1.097	1.07	1.49	1.13	1.13	1.26
	(0.35)	(0.40)	(0.41)	(0.43)	(0.56)	(0.31)	(0.46)	(0.27)
18:2n-6	8.86	9.88	10.30[c]	9.56	10.47	10.17	11.33	10.49
	(1.30)	(1.86)	(2.15)	(1.60)	(2.10)	(1.57)	(2.17)	(1.95)
18:3n-6	0.14	0.11	0.15	0.11	0.18	0.16	0.18	0.17
	(0.31)	(0.09)	(0.08)	(0.09)	(0.09)	(0.07)	(0.10)	(0.03)
20:2n-6	0.57	0.54	0.42[a]	0.39[b]	0.31[a]	0.32[c]	0.30	0.32
	(0.12)	(0.08)	(0.06)	(0.06)	(0.06)	(0.06)	(0.07)	(0.06)
20:3n-6	0.53	0.50	0.43[b]	0.45	0.38	09.41	0.38	0.41
	(0.13)	(0.15)	(0.11)	(0.05)	(0.06)	(0.04)	(0.08)	(0.08)
20:4n-6	0.72	0.74	0.59[a]	0.64[b]	0.50[a]	0.51[a]	0.45	0.48
	(0.07)	(0.12)	(0.09)	(0.08)	(0.06)	(0.04)	(0.07)	(0.03)
22:4n-6	0.23	0.24	0.15[a]	0.14[a]	0.10[a]	0.11[a]	0.08	0.11
	(6.00)	(0.08)	(0.03)	(0.03)	(0.02)	(0.02)	(0.02)	(0.02)
Total n-6 LCPUFA	2.15	2.13	1.59[b]	1.69[b]	1.35[a]	1.35[a]	1.28	1.31
	(0.34)	(0.32)	(0.20)	(0.19)	(0.17)	(0.13)	(0.19)	(0.19)
Total n-6	11.57	12.20	12.25	11.11	12.09	11.64	13.06	12.02
	(1.94)	(2.34)	(2.17)	(1.75)	(2.32)	(1.70)	(2.50)	(1.50)
18:3n-3	0.65	0.67	0.81[b]	0.67	0.78	0.78	0.90	0.73
	(0.07)	(0.12)	(0.17)	(0.09)	(0.11)	(0.15)	(0.20)	(0.14)
20:3n-3	0.09	0.09	0.05[b]	0.00	0.05	0.05	0.05	0.00
	(0.03)	(0.05)	(0.03)	(0.04)	(0.04)	(0.04)	(0.04)	(0.00)
20:5n-3	0.04	0.00	0.00[c]	0.00	0.04	0.05	0.05	0.00
	(0.08)	(0.04)	(0.03)	(0.05)	(0.05)	(0.05)	(0.07)	(0.04)
22:5n-3	0.22	0.22	0.18[a]	0.15[a]	0.15	0.15	0.15	0.15
	(0.05)	(0.05)	(0.04)	(0.04)	(0.04)	(0.01)	(0.03)	(0.01)
22:6n-3	0.46	0.43	0.39[c]	0.35[c]	0.27[a]	0.24[c]	0.23	0.24
	(0.08)	(0.08)	(0.06)	(0.06)	(0.07)	(0.05)	(0.06)	(0.05)
Total n-3 LCPUFA	0.80	0.73	0.66[a]	0.60[a]	0.53	0.42	0.48	0.42
	(0.21)	(0.17)	(0.11)	(0.13)	(0.15)	(0.07)	(0.16)	(0.07)
Total n-3	1.51	1.38	1.51	1.30	1.43	1.13	1.52	1.13
	(0.32)	(0.20)	(0.26)	(0.16)	(0.32)	(0.07)	(0.45)	(0.07)
Total LCPUFA	3.08	2.85	2.34[a]	2.30[b]	1.90[a]	1.66[b]	1.80	1.66
	(0.46)	(0.55)	(0.28)	(0.30)	(0.33)	(0.16)	(0.30)	(0.16)
n6/n3 LCPUFA	2.60	2.71	2.69	2.71	2.56	2.88	2.58	2.88
	(0.55)	(0.42)	(0.63)	(0.68)	(0.63)	(0.72)	(0.61)	(0.72)

[a]Adapted from Genzel-Boroviczeny *et al.* (105). Capillary GLC column. German donors. For abbreviation see Table 14.
[a]$P < 0.001$.
[b]$P < 0.01$.
[c]$P < 0.05$ vs. prior study day.
[d]$P < 0.05$.
[e]$P < 0.05$ vs. term milk.

were identified as being synthesized solely in the mammary gland. Peaks and troughs in total lipid content occurred at the same time as in the unsaturated FA. Gravimetric data not provided in Reference 116 were sent to me by Dr. Villalpando. It appears that when the relative or real weights of unsaturated FA decreased postprandially, these amounts of 8:0–14:0 increased to compensate. However, the total lipid contents dropped at these times. These changes were seen in 8 of 10

TABLE 29
Influence of Time Postpartum on FA (wt% ± SD) in Milks from French[a] and Australian[b] Women

	Days postpartum[a]					Weeks postpartum[b]		
	2–5	15	30	60	120	6	16	30
FA	(n = 56)	(n = 33)	(n = 56)	(n = 132)	(n = 28)	(n = 23)	(n = 23)	(n = 23)
18:2n-6	11.70 ± 2.76	13.21 ± 4.77	12.83 ± 4.47	12.94 ± 4.17	13.23 ± 4.17	13.56 ± 2.84	13.92 ± 3.02	13.56 ± 2.93
20:4n-6	0.67 ± 0.30	0.53 ± 0.13[b]	0.41 ± 0.12[b]	0.41 ± 0.14[b]	0.50 ± 0.21[b]	0.45 ± 6.08[a]	0.40 ± 0.072	0.39 ± 0.07[c]
18:3n-3	0.66 ± 0.24	0.60 ± 0.21	0.61 ± 0.19	0.65 ± 0.16	0.57 ± 0.19	0.89 ± 0.27	0.94 ± 0.25	0.85 ± 0.25
20:5n-3	—	—	—	—	—	0.07 ± 0.04	0.07 ± 0.04	0.06 ± 0.03
22:6n-3	0.55 ± 0.29	0.43 ± 0.18[a,d]	0.32 ± 0.13[b]	0.37 ± 0.19[b]	0.38 ± 0.17[b]	0.26 ± 0.13[a]	0.21 ± 0.13[b]	0.19 ± 0.10[b]

[a]Adapted from Guesnet *et al.* (107). Fifty-meter capillary GLC column coated with CB WAX 52 CB. Values in same row with different superscript roman letters are significantly different, $P < 0.01$. French donors. For abbreviations see Tables 8 and 14.
[b]Adapted from Makrides *et al.* (108). Fifty-meter capillary column coated with BPX-70. Values with common superscript roman letters are significantly different, $P < 0.01$. Australian donors. Each analysis represents a pooled breast milk sample collected over seven consecutive days in the 6th, 16th, and 30th week of lactation, term infants.

subjects. The curves were flat in the other two, i.e., very little postprandial response. When the FA were grouped according to time and types (8:0–14:0, 16:0, 18:0, and 16:1, 18:1, 18:2), the peaks and troughs were not seen. This is the first time that these variations in milks from marginally malnourished women have been reported. Investigators face a difficult problem in obtaining a representative milk sample from these women. It could be done as in Reference 116, the portions pooled, and the FA in all of these analyzed to find the best match in profiles.

The effects of Western and non-Western maternal diets on the FA profiles of milk (published March 1995 to December 1997) are summarized in Table 31. The means are averages of the sums. Similar earlier data (1) are included for comparison. The major differences between Western and non-Western milks are less 18:2n-6 and LCPUFA in the Western milks and less *trans* FA in the non-Western milks. The availability of seafood (Table 23) greatly increased the contents of LCPUFA, and the lack of partially hydrogenated fats and oils was responsible for these differences in non-Western milks. High carbohydrate–low fat diets increased 10:0, 12:0, and 14:0, as can be seen in the older non-Western data and in the results from Nigeria (Table 20). The pooling I have done for Table 31 eliminates the effects of regional diets. These should be evaluated on an individual basis. Notably missing from Tables 8–31 are any data on milks from U.S. mothers.

COMMENTS ON MONOUNSATURATED FA

The major monounsaturated FA; *c*9-18:1 (oleic acid), in addition to the usual roles of FA (source of energy and structural components), reduces the melting point of TG, thus providing the liquidity required for the formation, transport, and metabolism of milk fat globules. Other *cis* isomers are present (Table 16). Desaturation of 18:0 to 18:1 occurs in the mammary gland of cows and rats (117). This step helps maintain the liquidity of milk fat. The reaction has not been detected in the human mammary gland.

All *trans* FA found in milk originate from the maternal diet. Some of the *trans* monomers, which present different profiles when derived from partially hydrogenated oils or from bovine milk fat, may cause problems. These will be discussed later.

Amounts of unsaturated FA in milk. The mean contents and ranges of unsaturated FA can be seen in Table 31. The changes observed are due to variations in maternal diet. This is indicated by the differences in 18:1*t* and 18:2 between

TABLE 30
Effects of Maternal n-3-Enriched Egg[a] or Docosahexaenoic Acid (22:6n-3)[b] Supplementation on the Fatty Composition (wt%) of Human Milk

	n-3-Enriched eggs, means[a]		Maternal supplementation with 22:6n-3[b] (g), mean ± SD				
FA	Pre	Week 6	Placebo	0.20	0.40	0.90	1.30
n	8	8	12	19	12	9	8
Lipid (%)	3.50	3.60					
Total saturates	40.30	40.80	43.32 ± 2.95	44.06 ± 3.21	44.13 ± 3.72	46.52 ± 4.94	44.71 ± 2.22
Total monounsat.	45.00	44.70	37.61 ± 3.62	37.08 ± 2.09	38.92 ± 2.54	36.79 ± 2.71	36.93 ± 2.24
18:2n-6	11.80	9.10	14.06 ± 3.38	13.47 ± 2.28	11.83 ± 2.25	10.93 ± 3.91	12.17 ± 3.13
20:4n-6	0.40	0.50	0.41 ± 0.06[a]	0.35 ± 0.07[a,b]	0.39 ± 0.07[a,b]	0.33 ± 0.06[b]	0.36 ± 0.04[a,b]
18:3n-3	1.50	2.40*	0.97 ± 0.32	1.06 ± 0.16	0.90 ± 0.30	0.92 ± 0.28	0.98 ± 0.33
20:5n-3	0.10	0.20	0.08 ± 0.03	0.07 ± 0.01	0.07 ± 0.03	0.08 ± 0.02	0.09 ± 0.03
22:6n-3	0.30	0.60	0.21 ± 0.07	0.35 ± 0.04[a,b]	0.46 ± 0.16[b]	0.86 ± 0.24[c]	1.13 ± 0.43[d]

[a]Adapted from Cherian and Sim (110). Thirty-meter capillary GLC column, coatings not given. Canadian donors ate two n-3-enriched eggs per day which contained up to 690 mg of n-3 FA per egg. *Significantly different, $P < 0.05$. For abbreviations see Table 8 and 14.
[b]Adapted from Makrides *et al.* (111). Fifty-meter capillary GLC column coated with BPX-70, Australian donors. Term infants. Values with different superscript roman letters indicate significant differences, $P < 0.05$.

TABLE 31
FA Composition (wt%) of Milks from Women Consuming Western and Non-Western Diets

	Western				Non-Western			
FA	Mean[a]	Range[a]	Mean[b]	Range[b]	Mean[a]	Range[a]	Mean[b]	Range[b]
10:0	1.42	0.05–2.21	1.01	0.06–2.39	1.00	0.00–1.73	1.63	0.50–3.42
12:0	5.67	2.01–11.77	4.94	1.70–12.32	6.14	1.59–12.16	8.12	2.40–16.51
14:0	6.58	2.26–11.68	5.63	1.98–11.78	7.22	4.27–17.72	9.59	5.30–15.90
16:0	21.58	12.90–27.50	20.33	19.25–25.10	19.60	16.05–29.77	21.46	14.10–25.77
18:0	6.04	3.49–10.65	7.54	5.83–9.70	5.90	5.00–10.95	5.61	0.80–8.20
18:1	31.08	23.55–55.25	34.33	25.83–42.48	27.10	21.49–39.21	32.89	20.24–50.83
18:1*t*	2.78	0.10–15.45	3.63	3.12–4.72	0.43	0.14–1.09	—	—
18:2	11.73	5.79–27.55	12.55	9.57–16.80	18.14	9.31–32.70	13.78	8.84–23.80
18:2*t*	0.61	0.00–2.41	—	—	0.04	0.04	—	—
20:4n-6	0.42	0.05–0.87	0.47	0.36–0.68	0.92	0.28–1.12	0.48	0.09–0.70
18:3n-3	1.08	0.25–1.90	0.69	0.31–1.85	2.07	0.20–3.03	0.52	0.10–0.98
20:5n-3	0.09	0.00–0.71	0.07	0.00–0.1	0.52	0.00–1.07	0.24	0.05–1.10
22:6n-3	0.45	Tr–1.03	0.23	0.64–2.20	0.88	0.00–2.78	0.57	0.10–1.40

[a]From Tables 14–19, 25, 26, 28–30 for Western diets; Tables 12, 13, 20–23 for non-Western diets. Term mature milks used. Means are the averages of all amounts for each acid.
[b]From Tables 35–37 in Reference 1 for comparison. For abbreviation see Table 14.

Western and non-Western women. Western women ingest more partially hydrogenated oils, hence more *trans* FA, than non-Western women.

Monounsaturated FA: trans *isomers*. The major problems here may be the *trans* isomers. Their effects on infant and fetal development were reviewed by Carlson *et al*. (118). They will not have been resolved and identified unless long (100 m) capillary columns with polar coatings (CP-88 or SP-2560) are used (119).

We know the following about the sources and transport of *trans* FA and early development. (i) They are produced in foods by biohydrogenation in the rumen and appear in ruminant milks and their products and by processing of oils, partial hydrogenation, and heating. The procedures produced different FA profiles (97). (ii) They originate from the diet. Geometric isomerization (elaidinization) is not an inherent mechanism in humans. (iii) They are transferred to the fetus *via* cord blood and to the infant through milk. The amounts are related to the quantities consumed in the maternal diet.

Craig-Schmidt *et al*. (120) derived the following equation relating the amounts of *trans* FA 18:1 in milk to the quantities in the maternal diet:

$$y = 1.49 + 0.42\,x \qquad [2]$$

where y = % *trans* 18:1 in milk fat and x = % *trans* 18:1 in dietary fat. With this formula, Chen *et al*. (97) found that 90% of the Canadian women whose milks they analyzed were consuming moderate (14.2%) to large (26% of total fat) amounts of *trans* FA. The total per capita *trans* FA content of the U.S. diet has been estimated as 8.0 g/d with 6.4–6.7 g from partially hydrogenated vegetable oils (118). Reference 118 has a table presenting the *trans* FA contents of many foods. Data can also be obtained from http://www.nal.usda.gov/fnic/foodcomp.htlm.

Trans FA in milk may have deleterious effects. To evaluate the possibility, we must know how much and what kinds of *trans* FA are in human milk. Data on the former are given in Tables 32 and 33 (121). The differences in quantities are

TABLE 32
***Trans* FA Contents (wt%) of Human Milk Determined by GLC with Packed Columns, U.S. Donors[a]**

	Range	Mean
Aitchison *et al.* (122)[b]	3.2–5.4	4.1
Picciano and Perkins (123)	2.1–4.0	3.1
Craig-Schmidt *et al.* (120)[c]	—	4.8
Clark *et al.* (124)	—	4.7
Hundrieser *et al.* (125)		3.4
Finley *et al.* (126)	—	4.3

[a]Adapted from Reference 121.
[b]Prior separation with Ag^+-TLC. See Tables 6,8 and 14 for abbreviations.
[c]Average of high (11.2%) and low (1.8%) *trans* in maternal diets.

TABLE 33
***Trans* FA Contents (wt%) Determined by GLC with Capillary Columns[a]**

	Trans acids		
Reference	Country	Range	Mean
Homer (127)	Finland	—	2.0
Chappell *et al.* (128)	Canada	—	2.9
Koletzko *et al.* (100)	Nigeria	0.79–10.29	1.2
Koletzko *et al.* (129)	Germany	2.17–6.04	4.4
Dotson *et al.* (130)	United States	—	4.7
Boatella *et al.* (131)	Spain	—	1.3
Wolff *et al.* (132)	France	—	2.0
Jorgensen *et al.* (133)	Denmark	—	2.5
Laryea *et al.* (101)	Sudan	—	0.6
Chen *et al.* (97)[b]	Canada	0.1–17.2	7.2
Chardigny *et al.* (98)[b]	France	1.2–3.0	2.0
Sampugna and Teter[c]	United States	—	1.5
Genzel-Boroviczeny *et al.* (105)	Germany	—	1.5

[a]Adapted from Reference 121.
[b]Contain amounts of 18:1*t* positional isomers.
[c]Personal communication, 1997. For abbreviations see Tables 8 and 14.

due to maternal diet. Consumption of oils containing *trans* FA has been increasing. The older analyses (Table 32), done with packed GLC columns, did not resolve the *trans* FA as well as the capillary columns (Table 33). We do not know the amount of *trans* FA in the diet that could initiate difficulties. A list of the effects associated with intakes of *trans* FA is provided in Table 34. While the findings are persuasive, the Expert Panel on *trans* fatty acids and fetal and infant development (138) stated that an influence on the normal metabolism of other acids by the amounts of trans FA consumed by the infant in milk during early growth has not been definitively established. Feeding studies that would provide answers are difficult because ethics committees would never authorize a trial that might have even a remote possibility of causing problems with infant or mother. During the interval, what should we recommend to mothers who are nursing their infants about *trans* FA in the diet? At this time we do not have data upon which advice can be given, except for the information below.

Ascherio and Willett (139) reemphasized their belief that consumption of *trans* fatty acids should be decreased. Conversely, Shapiro (140) doubts that the epidemiological data used in Reference 139 are sufficient to support a decrease in consumption. Nevertheless, a degree of risk may possibly be present and could be avoided. Reduction of intake might reduce risk of coronary heart disease in women (139) and possibly their infants.

COMMENTS ON PUFA

Linoleic (18:2n-6) and linolenic (18:3n-3) acids. Linoleic acid (144) and 18:3n-3 or α-linolenic acid (145) are essential, that is, they are required because they cannot be produced in the human. Innis (146) reviewed the roles of these acids in growth and development. Linoleic acid is the precursor of 20:4n-6 and 18:3n-3 of 20:5n-3 and 22:6n-3. These acids are formed from their precursors by elongation–desaturation and, for 22:6n-3, an oxidation reaction converting 24:6n-3 to 22:6n-3 and by recycling (147,148).

The quantities of 18:2n-6, 18:3n-3 and the LCPUFA derivatives in milk can be found in Table 31. The amounts in milk respond to their quantities in the maternal diet. This was shown by Insull *et al*. (149), who increased the contents of 18:2 in milk to 43% by feeding large amounts of corn oil to their subject.

From 1978, when our first review appeared (150), the av-

TABLE 34
Effects of *trans* FA[a]

1. Reduction in fat content of mice (134) and dairy cattle (135,136) but possibly not in lactating women (97).
2. Linear correlation between amounts of 18:1*t* in human diet and milk; $y = 1.49 + 0.42x$ where y = 18:1*t* in diet of previous day; % of total acids and x = % in milk (120).
3. Hypercholesterolemic in adults (137–139). Not considered high risk according to References 138 and 140.
4. Negative correlation between milk contents of 18:1*t* and of 18:2 and 18:3 (97).
5. None on amounts of prostaglandins in milk (120).
6. Impairment of biosynthesis of LCPUFA in premature infants (141) and children (142) and of growth in premature infants (143). However a causal relationship between *trans* FA intake and changes in early development not established (118).
7. *Trans* fatty acids found in plasma triglycerides, sterol esters, and phospholipids of premature infants (143). *Trans* negatively correlated with birth weight and with the biochemical reactions 18:3n-3 to 22:6n-3 and 18:2n-6 to 20:4n-6. Both LCPUFA required for maturation of brain and nervous system of infants (see Ref. 6).
8. Effects in 7 seen in children, 1–15 yr where applicable (143).
9. Vaccenic acid, *t*11-18:1, from ruminant fats may have less or no effect on coronary heart disease in women (140). Inferred from Retrospective Dietary Study.
10. *Trans* FA in plasma of premature infants may be inversely associated with LCPUFA in membrane lipids (118).

[a]Adapted from Reference 121. For abbreviations see Tables 10 and 14.

TABLE 35
Effects of Conjugated Linolenic Acids (CLA) Containing *c*9,*t*11-18:2[a]

1. Anticarcinogenic for carcinogen-induced tumors and neoplasias in stomachs and colons of mice and rats (186,187), mammary tumors of rats (188), and cultured human mammary cells (189).
2. Overcame catabolic responses to endotoxin injection (190).
3. Improved weight gain and feed efficiency in rats (191).
4. Enhanced select immune functions in several animals (192).
5. Antiatherosclerotic in rabbits (193). Reduced total LDL cholesterol and triglycerides. Reduced total cholesterol and early atherosclerosis in hypercholesterolemic hamsters (194).
6. Is a potent antioxidant (195) although this has been questioned (196).
7. Modulated bone metabolism (197).
8. Altered lipid composition in mouse liver (198). Reduced body weight; increased feed efficiency.
9. Decreased body fat and increased lean body mass in mice (199).

[a]Adapted from Jensen and Lammi-Keefe (121).

TABLE 36
Amounts of Conjugated Linoleic Acid (18:2Δ 9*c*,11*t*) in Various Foods

Sample	9*c*,11*t*-18:2 mg/100 g sample	9*c*,11*t*-18:2 mg/g fat
Ground beef, fresh[a]	—	3.70
Lamb	—	5.20
Pork	—	0.50
Chicken	—	0.80
Salmon	—	—
Sharp Cheddar cheese[b]	161.20	4.59
Cottage cheese	19.60	4.80
Mozzarella cheese	91.40	4.31
American processed cheese	91.10	3.64
Homogenized milk	14.20	4.49
Yogurt	7.40	3.82
Bovine milk[c] (n = 238)	—	8.1
Human milk		
Australian[d]		
Normal diet	22.60	5.80
Hare Krishna diet	43.70	11.20
United States, normal diet		
Idaho[e]	11.00	3.64
Connecticut[f]	4.00	1.80

[a]Meat data from Chin *et al*. (181).
[b]Dairy products data from Lin *et al*. (200).
[c]Precht and Molkentin (201).
[d]Fogerty *et al*. (202), sample weight based on 3.9% fat content here and in d and e.
[e]McGuire *et al*. (203)
[f]Jensen *et al*. (204).

TABLE 37
Fatty Acids in Human Milk Lipids[a]

Number	Type	Identity
	Saturates	
11	Normal, even	4:0–24:0
7	Normal, odd	11:0–23:0
49	Monobranched	10:0–18:0
5	Multibranched	12:0–13:0
72	*cis* monoenes	10:1–18:1, 20:1, 23:1–24:1–26:1. 7–16 positional of 18:
14	*Trans* monoenes	14:1, 16:1, 18:1 6–16 positional isomers
24	Dienes	12:2–22:2 all even, *cis,cis*; *cis,trans*; *trans,cis*; *trans,trans*; and positional isomers
9	Trienes	18:3, 20:3, 22:3, geometric and positional isomers
3	Tetraenes	20:4, 22:4
3	Pentaenes	20:5, 22:5
1	Hexaene	22:6
1	Cyclic hexane	11-terminal hexane
6	Cyclic 18*c*	Butyl and propylpentyl and hexyl 9 and 10
9	Hydroxy-	16:0, 18:0, 20:0, 22:0, 23:0, 24:0, 24:1, 25:0, 26:0
Total 214		

[a]Adapted from Jensen (1).

erage content of 18:2n-6 in milk has increased from about 8 to 14–16%. Has this caused an imbalance with the other unsaturated FA? The report by Sheaff *et al*. (151) indicated that high levels of 18:2n-6 did not inhibit the conversion of 18:3n-3 to 22:6n-3 in rats. Nevertheless, Lands has expressed concern about the imbalance (152). He hypothesized that an overproduction of n-6 prostaglandins produced from high levels of 20:4n-6 arising from large amounts of 18:2n-6 results in chronic inflammatory disorders. It may be that maternal diets are being consumed that will result in abnormally high levels of 18:2n-6 compared to 18:3n-3 in milk. A desirable ratio of 5:1 to 15:1 has been established (153). However, the ratio in many milks is greater than 15:1 (Table 31).

Sauerwald *et al*. (154) checked the effects of varying amounts of 18:3n-3, 0.4, 1.0, or 3.2%, with a fixed quantity, 16%, of 18:2n-6. They found greater fractional incorporation rates of 18:3n-3 into the plasma PL pool at 3.2% than at 1.0 or 0.4% levels. Yet, the fractional rates of conversion of 18:2n-6 to 20:4n-6 and incorporation of 20:4 into the plasma PL pool were lower at 3.2% 18:3n-3. According to the authors (154), their data cannot be used to estimate the amounts of LCPUFA synthesized daily. Obviously, without these amounts the adequacy of endogenous availability cannot be determined. The high intakes of 18:2n-6 did not inhibit the conversion of 18:3n-3 to 22:6n-3 (154). Furthermore, the premature infant with a very low birth weight is capable of synthesizing 20:4n-6 from 18:2n-6 and 22:5n-3 from 18:3n-3 (155). In term infants, 23% of plasma 20:4n-6 originates from 18:2n-6 (156). Microsomes from infant livers are capable of desaturating 18:2n-6 and 18:3n-3 (157).

A question remains: Is endogenous synthesis sufficient for the infant's requirements in growth and maturation of the brain, nervous system, and visual process? We do not know the answer, but it may be that LCPUFA are conditionally essential for preterm infants, and term babies may require them in their diets, but usually do not (158).

The functions of LCPUFA in human milk. The functions of LCPUFA have been reviewed by Gibson and Makrides (158) and I will use their paper as a guide for this presentation. Others who have recently reviewed all or parts of the literature are Innis (146), Nettleton (159), and Carlson and Werkman (160). Most of the recent papers describe studies on the effects of supplementation with FA on the development of preterm infants, e.g., Uauy and de Andraca (161). Many papers on the subject have appeared and the ones discussed below are useful for historical purposes. Beginning in 1973 and continuing, Crawford *et al*. (162) noted that the brain and nervous systems of infants, as well as human milk, contained LCPUFA. Crawford *et al*. (163) stated that the contents in brain and nerve tissues "probably indicated a remarkable thirst for long chain n-6 and n-3 fatty acids for fetal development." In 1993, Crawford *et al*. (164) emphasized the necessity for including 20:4n-6 and 22:6n-3 in formulas for preterm infants. Later, Crawford *et al*. (165) reviewed the requirements of preterm infants. They concluded that the amounts of these acids in formulas for preterm infants should be increased.

It has been known for years that human milk contains LCPUFA that were not being added to formulas. Nonetheless, the significance was not apparent until the results of feeding trials using diets containing formula-type fats done in 1984 and 1986 by Neuringer *et al*. (166,167) with rhesus monkeys showed the effects of a deficiency in 18:3n-3. The monkeys' tissues were low in 22:6n-3 and their vision was impaired. These findings led to investigations on the effects of 20:4n-6 and 22:6n-3 (168–170). However, some of the results with formulas for preterm infants were contradictory. The acids were known to be components of cellular membrane PL, but their importance was clarified when it was discovered that 18:2n-6 and 18:2n-3 were the precursors *via* 20:4n-6 and 22:6n-3 of eicosanoids. These bioactive compounds mediate many metabolic reactions and are regarded as hormones.

Is 22:6n-3 essential for infants? The amounts in milk range from 0 to 2.78 wt% with means of 0.45 for Western women and 0.88 for non-Western women (Table 31). Amounts of 0.2 to 0.3% are generally accepted as representative. The quantities in milk respond to the amounts of DHA in foods, primarily fish, in the maternal diet. It is almost always present in milk. An apparent absence may be due to analytical problems, such as failure to inject enough sample into the gas–liquid chromatograph. However, presence is not an indicator of essentiality. Gibson and Makrides (158) observed that the data are equivocal for specific health benefits of DHA supplementation to healthy term infants. Innis *et al*. (168) found in a study of term infants fed formula (no LCPUFA) as compared to those receiving human milk that the plasma and red blood

cell PL, docosahexaenoic acid (DHA), arachidonic acid, C, and apolipoprotein B were lower. There were no differences in visual acuity or growth between the groups. There was no evidence on the basis of these parameters suggesting the formula contained inadequate n-6 and n-3 fatty acids. The infants fed formula were assumed to be able to convert 18:2n-6 and 18:2n-3 to sufficient 20:4n-6 and 22:6n-3 for normal growth and visual acuity. This is because after about 2 wk postpartum, they may be able to synthesize the amounts required. Therefore, 22:6n-3 is not essential, because while needed, the infant can probably synthesize the amount required if precursor is available. In contrast, 18:2n-6 and 18:3n-3 are essential, because they are not synthesized.

Preterm infants do not have normal access to a uterine supply of LCPUFA; they also have very little adipose tissue and decreased reserves of essential FA. Therefore, the preterm infant may require more dietary DHA than the term infant. There is evidence that for infants born at less than 32 wk gestation, dietary DHA provides a measurable benefit on visual outcomes. However, DHA is still not essential as defined classically.

Gibson and Makrides (158) commented on the essentiality of 20:4n-6. There is no evidence of a dietary need of 20:4n-6 for healthy term infants. There have not been any reports of reduced growth related to a low 20:4n-6 status induced by n-3 LCPUFA. This has been observed in some preterm infants but not others (170). Carnielli *et al*. (155) showed that the very low birth weight infant can synthesize 20:4n-6 and 22:6n-3 from 18:2n-6 and 18:3n-3. Their data reveal that these infants have adequate levels of Δ-6 desaturation. Demmelmair *et al*. (156) found that 18:2n-6 conversion in term infants produced 23% of the 20:4n-6 in the infant's plasma. It appears that 20:4n-6 is not an essential FA in the diets of term or preterm infants.

Johnson *et al*. (171) identified free 9- and 13-hydroxyoctadecadienoic acids (9- and 13-HODE and their *R* and *S* isomers) in mammary gland tissue from mice. These oxygenated products of 18:2 are produced by free-radical (autooxidation) and/or enzymatic mechanisms. The amounts were increased by feeding a diet high in 18:2. Consumption of increased amounts of dietary fats high in 18:2, e.g., corn oil, increases the development of mammary tumors. Johnson *et al*. (171) proposed that 9- and 13-HODE may contribute to mammary tumorigenisis. They found that most of the hydroxy acids were produced by enzymatic processes and small quantities by autooxidation. To summarize, a diet high in 18:2 increases enzymatic production in the mammary gland of oxidation products that have been proposed to be associated with tumorigenesis. These products should appear in milk but the usual GLC analysis will not detect them. They would not appear on the chart because the highly polar hydroxy groups retard elution and are not methylated by esterification methods. Johnson *et al*. (171) converted the hydroxy groups in the methylated acids to trimethylsilyl ethers for GLC analysis. The authors also noted that there was no autooxidation when the mammary tissues were stored at –80°C for up to 1 mon.

Two excellent reviews on LCPUFA have recently been published by Hamosh and Salem (172) and Bendich and Brock (173). The latter authors describe the rationale for inclusion of LCPUFA in infant formulas as well as an increase in vitamin E levels.

Conjugated linoleic acid (CLA). CLA is the collective name of the positional and geometric isomers of octadecadienoic acid with conjugated double bonds. Such bonds are found at positions 9 and 11 or 10 and 12. The geometric configurations of each double bond can be *cis* or *trans*. The CLA are produced by biohydrogenation of PUFA in the rumens of dairy and beef cattle, sheep, etc. Meats, milk, and milk products from these animals contain CLA. Smaller amounts are found in processed oils, presumably as the result of heating and oxidation. The properties of CLA have been reviewed (121,174–181). The CLA have been reported to have a variety of beneficial effects including anticarcinogenicity. Because the properties of the acid have stimulated research and publications, Kramer *et al*. (182) have recommended that the trivial name rumenic acid be applied to *c*9,*t*11-18:2 to identify the source. Rumenic acid is the major and occasionally the only one of these acids in ruminant-derived foods; and it is believed, but not proven, to be the bioreactive fatty acid of CLA. CLA were known to exist, primarily in dairy products, for many years. Riel (183) analyzed bovine milk fat spectrophotometrically for CLA, finding a seasonal range of 0.24 to 2.81% with the highest values occurring when the cows were on pasture during the summer. Parodi (184) identified the major acid as *c*9,*t*11-18:2. The acid is not a normal constituent in the feed of dairy cattle. CLA are produced by ruminal biohydrogenation. The acid was identified earlier by Kepler *et al*. (185) as an intermediate when 18:2 was incubated with the rumen microorganism, *Butyrivibrio fibrisolvens,* an obligate anaerobe. Other isomers are also produced in the rumen, primarily *t*10,*t*-12-18:2.

The CLA are resolvable by GLC with an SP-2560 fused-silica capillary column, 100 m × 0.25 mm i.d. with a 0.2-mm film thickness (85) as previously mentioned. However, acid catalysts, HCl, BF_3, acetyl chloride, or H_2SO_4, employed for preparation of FAME, reduce the quantities of *c*9,*t*11-18:2 in bovine milk (85). The isomer is converted to *t*9,*t*11-18:2 and allylic methoxy artifacts (85). Base catalysts, for our purposes, sodium methoxide, do not affect rumenic acid and therefore must be used.

Attention has centered on CLA because they inhibited mouse epidermal tumors induced by 7,12-dimethylbenz(α)-anthracene (186). Several additional beneficial attributes have since been reported. These and other points are listed in Table 35. Since any FA in the maternal diet, even in trace amounts, will appear in human milk, the acid has been detected therein. These results are shown in Table 36. All of the investigators used polar capillary GLC columns and standards to separate and identify the acid. We confirmed identity with mass spectrometry (204). I noted in several papers that the acid was separated and identified in some as 18:2 *ct*, but not as rumenic acid. Fogerty *et al*. (202) observed that milk from members of

a Hare Krishna group had two times more rumenic acid than subjects on normal diets. This group consumed large quantities of butter and ghee (heated, clarified butter oil), which, based on a high fat content (at least 80%) contain large amounts of the acid. Huang *et al*. (205) fed 112 g (4 oz) of Cheddar cheese per day to male subjects. Plasma contents of rumenic acid were increased 19–27% without affecting C contents. While 4 oz is more cheese than one would usually consume during a meal or snack, these results indicate the content of rumenic acid in serum (and likewise in milk) can be increased by manipulation of the (maternal) diet. Park *et al*. (206) showed an increase in milk contents of rumenic acid when large amounts of foods containing CLA were ingested.

One of the most attractive properties of CLA listed in Table 35 is anticarcinogenicity. Parodi (179) discussed this property in a paper with the provocative title, "Milk fat conjugated linoleic acid: Can it help prevent breast cancer?" He noted that physiological concentrations of CLA suppressed cultured cell growth in human malignant melanoma, colorectal, breast, lung, prostate, and ovarian cell culture lines. Growth in all lines of leukemia, mesothelioma, glioblastoma, and hepatoma was also inhibited. Linoleic acid, the precursor of CLA, did not inhibit growth and was often stimulatory to these cell lines. CLA is an excellent inhibitor of mammary tumors in rats. Supplementations of the diet by 1% by weight or less are protective. When feeding occurs during the period of mammary gland development to adult stage morphology only, protection for life results. The type of dietary fat does not affect protection.

The CLA content of ruminant meat and milk can be increased by manipulation of the cow's dietary regimen. Consumption of these foods by humans increases the CLA content of breast milk and blood plasma. By extrapolation from rat studies, more CLA in the diet might protect against breast cancer. Ip *et al*. (188) calculated that 0.1% dietary CLA, the preventive amount for rats, would be equivalent to a daily intake of 3 g for humans. The estimated consumption in the United States is 1 g/d. However, these amounts are based on the mixture of isomers in the CLA used. The mixture contained about 42% rumenic acid, and this was the only isomer incorporated into membrane PL. McGuire *et al*. (178) gave an average consumption in the United States as 50 to 180 mg/d for adults and 224 mg/d for lactating women. We must have more reliable data on how much of the acid is required and how much of the isomer-rich foods should be consumed to attain this level. McGuire *et al*. (178) noted that consumption of only two glasses (16 oz, or 469 mL) of milk/d (55 mg/d of rumenic acid) by Finnish women decreased the risk of breast cancer. An increase of 55 mg/d would require modest increases in beef and dairy product consumption. Even so, the intake of fat, saturated FA, and C would also be increased slightly. Since this is counter to current dietary recommendations, only women with a family history of breast cancer and none of heart disease should consider a high rumenic acid diet after consulting their physicians. The acid is fat-soluble and increases with the fat content. Remember that CLA are antiatherogenic in test animals and some cultured cancer cells and that few physicians and nutritionists are knowledgeable about rumenic acid.

Ip (207), who with his colleagues has evaluated the dietary aspects of CLA, states that CLA are unique. They are the only mixture of FA, with rumenic acid (presumably anticarcinogenic), that significantly inhibits tumors. Paradoxically, the public relations effect of a nutrient can be gauged by how rapidly it is adopted by groups who hope for beneficial results beyond those described above. There are anecdotal reports that body builders, attracted by the apparent ability of CLA to shift nutrients from adipose to muscle tissue, are consuming CLA. Unfortunately, the amounts of rumenic acid in these preparations are not subjected to quality control. One commercial sample contained these *cis,trans* isomers: 8,10 (84%), 9,11 (30 %), 10,12 (31 %), and 11,13 (24 %) (208). This reference described a discriminating analysis of CLA isomers. Much more information is needed, but it is tempting to believe that women who do not have a family history of heart disease should increase their ingestion of foods containing rumenic acid while nursing their babies and protect themselves from breast cancer. Milk and its products contain many other desirable nutrients, e.g., calcium, high-quality proteins, B vitamins, etc. Preparations containing mostly rumenic acid are only now becoming available for feeding studies. Some of the many questions that have arisen should be answered.

FA IN HUMAN MILK

In Table 37 I have listed the FA known to be present in human milk. The number has increased from 199 in my last review (1) to 214. The new acids represent nine positional isomers of oleic acid, *c*9-18:1, and six cyclic pentyl and hexyls at C-9 and C-10 terminated by butyl and propyl chains to total 18 carbons. Many more are certainly present, awaiting the attention of the FA taxonomists. These minor acids should not be ignored. Rumenic acid is found in small amounts, but it has major physiological effects.

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ARTICLE

Cerebrospinal Fluid Lipoproteins Are More Vulnerable to Oxidation in Alzheimer's Disease and Are Neurotoxic When Oxidized *ex Vivo*

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ABSTRACT: Brain regional oxidative damage is thought to be a central mechanism in the pathogenesis of Alzheimer's disease (AD). Recent studies of cerebrospinal fluid (CSF) have suggested that increased lipid peroxidation of CSF and CSF lipoproteins also may occur in AD patients. In the present study, we determined the susceptibility of human CSF to *ex vivo* lipid peroxidation and tested the hypothesis that oxidized CSF lipoproteins may be neurotoxic. Whole CSF or a CSF lipoprotein fraction (d < 1.210 g/mL) was oxidized with 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH), a hydrophilic free-radical generator. Kinetics of CSF lipid peroxidation were followed by a standard fluorescence product accumulation assay. Oxidation of AD CSF yielded significantly shorter fluorescent lag times than controls, indicating reduced antioxidant capacity. Electrophoretic mobilities of CSF apolipoproteins were specifically reduced upon oxidation of CSF with AAPH, suggesting that lipoproteins are primary targets of CSF lipid peroxidation. Cultured neuronal cells were exposed to physiological concentrations of isolated CSF lipoproteins oxidized with increasing concentrations of AAPH; the resulting neurotoxicity showed a significant linear AAPH concentration-response relationship. These results suggest that oxidized CSF lipoproteins may contribute to the pathogenesis of neurodegeneration in AD.

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Alzheimer's disease (AD) is a devastating and common neurodegenerative disease that currently afflicts about 4 million Americans, a number predicted to approach 12 million by the year 2020 (1). AD is a clinical-pathological entity that probably derives from different causes. Autosomal dominant AD is relatively rare, accounting for less than 5% of all patients with AD. The majority of autosomal dominant AD is caused by mutations in one of three different genes: amyloid precursor protein, presenilin 1, or presenilin 2 (2). In contrast, sporadic AD is by far the most common form of the disease, but its causes have not been clearly defined. Several risk factors for sporadic AD have been identified, including advancing age, previous head trauma, and inheritance of the ε4 allele of the apolipoprotein E gene (*APOE*) (3). Despite differences in etiology, clinical and pathological data indicate that both autosomal dominant and sporadic forms of AD share common pathogenic steps (4). One pathogenic step proposed to be important in the progression of AD is regionally increased brain oxidative damage (5).

Oxidative damage results when oxidative stress exceeds the antioxidant capacity of tissue. Although several different classes of molecules may be affected, lipid peroxidation is thought to be a prominent and especially deleterious form of oxidative damage in brain due to this organ's relative enrichment in polyunsaturated fatty acids (6). Regionally increased brain lipid peroxidation has been demonstrated consistently in postmortem studies of AD patients compared with age-matched controls (5). Recent work also has shown increased central nervous system (CNS) lipid peroxidation in the early stages of AD in living patients compared to controls (7). Moreover, several experimental studies have demonstrated that products of lipid peroxidation may cause neurodegeneration that mimics some of the features of AD (8). The causes of increased brain oxidative damage in AD are not fully resolved but may involve both increased oxidative stress from several potential sources and reduced antioxidant defenses.

Human cerebrospinal fluid (CSF) contains lipoproteins that are distinct from their counterparts in blood (9). CSF lipoproteins are small high density lipoprotein-like particles that are enriched with either apolipoprotein E (apoE) or apoAI (10–13). CNS apoE is derived primarily from astrocytes, whereas apoAI is derived from hepatocytes and enterocytes; there is no apoB in the CNS (10,12–17). Therefore, apoE is thought to be the major protein for receptor-mediated lipoprotein trafficking in the CNS (9). Recently, we showed that CSF lipoproteins from AD patients contain reduced concentrations of polyunsaturated fatty acids compared to age-matched controls (16); however, the importance of these changes in the

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Abbreviations: AAPH, 2,2′-azobis(2-amidinopropane)dihydrochloride; AD, Alzheimer's disease; ANOVA, analysis of variance; apo, apolipoprotein; *APOE,* human apolipoprotein E gene; APOE4, ε4 alleles of *APOE*; BCA, bicinchoninic acid; CNS, central nervous system; CSF, cerebrospinal fluid; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; LDL, low density lipoprotein; PBS, phosphate-buffered saline at pH 7.4; RFU, relative fluorescence units.

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pathogenesis of AD is not known. One explanation for this finding is that lipoproteins in AD brain extracellular fluid are more vulnerable to oxidation than controls. Here we have tested this hypothesis by quantifying the susceptibility of CSF from AD patients and controls to lipid peroxidation. In addition, we have tested directly the hypothesis that oxidized CSF lipoproteins may contribute to neurodegeneration.

EXPERIMENTAL PROCEDURES

Collection of CSF. CSF from seven patients with AD and five control subjects was obtained from autopsies performed at Vanderbilt University Medical Center or the University of Kentucky Medical Center between 1996 and 1998. All AD patients carried a clinical diagnosis of probable AD during life and were verified as definite AD postmortem using standard criteria (18). Control subjects were age-matched individuals without clinical evidence of dementia or other neurological disease and who underwent annual neuropsychological testing with all test scores in normal ranges. All control subjects also underwent postmortem examination and were demonstrated to have age-related changes only. The histopathologic staging protocol of Braak and Braak was applied to all individuals (19). CSF was removed postmortem from the lateral ventricles, sedimented at 1,000 × *g* for 10 min, and frozen at –80°C until analyzed. Freezing at –80°C with a single thaw does not significantly alter CSF lipoprotein lipid or protein content (16,17). *APOE* genotype was determined postmortem in all cases (20).

Oxidation of CSF. Oxidation of CSF was initiated by the addition of 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH; Wako Chemicals USA, Richmond,VA) dissolved in phosphate-buffered saline (PBS) at pH 7.4 at 37°C under air.

Fluorescence of oxidized CSF. Oxidation of CSF was measured by spectrofluorometry (excitation 360 nm, emission 430 nm) as described for low density lipoprotein (LDL) oxidation (21,22). Relative fluorescence units (RFU) were measured continuously for 24 h with readings taken every 15 min. Lag time, maximal change in fluorescence (ΔRFU), and propagation rate were calculated for each sample from a plot of RFU vs. time (h).

Electrophoresis and immunoblotting. Following a 24-h incubation with AAPH, aliquots of native or oxidized CSF were boiled for 5 min in 2× Laemmli sample buffer (1:1 vol/vol dilution), and samples were electrophoresed on a 10% polyacrylamide gel (60 min at 150 V). CSF proteins were visualized by Coomassie staining or by immunoblotting with rabbit antihuman apoE antibody (Dako Corp., Carpinteria, CA) or rabbit antihuman apoAI (Calbiochem, La Jolla, CA) according to previously published methods (17). Electrophoretic mobility was determined by measuring the distance traveled by the monomeric protein from the origin.

Cell culture. Mouse Neuro2A cells were obtained from the American Tissue Culture Collection (Rockville, MD) and grown according to established methods (23). Cells were incubated in medium (DMEM/F12 with N2 supplement; Gibco, Grand Island, NY) in a humidified incubator at 37°C with an atmosphere of 5% CO_2/95% air. Neuro2A cells were plated onto 10-mm slides in cell culture medium at a cell density of 5×10^4 cells per slide for both fluorescent labeling and cytotoxicity experiments.

CSF lipoprotein isolation. CSF lipoproteins were prepared by ultracentrifugation and isolation of the $d < 1.210$ g/mL fraction exactly as previously described (16). The CSF lipoprotein fraction was then dialyzed against PBS at 4°C, and protein concentration determined using the bicinchoninic acid (BCA) method (Pierce, Rockford, IL).

Fluorescent labeling. CSF lipoproteins were diluted to 50 µg protein/mL and then labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) based on published methods used for plasma lipoproteins (24). Briefly, DiI (3 mg/mL in dimethylsulfoxide) was incubated with CSF lipoproteins for 18 h at 37°C and then filter-sterilized with a 0.22-micron filter to prepare the solution for cell culture and to remove unbound DiI. The labeled lipoproteins were then diluted 1:1 (vol/vol) with culture medium (final CSF lipoprotein concentration of 25 µg protein/mL) and incubated with cells for 1 h. The DiI-containing medium was removed, the cells washed, and then fixed in 4% paraformaldehyde/PBS for 30 min. DiI incorporation into cells was visualized with a Zeiss Axiovert 135 fluorescence microscope equipped with rhodamine filters (23).

Cytotoxicity assay. CSF lipoproteins were diluted to a protein concentration of 50 µg/mL in PBS. Diluted lipoprotein samples or PBS alone was oxidized with AAPH exactly as described above for whole CSF. Following oxidation, all samples were dialyzed against PBS at 4°C. CSF lipoprotein-containing samples were diluted into cell culture medium to give a final concentration of 25 µg protein/mL; samples without CSF were similarly diluted with cell culture medium. Cells were incubated with the oxidized samples for 24 h immediately after dilution into cell culture medium. Cell viability was determined using the Live/Dead Kit (Molecular Probes, Eugene, OR). Cytotoxicity was quantified by counting over 500 cells per culture (23).

Fatty acid quantification. Fatty acid composition of CSF lipoproteins was determined exactly as previously described (16). Briefly, after dialysis of the $d < 1.210$ g/mL fraction and determination of protein concentration by the BCA method, total fatty acids were quantified by gas chromatography following transmethylation using BF_3/methanol (25). Fatty acids were identified by comparison with known standards.

Statistics. Statistical analyses were performed using Prism 2.0 (GraphPad Software Inc., San Diego, CA) software.

RESULTS

Descriptive data as well as *APOE* genotype for the 12 individuals whose CSF was analyzed in this study are presented in Table 1. There was no significant difference between AD patients and controls when compared for age, gender, or postmortem interval. AD patients did have significantly lower

TABLE 1
Descriptive Data for AD Patients and Control Individuals

	Number	Age (yr)[a]	*APOE* (*n*)	Female/male (*n*)	Postmortem interval (h)[a]	Brain weight (g)[a]
AD	7	82 ± 2	3-3 (3) 3-4 (3) 4-4 (1)	4 : 3	2.7 ± 0.8	1089 ± 48*
Control	5	83 ± 1	3-3 (3) 3-4 (2)	3 : 2	2.6 ± 0.3	1229 ± 18

[a]Values are numbers or means ± SEM. *Significantly different from control ($P < 0.05$). Abbreviations: *APOE*, human apolipoprotein E gene; AD, Alzheimer's disease.

brain weights than controls ($P < 0.05$), a well-described consequence of cerebral atrophy in AD.

AAPH is a hydrophilic compound that generates a constant radical flux under the conditions used in these experiments, and that property has been used extensively in the study of lipoprotein oxidation (21). Oxidation of CSF was monitored using a fluorescence-based assay that has been widely employed to study plasma lipoprotein oxidation in both isolated lipoproteins and in whole plasma (21,22,26). This method detects fluorophores formed by reactions of lipid peroxidation products with amino groups on proteins and is characterized by three phases: a lag phase when there is no change in RFU, a propagation phase with steep change in RFU vs. time, and a plateau phase with no further increase in RFU (21,22). The concentration–response relationships for CSF oxidation by AAPH are presented in Figure 1. Oxidation of CSF from the 7 AD patients with 1, 5, and 10 mM AAPH significantly increased the RFU of CSF. Incubation of CSF with increasing concentrations of AAPH shortened lag times, an indicator of antioxidant capacity (ANOVA: $P < 0.001$, Fig. 1A). Lag times significantly decreased from 1 to 5 mM AAPH (paired analysis with Bonferroni correction, $P < 0.01$), but not from 5 to 10 mM AAPH. Lag times could not be determined for CSF samples not exposed to AAPH because there was no significant change in RFU over time in these samples. Propagation rate is an index of oxidizable fatty acid content and depends on the lipid composition of lipoproteins. The propagation rate of fluorescence did not change significantly with increasing AAPH concentration from 1 to 10 mM (Fig. 1B). Maximum change in RFU (ΔRFU) is the difference between RFU at the plateau and lag phases. All oxidized samples reached plateau by 18 h. ΔRFU significantly increased from 1 to 10 mM AAPH (ANOVA: $P < 0.05$); paired analyses with the Bonferroni correction showed a significant difference for 1 and 5 mM AAPH but not for 5 and 10 mM AAPH (Fig. 1C).

Comparison of the concentration-response relationships between controls and AD patients revealed significant differences in lag times, but not ΔRFU or propagation rate. Oxidation of AD CSF yielded significantly shorter lag times compared to CSF from controls at both 5 and 10 mM AAPH ($P < 0.05$, Fig. 2A). Lag times among CSF samples from AD patients and controls were inversely correlated with Braak stage (Spearman's ranked correlation: $P < 0.01$, data not shown). Lag times did not correlate with gender, age, or the number of ε4 alleles of *APOE* (APOE4). Although ΔRFU at both 5 and 10 mM AAPH tended to be greater in AD patients than in controls, these were not statistically significant differences (Fig. 2B). There was no significant difference in the propagation rates between AD and control CSF (data not shown).

In addition to generating fluorophores, extensive modification of protein with lipid peroxidation products can increase apparent molecular mass and in some cases lead to protein cross-linking (27). Such changes in the electrophoretic mobilities of apoAI and apoE were observed after oxidation of CSF with 5 and 10 mM AAPH (Fig. 3A and 3B). The apparent molecular masses of apoAI and apoE monomers increased with increasing oxidation by AAPH. Nonreducible abnormally high molecular weight species that were immunoreactive for apoAI and apoE also were observed with 5 and 10 mM AAPH (data not shown). The electrophoretic migrations of apoAI and apoE were significantly altered from the control at 5 and 10 mM AAPH (ANOVA: $P < 0.001$, Fig. 3C). In contrast to these two major CSF apolipoproteins, there was no change in the migrations of the two major CSF proteins not contained in lipoproteins, albumin and immunoglobulin, after oxidation with AAPH. Changes in electrophoretic mobilities of apoAI and apoE did not differ significantly between AD and control CSF, nor was there any relationship to the number of APOE4 alleles. These data suggest that CSF lipoproteins were a target for lipid peroxidation when CSF was incubated with the hydrophilic radical generator, AAPH.

The previous experiments were performed on whole CSF rather than isolated CSF lipoproteins because of the likely interactions between lipid- and water-soluble antioxidants in CSF. The next goal was to determine whether oxidized CSF lipoproteins carry some pathological significance. To accomplish this, CSF lipoproteins ($d < 1.210$ g/mL fraction) were isolated and oxidized with AAPH exactly as described for whole CSF. The alterations in electrophoretic mobilities of apoAI and apoE following incubation with AAPH under these conditions were identical to those described for whole CSF (data not shown). CSF lipoproteins were incubated with Neuro2A cells to assess neurotoxicity. Neuro2A cells were selected because this neuronal cell line has been used extensively to study the neurotrophic effects of human apoE-containing lipid particles, and Neuro2A cells have been shown to bind and internalize artificial lipid particles bearing human apoE isoforms (28).

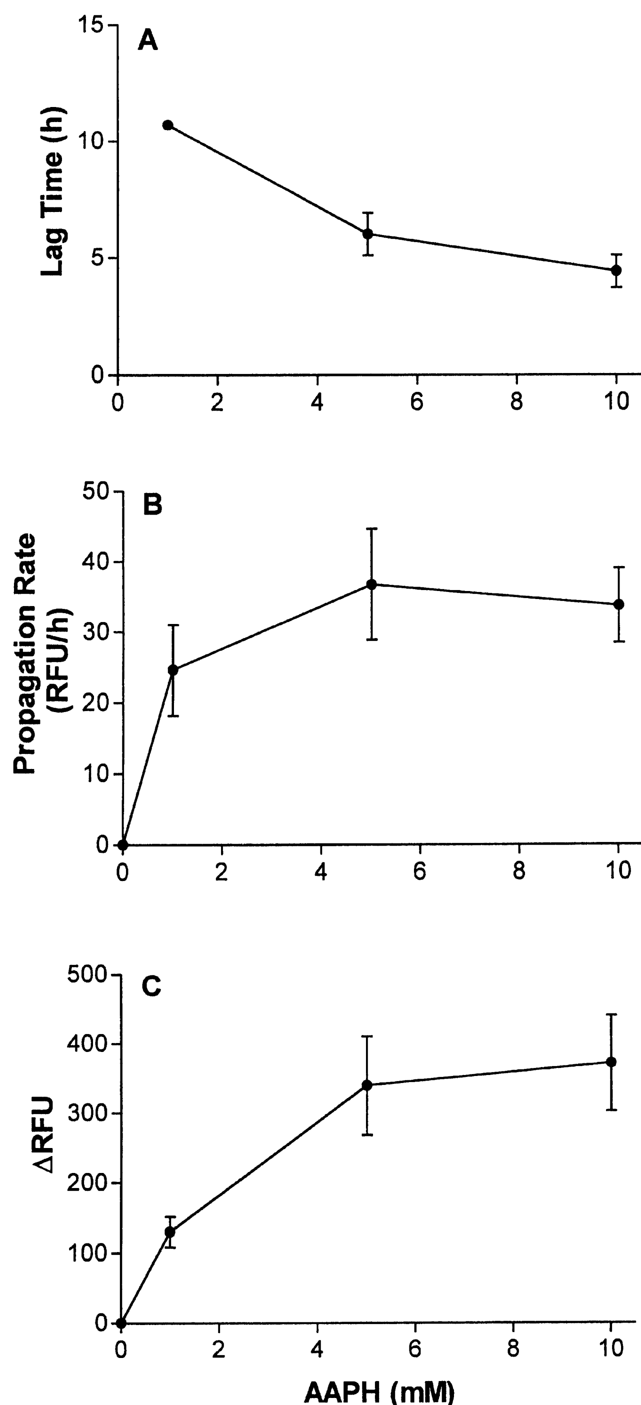

FIG. 1. Cerebrospinal fluid (CSF) oxidation by 2,2′-azobis(2-amidinopropane)dihydrochloride (AAPH). Alzheimer's disease (AD) CSF samples (n = 7) were incubated with 0, 1, 5, or 10 mM AAPH at 37°C for 24 h. Fluorescence (excitation at 360 nm, emission at 430 nm) was measured every 15 min. Plots of relative fluorescence units (RFU) vs. time were used to compute (A) lag time (h), (B) propagation rate (RFU/h), and (C) ΔRFU for each CSF sample. Values are presented as mean ± SEM of triplicate determinations. Analyses of variance were significantly different for lag time ($P < 0.001$) and ΔRFU ($P < 0.05$) over the concentration range 1 to 10 mM AAPH. Propagation rate did not change significantly over these concentrations of AAPH. There were no differences between 5 and 10 mM AAPH for any of the three variables.

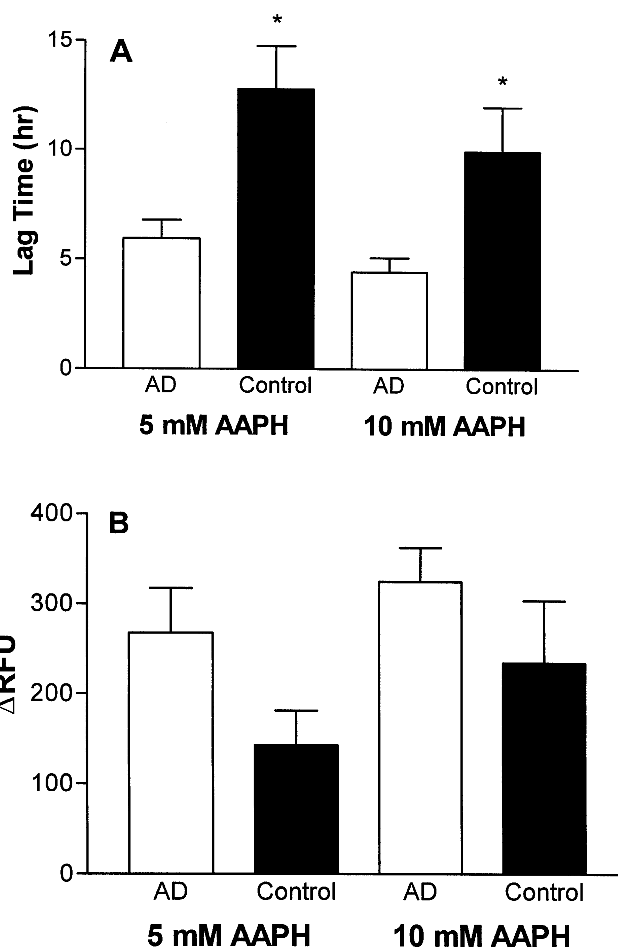

FIG. 2. Comparison of CSF oxidation between AD patients and controls. CSF from AD patients (n = 7) and controls (n = 5) was incubated with 5 or 10 mM AAPH at 37°C for 24 h. Fluorescence (excitation at 360 nm, emission at 430 nm) was measured every 15 min. Plots of RFU vs. time were used to compute (A) lag time (h) and (B) ΔRFU for each sample. Values are presented as mean ± SEM. Lag times for AD patients were significantly shorter than controls at both 5 and 10 mM AAPH (*, $P < 0.05$). Neither lag time nor ΔRFU was significantly different between 5 and 10 mM AAPH in AD patients or controls. For abbreviations see Figure 1.

Uptake of human CSF lipoproteins by mouse Neuro2A cells was confirmed by labeling CSF lipoproteins with DiI and visualizing incorporation into cells by fluorescence microscopy. Incubation of cells with DiI-labeled CSF lipoproteins led to rapid incorporation of fluorescent label into cells (Fig. 4). The vesicular pattern of fluorescence observed in Neuro2A cells following incubation with DiI-labeled CSF lipoproteins is characteristic of lipoprotein uptake into the endosomal-lysosomal system. A similar pattern of DiI uptake was observed with CSF lipoproteins oxidized with 10 mM AAPH. No DiI incorporation into cells was observed with lipoprotein-free medium (data not shown).

Nonoxidized and oxidized CSF lipoproteins were incubated with Neuro2A cells at physiologic concentrations and cell death was quantified. Sufficient CSF lipoproteins were

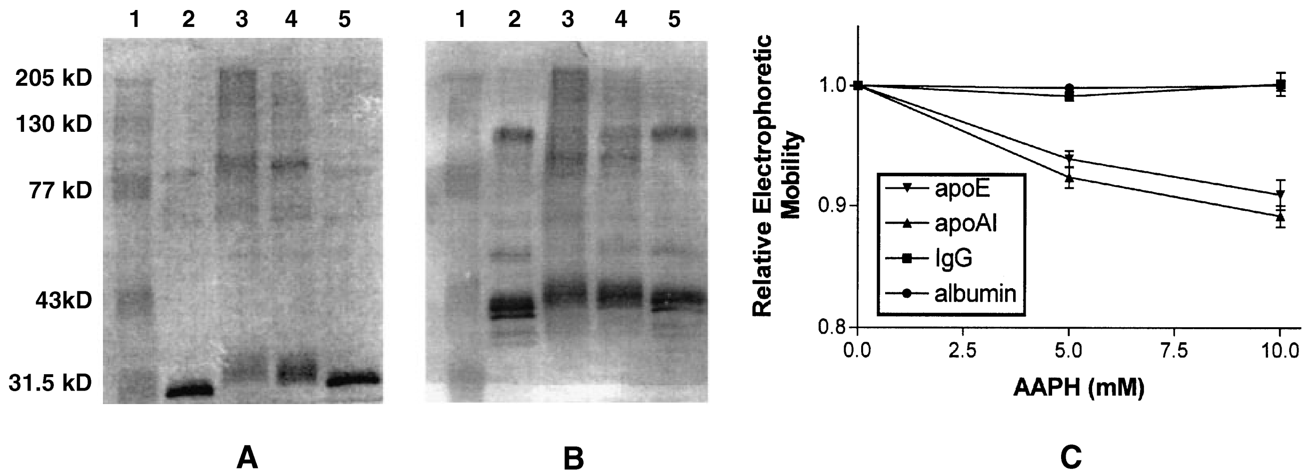

FIG. 3. Effects of AAPH on electrophoretic mobilities of CSF proteins. CSF from all 12 individuals were incubated with increasing concentrations of AAPH at 37°C for 24 h and then aliquots were taken, diluted 1:1 (vol/vol) in 2× Laemmli sample buffer, and boiled for 5 min. Proteins were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and either stained with Coomassie Blue or transferred to nitrocellulose and probed with anti-apoprotein (apo)AI or anti-apoE antibodies. (A) Anti-apoAI immunoblot and (B) anti-apoE immunoblot demonstrating the apparent increase in molecular mass of apoAI and apoE and the appearance of abnormally high molecular weight immunoreactive species. Lane 1 is molecular weight markers; lane 2 is CSF; lanes 3 to 5 are the same individual's CSF oxidized with 10, 5, or 1 mM AAPH, respectively. (C) Electrophoretic mobilities were determined by measuring the distance that the monomeric proteins migrated from the gel origin. Results from AAPH-exposed CSF are expressed relative to unexposed CSF. Electrophoretic mobilities were determined for apoE, apoAI, albumin, and immunoglobulin (IgG). Values are presented as mean ± SEM of triplicate determinations of all CSF samples. Eletrophoretic mobilities of apoE and apoAI, but not albumin and IgG, changed significantly over this concentration range (analysis of variance: $P < 0.001$). There was no difference between AD patients ($n = 7$) and controls ($n = 5$). For other abbreviations see Figure 1.

available to perform these experiments with samples from four individuals, two AD patients and two controls. Incubation of Neuro2A cells with nonoxidized CSF lipoproteins from control or AD patients did not produce significant neurotoxicity. Oxidized preparations using PBS rather than CSF lipoproteins were not neurotoxic. Incubation of Neuro2A cells with increasingly oxidized CSF lipoproteins resulted in a highly significant linear relationship between the extent of lipoprotein oxidation and neurotoxicity ($P < 0.001$, Fig. 5). There was no apparent difference in CSF lipoprotein-mediated neurotoxicity between AD patients and controls; however, the number of individuals is too small to allow a valid comparison between groups.

A final series of experiments was performed to determine whether the extent of CSF lipoprotein oxidation by AAPH correlated with degree of neurotoxicity. The concentration–response relationship for AAPH-mediated consumption of fatty acids in CSF lipoproteins was determined in two individuals from whom there was the largest volume of CSF (Fig. 6). Absolute concentrations of CSF lipoprotein fatty acids were similar to those previously reported (16). There was a significant concentration–response relationship between AAPH and reduction in total fatty acids, arachidonic acid, and docosahexaenoic acid ($P < 0.001$ for each measure). As expected, docosahexaenoic acid was most vulnerable, arachidonic acid was intermediate, and total fatty acids were least vulnerable to oxidation by AAPH (29).

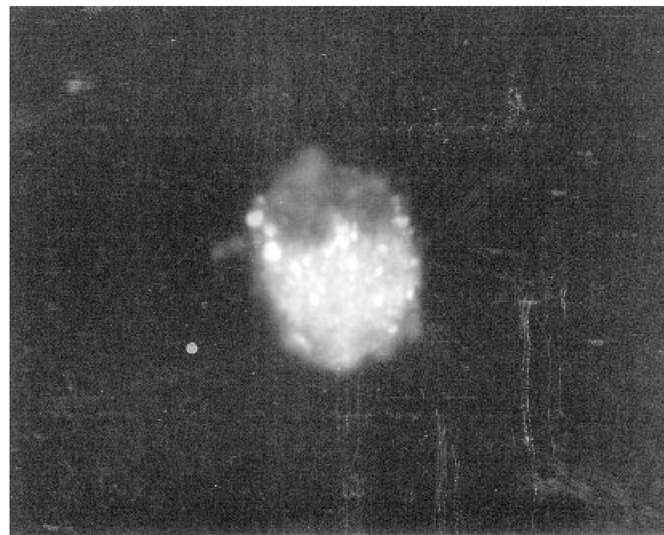

FIG. 4. Incorporation of fluorescent-labeled CSF lipoproteins into Neuro2A cells. CSF lipoproteins ($d < 1.210$ g/mL fraction) were labeled with 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate, filter sterilized, diluted in growth medium (1:1 vol/vol), and then incubated with Neuro2A cells for 1 h at 37°C. Cells were then washed, fixed, and examined by fluorescence microscopy using rhodamine filters (× 1000). For abbreviation see Figure 1.

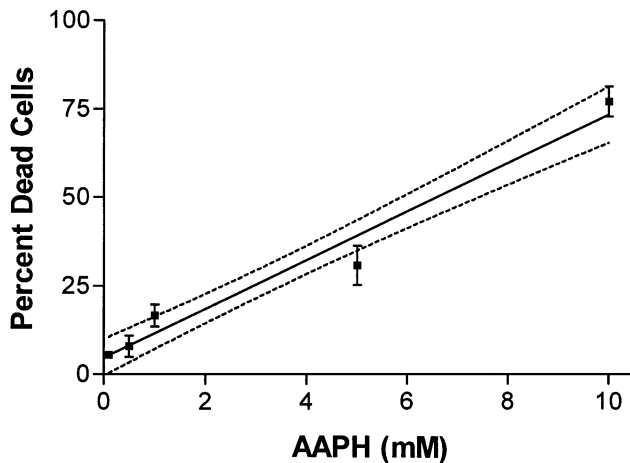

FIG. 5. Cytotoxicity of oxidized lipoproteins in Neuro2A cells. CSF lipoproteins (d < 1.210 g/mL fraction) were prepared from four different individuals (two control subjects and two AD patients). CSF lipoproteins or phosphate-buffered saline (PBS) alone was oxidized by incubation with increasing concentrations of AAPH for 18 h at 37°C and then dialyzed against PBS. CSF lipoprotein-containing samples were diluted with culture medium to give a final concentration of 25 μg protein/mL; samples without CSF lipoproteins were similarly diluted. All samples were incubated with Neuro2A cells for 24 h. Cytotoxicity was determined using the Live/Dead Kit (Molecular Probes, Eugene, OR) and counting over 500 cells per culture. Duplicate experiments were performed with each sample, and the average value for each of the four patients was then used to compute the mean and SEM. Oxidized PBS without CSF lipoproteins was nontoxic. Drawn are the best fit regression line (P < 0.001) and the 95% confidence intervals for CSF lipoprotein-containing samples. For abbreviations see Figure 1.

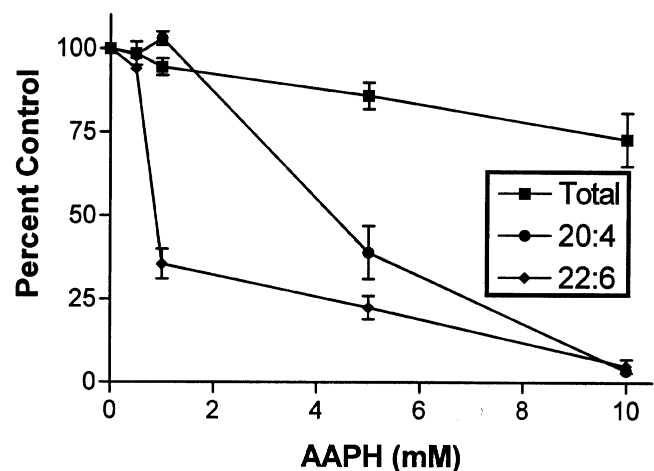

FIG. 6. Changes in CSF lipoprotein fatty acids following oxidation with AAPH. CSF lipoproteins (d < 1.210 g/mL fraction) were prepared from two different individuals (one control subject and one AD patient) and then incubated with 0, 0.5, 1, 5, or 10 mM AAPH for 18 h at 37°C. The concentrations of fatty acids (μg/mg protein) were determined twice for each sample using gas chromatography. Data are presented as a percentage of nonoxidized fatty acid levels. For abbreviations see Figure 1.

DISCUSSION

Regionally increased brain lipid peroxidation has been a consistent finding in postmortem studies of the AD brain (5), and increased CNS lipid peroxidation recently has been demonstrated in probable AD patients early in the course of dementia (7). In combination with these clinical studies, several experimental investigations have shown that lipid peroxidation products may cause neurodegeneration that mimics some of the features of AD (8). Here we have shown that CSF from AD patients was more vulnerable to lipid peroxidation than CSF from age-matched controls, that CSF lipoproteins were a target of oxidative damage to CSF, and that oxidized CSF lipoproteins were neurotoxic. These results suggest that oxidized CSF lipoproteins could play a role in the pathogenesis of neurodegeneration.

Several sources of increased free radical production have been proposed in AD. Recent attention has focused on extracellular sources of free radicals such as aggregated Aß peptides and products released by activated glia and inflammatory cells (8,30). These data would predict that brain extracellular fluid, and not just intracellular contents, could be exposed to oxidative stress in AD. We selected AAPH *ex vivo* oxidation of CSF to model the excess free radical generation in brain extracellular fluid that may occur in AD because CSF derives in large part from brain extracellular fluid and there is extensive literature on AAPH-mediated plasma lipoprotein oxidation (21,31). Earlier studies have shown that AAPH is a hydrophilic radical generator that, under the conditions used here, generates a constant low radical flux in the aqueous phase (32). Thus, AAPH-mediated oxidation of CSF *ex vivo* is a reasonable model of excess free radical generation in brain extracellular fluid *in vivo*.

Our results showed significantly shortened lag times for CSF oxidation by AAPH in AD patients compared with controls, indicating reduced antioxidant capacity in CSF from AD patients. Although this is the first demonstration of reduced antioxidant capacity in CSF, others have shown that brain tissue from AD patients may have reduced antioxidant capacity compared to controls (33–35). Moreover, recent clinical trials have shown a therapeutic benefit from α-tocopherol supplementation in AD patients (36). In combination, these data suggest that AD brain oxidative damage results in part from reduced antioxidant defenses. A potential application of our finding of reduced lag times in AD CSF resides with the ability to obtain CSF during life, thus raising the possibility of measuring CNS antioxidant status and assessing the efficacy of antioxidant therapeutics in living AD patients.

Braak staging is a histopathological grading system for AD; increasing Braak stage from I to VI indicates increasing severity of AD pathological changes (19). Shorter lag times for CSF oxidation, indicating reduced antioxidant capacity, were significantly associated with more severe AD pathological changes. It is noteworthy that lag times did not correlate with the number of APOE4 alleles. *In vitro* evidence has suggested that apoE may act as an antioxidant and that apoE4 is the least effective of the human apoE isoforms in this role (37). Investigation of apoE isoforms was not a goal of this study, and our results are limited by a relatively low number patients with different *APOE* genotypes and only one homozygous APOE4 individual. Despite these limitations, our data do not support a major

difference in the antioxidant function of native apoE3 vs. apoE4 in CSF under these experimental conditions.

The fluorescent products generated during lipoprotein oxidation are formed primarily by complex protein adducts from lipid peroxidation products (38). Some of these lipid peroxidation products covalently cross-link apolipoproteins (21,27). For example, oxidation of plasma LDL or exposure of LDL to lipid peroxidation products results in changes in electrophoretic mobility of apoB and covalent cross-linking of apoB (22,39). There is no apoB in human CSF (10,12,13,17). Rather, apoE and apoAI are the two major apolipoproteins in CSF. Protein adduct formation was confirmed on CSF apolipoproteins by the progressive increase in molecular masses of apoE and apoAI with increasing concentrations of AAPH. The very high molecular weight species containing epitopes for apoE and apoAI likely represent proteins that have been covalently cross-linked. Our results also showed that CSF proteins not contained within lipoproteins were not significantly modified by incubation with the hydrophilic agent, AAPH. These data suggested that lipoproteins were a major target for lipid oxidation in CSF. This is not surprising given that most of the lipid in CSF is contained within lipoprotein particles. However, these data are important because they underscore the potential for CSF lipoproteins to harbor a disproportionate load of lipid peroxidation products following oxidation of CSF.

Oxidized plasma lipoproteins, especially LDL, are thought to contribute importantly to the pathogenesis of another age-related disease, atherosclerosis. Our results with oxidized CSF and CSF lipoproteins parallel several findings with oxidized plasma and LDL. First, the vulnerability to oxidation of plasma and LDL is greater in patients with coronary artery disease, atherosclerosis, and hyperlipidemia compared to controls (40–45). Here we showed that CSF from AD patients is more susceptible to lipid peroxidation than controls. Second, oxidation of plasma or LDL results in modifications of apoB (21,22). Our study demonstrated that CSF apolipoproteins are relatively selectively modified under oxidizing conditions. Finally, oxidized plasma lipoproteins are cytotoxic to a variety of cell types (21), including neurons (46–49). We showed for the first time that oxidized CSF lipoproteins at physiologic concentrations are cytotoxic to neurons.

Despite these similarities, plasma lipoproteins and CSF lipoproteins have several important differences in their concentrations, lipid content, and apolipoprotein composition (10–13,16,17). It is important to note that, despite using physiologic concentrations of CSF lipoproteins, the extent of CSF lipoprotein oxidation necessary to induce significant neurodegeneration in our assay (i.e., 5 or 10 mM AAPH) produced changes in CSF lipoproteins that exceed those observed in AD patients (16,17). A similar situation exists for LDL oxidation and atherosclerosis; numerous experiments have shown that *ex vivo* oxidized LDL may contribute to atherogenesis, but appropriately modified LDL has been difficult to demonstrate in plasma (21). One explanation for this apparent discrepancy is that oxidized LDL is generated locally in atherosclerotic plaques and does not reach significant concentrations in the general circulation. This argument also may apply to oxidized lipoproteins in brain extracellular fluid vs. CSF and potentially explains why we did not observe neurotoxicity with nonoxidized CSF lipoproteins from AD patients. Moreover, we measured relatively acute neurotoxicity, in contrast to a disease that typically spans a decade. Protracted exposure to less extensively oxidized CSF lipoproteins possibly could contribute to neurodegeneration *in vivo*.

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Effect of the Nonenzymatic Glycosylation of High Density Lipoprotein-3 on the Cholesterol Ester Transfer Protein Activity

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ABSTRACT: This study examines the relationship between high density lipoprotein-3 (HDL-3) glycation and cholesteryl ester transfer mediated by cholesteryl ester transfer protein (CETP). HDL-3 were glycated with various glucose concentrations (0–200 mM) for 3 d at 37°C with sodium cyanoborohydride as reducing agent and antioxidants. About 47% of the lysine residues were glycated in the presence of 200 mM glucose, resulting in an increase in the cholesterol ester (CE) transfer of about 30%. Apparent kinetic parameters [expressed as maximal transfer (appT_{max}) and CE concentration at half of T_{max} (appK_H)] of CETP activity with glycated HDL-3 showed conflicting and paradoxical data: an increase in CETP activity associated with a decrease of CETP affinity. These alterations were not due to a change in HDL-3 lipid and protein composition nor to a peroxidative process but were associated with an increase in HDL-3 electronegativity and a decrease of HDL-3 fluidity. This study suggests that glycation modifies the apolipoprotein's conformation and solvation which are major determinants of interfacial properties of HDL-3. These modifications in turn affect CETP reactivity.

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The glycation of proteins in the pathogenesis of diabetes has been extensively investigated (1–4). As regards high density lipoproteins (HDL), recognized as protective against the atherosclerotic process (5), previous studies have shown that HDL glycated *in vitro* are functionally abnormal. They impair HDL-receptor-mediated cholesterol efflux (6), inhibit the high-affinity binding to cultured cells (7), and alter lecithin-cholesterol acyl transferase (LCAT) reactivity (8,9).

Cholesteryl ester (CE) transfer, a central step in reverse cholesterol transport, is mediated by the cholesteryl ester transfer protein (CETP), which is responsible for all of the neutral lipid transfer activity between lipoproteins (10–12). Furthermore, CETP is one of the major factors that remodels the concentration of HDL in circulation by mediating the heteroexchange of CE from HDL for triacylglycerols (TG) from very low density lipoproteins, which results in an enrichment of apoprotein (apo) B-containing lipoproteins with CE and HDL with TG. Bagdade *et al.* (13,14) have reported that the cholesteryl ester transfer is increased in both insulin-dependent diabetes mellitus and noninsulin-dependent diabetes mellitus (NIDDM). They have suggested that the compositional modifications of lipoproteins may enhance their interaction with CETP. In contrast, Ahnadi *et al.* (15) observed low *in vitro* rates of CE transfer using HDL from NIDDM patients and concluded that HDL from these patients have a decreased ability to transfer CE. As proposed by Van Tol (16), the reason for this low transfer could be that an accelerated *in vivo* transfer makes these HDL an inferior CE donor *in vitro*. Recently, Passarelli *et al.* (17) observed greater transfer rates with glycation of the donor and acceptor lipoproteins simultanously and suggested that glycation of HDL alone plays the major role in enhancing the transfer rate. Given the extreme complexity involved in CETP-mediated lipid exchanges, we chose to investigate the effects of *in vitro* glycation of HDL-3 alone (the more homogeneous of the HDL subfractions) on CE transfer. We demonstrate that *in vitro* glycation of HDL-3 affects the apparent kinetic parameters (appT_{max} and appK_H) of CETP in a paradoxical way.

MATERIALS AND METHODS

Isolation of HDL-3 particles. Fresh citrated plasma from nondiabetic donors was provided by the Centre de Transfusion Sanguine (Hôpital Larrey, Angers, France). HDL-3 were isolated as the plasma fraction of density 1.125–1.21 g/mL by sequential ultracentrifugation at 130,000 × g for 40 h. The HDL-3 fraction was subsequently washed with one 40-h spin at its upper density. The isolated lipoproteins were dialyzed 48 h against 10 mM Tris-HCl buffer (pH 7.4) containing 150 mM NaCl, 0.01% NaN_3 (wt/vol), and 0.01% EDTA (wt/vol).

CETP preparation. CETP was purified from normolipidemic human plasma as described by Lagrost *et al.* (18). Briefly, the plasma protein fraction precipitated with ammonium sulfate between 35 and 65% of saturation was then subjected to ultracentrifugation at a density of 1.21 g/mL. The re-

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Abbreviations: apo, apoprotein; CE, cholesterol ester; CETP, cholesterol ester transfer protein; DPH, 1,6-diphenyl-1,3,5-hexatriene; gHDL-3, glycated HDL-3; ^{3}H-CE, tritiated cholesterol ester; HDL, high density lipoproteins; K_H, CE concentration at half T_{max}; LCAT, lecithin-cholesterol acyl transferase; LDL, low density lipoprotein; NIDDM, noninsulin-dependent diabetes mellitus; r, fluorescence anistropy; rHDL, recombinant HDL; T_{max} maximal transfer velocity; TBARS, thiobarbituric acid-reactive species; TG, triacylglyerols; TNBS, trinitrobenzene-sulfonic acid; UC/PL, unesterified cholesterol/phospholipid ratio.

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sulting d >1.21g/mL fraction was then subjected successively to hydrophobic interaction chromatography on a phenyl-Sepharose column (CL-4B; Pharmacia Biotech, Uppsala, Sweden), to cation exchange chromatography on a carboxymethyl-cellulose column (CM52; Whatman, Maidstone, England), to affinity chromatography on an heparin hyper-D column (Biosepra), and to anion exchange chromatography on a DEAE-Trisacryl column (Biosepra, Villeneuve La Garenne, France). All chromatographic separations were carried out on a fast protein liquid chromatography system (Pharmacia Biotech) at 4°C. A linear gradient ranging from 0 to 0.5 M NaCl was used to elute the CETP from the DEAE-Trisacryl column. The active fractions, which were eluted with a Tris 20 mM–NaCl 150 mM (pH 7.4) buffer were pooled, aliquotted and stored at −80°C. The CETP preparation was deprived of apo A1 and of both LCAT and phospholipid transfer protein. Its activity was increased by about 2,000×.

Preparation of radiolabeled lipoproteins. HDL-3 labeled with ^{3}H-CE were prepared by combination of the LCAT and CETP reactions (9). ^{3}H cholesterol (50 µCi) dissolved in 100 µL absolute ethanol was added to 10 mL of a d > 1.125 g/mL fraction of human plasma and incubated at 37°C for 18 h. The density fraction 1.125–1.21 g/mL was isolated. Trace amounts of this fraction were incubated with a large excess of native HDL-3 and partially purified CETP at 37°C for 18 h. At the end of the incubation, CE-labeled HDL-3 was reisolated without CETP by ultracentrifugation at 1.21 g/mL; 94% of the radioactivity was in the form of CE, and the chemical composition of the labeled HDL-3 was very similar to that of native HDL-3. The specific radioactivity was about 3,500 dpm/nmol CE.

HDL-3 glycation. HDL-3 was glycated *in vitro* as previously described by Duell *et al.* (7). Briefly, ^{3}H-CE-HDL-3 were incubated 3 d at a concentration of 1 mg protein/mL in 0.9% NaCl/1 mM EDTA containing 0–200 mM glucose, sodium cyanoborohydride (12 mg/mL), and the antioxidants butylated hydroxytoluene (25 mM) and diethylenetriaminepentaacetic acid (50 mM). Control and glycated HDL-3 were dialyzed four times against 0.9% NaCl/1 mM EDTA before use. The amount of glycation was quantified with the trinitrobenzenesulfonic acid (TNBS) assay which specifically reacts with free amino groups of amino acid residues (19). Briefly, 50 µg glycated HDL-3 (1 mg/mL) was added to 1 mL 4% $NaHCO_3$, pH 8.4, mixed with 50 µL 0.1% TNBS, and incubated for 1 h at 37°C. The absorbance at 340 nm is a linear function of the concentration of trinitrophenyl derivatives. Hence, the relative reduction in absorbance for glycated HDL-3 (gHDL-3) compared with control HDL-3 is a direct indication of the relative loss of free lysine and $-NH_2$ terminal amino groups. The percentage of derivatized residues was calculated as follows:

$$\% = \frac{\text{absorbance of control HDL} - \text{absorbance of glycated HDL}}{\text{absorbance of control HDL}} \times 100 \quad [1]$$

CETP activity. CETP activity was assayed in duplicate using different CETP and HDL-3 preparations. Transfer rates were measured in conditions where transfer was linear with time of incubation; that is, less than 30% of HDL-3 radiolabeled CE (^{3}HCE) transferred. Transfer of ^{3}HCE was measured by incubating gHDL-3 (0.2 mM CE) with LDL (1 mM CE) and CETP. All incubations were carried out for 3 h at 37°C in Tris buffer pH 7.4, in a final volume of 250 µL. Then HDL and LDL were immediately separated by a single-spin centrifugation at d = 1.07 g/mL. Radioactivity in donor and acceptor lipoproteins was determined by liquid scintillation counting, and the percentage of cholesteryl ester radioactivity transferred was calculated. Incubations lacking CETP were used as control and were subtracted from that measured for the test. To investigate the effects of HDL-3 glycation on the CETP activity, the kinetic parameters that represented the maximal velocity of transfer T_{max} and the concentration required for half-maximal velocity K_H of the CE transfer were analyzed. gHDL-3 with an extent of glycation of about 21 and 47% and in a concentration range from 12.5 to 500 mM CE were incubated with LDL (1 mM CE) and CETP. As described by Sparks and Pritchard (20), we have determined values analogous to V_{max} and K_m as T_{max} and K_H.

Fluorescence polarization studies. Fluidity of lipoproteins was studied by determining the fluorescence anisotropy (r) of the probe 1,6-diphenyl-1,3,5-hexatriene (DPH). Lipoproteins were labeled with DPH following the procedure described by Dachet and coworkers (21). Briefly, a fresh dispersion of DPH was incubated in 1.5 mL of a buffer containing 10 mM Tris, 150 mM NaCl, 0.01% NaN_3 (wt/vol), and 0.01% EDTA (wt/vol), pH 7.4 with 50 µL of lipoprotein, for 20 min at 24°C with gentle agitation. The probe concentration was chosen so that the final probe/phospholipids ratio was always less than 1:1000, and incubation time was controlled in order to limit the probe diffusion into the lipoprotein core. Immediately after labeling, fluorescence measurements were carried out on a PerkinElmer MPF 66 spectrofluorimeter (Norwalk, CT) equipped with a fluorescence polarization accessory and a controlled temperature cell holder. The excitation and emission wavelengths were 360 and 430 nm, respectively. The fluorescence anisotropy was obtained from the fluorescence intensities parallel ($I_{\parallel}$) and perpendicular ($I_{\perp}$) to the polarization direction of excitation light using the equation of Schachter and Shinitsky (22):

$$r = \frac{I_{\parallel} - I_{\perp}}{I_{\parallel} + 2I_{\perp}} \quad [2]$$

Characterization of gHDL-3. Oxidation of control and gHDL-3 was measured as the amount of thiobarbituric acid-reactive species (TBARS) (23). Total and free cholesterol, phospholipids, and triacylglycerols were determined with enzymatic test kits from (total and free cholesterol: Boehringer-Mannheim; triglycerides: Ultimate, Roche; phospholipids: PAP150, Biomerieux). Protein concentrations were determined as described by Lowry *et al.* (24) using serum albumin as standard. In order to qualitatively check modifications elicited by glucose, electrophoretic mobility of gHDL-3 was analyzed on agarose gel stained with Oil red (Ciba Corning, Cergy Pontoise, France) and on 2–3% polyacrylamide gels stained with

Sudan black and Coomassie blue (Sebia, Issy les Joulineaux, France). The *z* potential was analyzed in distilled water with a DELSA 440 apparatus (Coultronics, Margency, France). Particles size was evaluated by nondenaturing gradient gel electrophoresis on 1.5–25% spiragel (Spiral, Couternom, France).

Statistical analysis. Means ± SD are given. Paired *t* tests (two-tailed) were used to determine the significance of differences. *P* values <0.05 were considered significant.

RESULTS

HDL-3 glycation. The extent of HDL-3 glycation assessed by the TNBS assay was positively related to the glucose concentration. about 21 ± 4.7% ($n = 4$) and 47 ± 8.9% ($n = 8$) of free lysine residues in HDL-3 were glycated after 3 d in the presence of 50 and 200 mM glucose, respectively, with sodium cyanoborohydride and antioxidants (Fig. 1). This result is in agreement with the results of Duell *et al.* (7) and Passarelli *et al.* (17), who reported that about 40 and 70% of free lysine residues in HDL-3 were glycated after incubation for 4 d with 150 and 200 mM glucose, respectively.

CETP activity. In experiments with 0.2 mM ^{3}HCE of control and gHDL-3 incubated with LDL (1 mM CE) and CETP, the CETP-mediated lipid transfer and the extent of HDL-3 glycation appeared to be positively related to the glucose concentration. Figure 1 shows that CETP activity increases whatever the level of HDL-3 glycation and that a low level of glycation (derived residues <10%) leads to a significant increase of CETP activity (13.7%). For high levels of HDL-3 glycation [derived residues 21 and 47%, a value near that obtained by Passarelli *et al.* (17) on diabetic HDL-3] the percentage of increase of CE transfer (compared with control HDL-3) was about 23 and 30%, respectively.

The curves presented in Figure 2 were the most representative of four saturation curves. The kinetic analysis of CETP activity with high levels of glycated HDL-3 shows that the CETP-mediated CE transfer in the presence of control or modified HDL-3 tends toward a saturable process. The apparent T_{max} and K_H calculated from experimental data using linear regression (1/*T* vs. 1/s) are significantly increased in gHDL-3 compared to control HDL-3 (Table 1). The T_{max}/K_H ratio which represents the CETP protein efficacy is identical for the control and gHDL-3.

Fluorescence polarization studies. The mean steady-state fluorescence anisotropy values at 24°C of DPH in control and

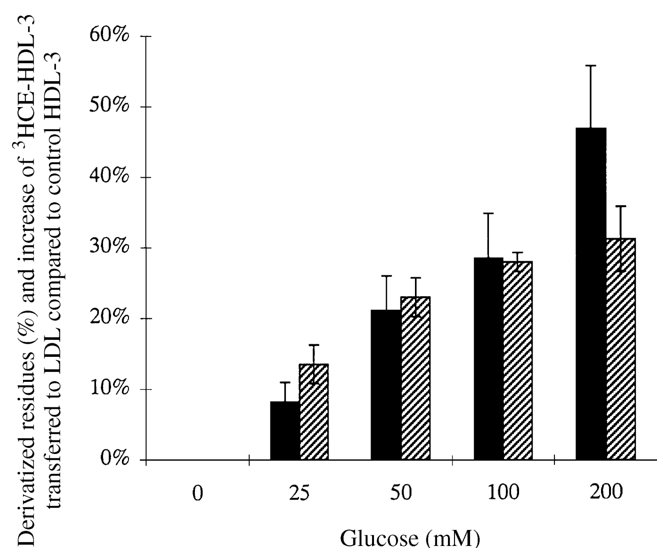

FIG. 1. Influence of glucose concentration on high density lipoprotein-3 (HDL-3) glycation level and on tritiated cholesterol ester (^{3}HCE)-HDL-3 transfer. Incubations were carried out with 0.2 mM CE-HDL-3 and 1 mM CE-low density lipoprotein (LDL) and cholesterol ester transfer protein (CETP) at 37°C for 3 h; solid block, % derivatized residues; diagonally lined block, % increase of ^{3}HCE-HDL-3 transferred by CETP to LDL as compared to control HDL-3. Data are means of four determinations (±SD) for 0, 25, and 100 mM glucose and eight determinations (±SD) for 50 and 200 mM glucose.

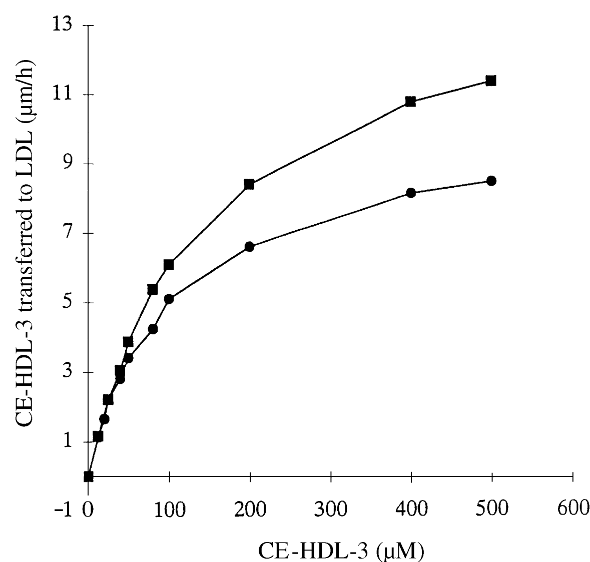

FIG. 2. Kinetic analysis of cholesteryl ester transfer by CETP from (●) control HDL-3 or (■) *in vitro* gHDL-3 (47%) to LDL during 1 h incubation, in a range of 12.5 to 500 µM CE-HDL-3. Concentration of acceptor lipoprotein (LDL) was 1 mM CE. The curve is the most representative of four different experiments made in duplicate. See Figure 1 for abbreviations.

TABLE 1
Kinetic Parameters of the CE Transfer from HDL-3 to LDL Mediated by CETP[a]

	appK_H (µM CE-HDL-3)	appT_{max} (µM CE-HDL-3/transferred/H)
Control-HDL-3	100 ± 17.2	10.1 ± 1.5
gHDL-3 (21%)	111 ± 18	11.6 ± 0.9
gHDL-3 (47%)	138.7 ± 40	14.5 ± 2.2
	($P < 0.05$)	($P < 0.01$)

[a]appK_H and appT_{max} values are means ± SD from four different gHDL-3 (47%) and two different gHDL-3 (21%). They were determined from 1/*T* vs. 1/*S* curves. Linear regression factors ranged from 0.98 to 0.99. CE, cholesterol ester; HDL-3, high density lipoprotein-3; LDL, low density lipoprotein; CETP, cholesterol ester transfer protein; gHDL-3, glycated HDL-3.

in vitro gHDL-3 (47%) were determined with the following results [glucose concentration (mM); fluorescence anisotropy (mean ± SD, $n = 4$; $*P < 0.05$ vs. control)]: 0, 0.181 ± 0.007; 25, 0.184 ± 0.007*; 50, 0.185 ± 0.004*; 100, 0.186 ± 0.004*; 200, 0.191 ± 0.005*. At all four glucose concentrations, values were significantly greater than those of the HDL-3 control (0.181 ± 0.007). As with the extent of glycation, most of the increase in fluorescence anisotropy was observed for the HDL-3 incubated with 200 mM glucose.

HDL-3 charge and composition. As shown in Table 2, the lipid and protein compositions of *in vitro* control and gHDL-3 (47%) are very similar to native HDL-3. However the determination of the ζ potential showed an increase of the electronegativity of gHDL-3 of about 49 ± 19%. Analysis of native and glucose-treated HDL-3 by electrophoresis showed that gHDL-3 moved faster on agarose gel and slower on acrylamide gel in proportion to glucose incorporation. Moreover, glycation had little influence on the staggering of the migration pattern (data not shown). These results did not agree with the observation of Fournier *et al.* (9) and Passarelli *et al.* (17) who found no change in the electrophoretic mobility of their *in vitro* glycated HDL.

On a polyacrylamide gradient gel (1.5 to 25%) a slight increase in size was observed according to the level of glycation: 6 to 11.5 nm for native HDL-3; 8 to 12.5 nm for control HDL-3; 8.15 to 13.2 nm for gHDL-3 (21%), and 9.3 to 16.3 nm for gHDL-3 (47%). The pattern of control HDL-3 is comparable to native HDL-3 one, and about 60% of gHDL-3 (47%) had a size less than 12 nm (data not shown).

DISCUSSION

The plasma activity level of CETP is a controversal subject in the development of atherosclerosis in diabetes. Recently, Passarelli *et al.* (17) observed greater transfer values between lipoproteins of diabetic subjects compared to those of control subjects and also observed that a high glycation level of HDL increases CETP activity. They also suggested that *in vitro* glycation of HDL-3 alone plays the major role in enhancing the transfer rate. This work showed that CETP activity increases with HDL-3 glycation level in a proportional way even at a low glucose concentration (25 mM), which could represent a plasma glucose concentration in a diabetic subject. In order to explain this result, we analyzed the kinetics parameters of CETP activity and some physicochemical properties of glycated HDL-3.

TABLE 2
Composition of Control and *in vitro* gHDL-3 (47%)[a]

	Control HDL-3 ($n = 4$)	gHDL-3 (47%) ($n = 8$)
Proteins	56 ± 3	58 ± 2
Phospholipids	25.4 ± 3.1	24.3 ± 2.1
Free cholesterol	1.9 ± 0.2	1.9 ± 0.3
Esterified cholesterol	14 ± 1.2	13.5 ± 1.7
Triglycerides	2.6 ± 1.1	2.1 ± 1.
ζ potential (mv)	–16.8 ± 5.5	–24.4 ± 4.9[b]
Size (nm)	8–12.5	9.3–16
Electrophoretic mobility (cm)	15.6 ± 0.6	17.2 ± 0.3

[a]The values are expressed as the percentage of each component (mean ±SD). [b]The values are expressed as the mean obtained with two different preparations of HDL-3. For abbreviations see Table 1.

The use of two levels of glycation (~21 and 47%) led to a significant increase of both appT_{max} (11.6 and 14.5 μM CE transferred/H) and appK_H (111 and 138.7 μM CE) as compared to control HDL-3 (appT_{max} = 10.1 μM CE transferred/H, appK_H = 100 μM CE), reflecting a reduced affinity of CETP for the glycated HDL-3 substrate and associated with a higher velocity of CE transfer. Similar paradoxical alterations of the LCAT activity were observed by Fournier *et al.* (9) for moderate *in vitro* glycated HDL-3 (derived residues <30%).

Our data, like those of Passarelli *et al.* (17), did not appear to be due to changes in HDL-3 chemical composition. No apparent alteration occurred in glycated HDL-3 in terms of lipid and protein composition or TBARS assay results. Glycation induced a slight increase in size, which could contribute to modifying interfacial properties of gHDL-3 without generating two distinct populations of small and large particles. However, the results obtained by electrophoresis (agarose and polyacrylamide gels) and measurements of potential showed an important increase in electronegativity of the modified HDL-3 due to decreased ionization of lysine residues. Masson and coworkers (25) reported that an optimal charge value is required for the maximal CETP transfer activity. Nishida *et al.* (26) proposed that the increased CETP activity observed with an increased lipoprotein negative charge was due to an increased affinity of CETP for lipoprotein and that above the optimal lipoprotein electronegativity, the decline in the transfer activity was caused by an excessive CETP–lipoprotein interaction. But in our glycation conditions, an increase in the extent of *in vitro* glycation corresponds to an increase in modified HDL-3 electronegativity and to a decrease in CETP affinity. This indicates that the CETP transfer activity is governed not only by electrostatic affinity but also by some other factor(s) such as the apo A-I conformation stability. In this way, Sparks and coworkers (27), who characterized structural spherical and discoidal recombinant HDL (rHDL), have demonstrated a strong relationship between the stability of apo A-I α helices and the surface charge characteristics of the apo A-I particles. It is known that glycation of apo A-I induces a decrease in its content of α helices (28) and a decrease in the stability of lipid–apolipoprotein complexes that affect the structural cohesion of HDL particles (29,30). We suggest that glycation, by increasing the soluble part of apoproteins as well as the surface electronegativity, alters the stability of apo A-I α helices and induces interfacial molecular reorganization of HDL-3. Such general events interfering with proteins functions are well described by Nylander (31).

Previous studies showed that addition of cholesterol to a discoidal rHDL directly affects both the conformation and charge of apo A-I by causing a significant reduction in the amount of α-helical structure in apo A-I and by modifying the physical properties of the rHDL particles (32). Increased cholesterol content has also been reported to affect LCAT activity by causing an increase of both appK_H and appV_{max} (33). Rajaram *et al.* (34) reported that the enhancement of CETP activity due to the

presence of unesterified cholesterol did not correlate with any increased binding of CETP to the emulsion surface. These elements led us to conclude that *in vitro* glycation affects CETP activity through a change in protein conformation, which causes a change of protein solvation in the lipoprotein envelope and an impaired association of lipids and apolipoproteins at the surface of the glycated HDL-3. This conclusion could be compatible with the work of Meng *et al.* (35) who propose a competition between apo A-1 and cholesterol for hydrophobic solvation by phospholipids and the possible existence of different pools of cholesterol within the lipoprotein whose distribution may vary in relation to apo A-1 domains. These events could induce a new repartition of lipids in HDL-3 and increase the solubilization of CE in the envelope, contributing to the reduced fluidity of glycated HDL-3 since cholesterol analogs like cholesterol-hemisuccinate are known as rigidifying agents (36). This agrees with the studies of Mac Ritchies (37), who has shown that interfacial pressure and electrical potential influence reaction rate by altering the distribution of species between bulk and interface. The mechanism responsible for the reduced fluidity of the glycated HDL-3 is not elucidated. It is not related to alterations in the cholesterol to phospholipids molar ratio but could be related to the extent of glycation as reported by Winocour and coworkers for platelet membranes from diabetic subjects (38). We think that this observed decrease of fluidity is a consequence of the decrease of the apo A-I helicity and the consecutive reorganization of the lipid–protein interactions. This supports earlier studies which showed that phospholipid order and apo A-I conformational stability are inversely related and together modulate the structural integrity of the discoidal rHDL (39–42).

The *in vitro* glycation of HDL-3, by increasing the hydrophobic part and the negative charge of the apolipoproteins, changes the apolipoproteins' conformation. This affects the lipidic solvation of the apoproteins and could increase the CE solubilization in the phospholipid envelope and create a better access of CE to CETP. Moreover, the curvature radius and the interfacial pressure are affected. These changes probably influence the CETP HDL-3 surface affinity. This is in good agreement with our recent work (43) in which we showed that CETP can penetrate a mixed lipid monolayer with a maximal penetration for unesterified cholesterol/phospholipid (UC/PL) ratio of 0.2, a value close to the ratio in native HDL-3, suggesting the presence of an optimal UC/PL ratio for a maximal CETP affinity. Glycation by inducing changes in packing density and in envelope composition modifies CETP activity.

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Effect of Eicosapentaenoic Acid on the Proliferation and Incidence of Apoptosis in the Colorectal Cell Line HT29

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ABSTRACT: Fish oil has been shown to reduce the induction of colorectal cancer in animal models by a mechanism which may involve suppression of mitosis, increased apoptosis, or both. We used the human colonic adenocarcinoma cell line HT29 to explore the effects of the long-chain n-3 polyunsaturated fatty acid eicosapentaenoic acid (EPA) on cell proliferation and death *in vitro*. Cells were cultured in media containing EPA at 5, 10, and 15 μg/mL. Cell number and thymidine incorporation were used to quantify proliferation, and cell cycle effects were studied using flow cytometry. Gel electrophoresis, annexin-V binding, and morphological criteria were used to characterize apoptosis. Adherent cells and freely floating detached cells were treated as two distinct populations. In the presence of EPA at 10 and 15 μg/mL there was a marked reduction in the growth rate of adherent HT29 colonies, owing to an increased detachment of adherent cells. After treatment with 10 or 15 μg/mL EPA the proportion of adherent cells in S-phase increased, indicating either a block in late S-phase or early G2. Floating cells showed evidence of extensive DNA cleavage, but the proportion of floating cells with sub G0 DNA content declined on treatment with 10 or 15 μg/mL EPA even though the number of floating cells increased. We conclude that EPA does not inhibit mitosis of adherent cells, but increases the rate at which they become detached from the substrate, probably at an early stage in the initiation of apoptosis. This mechanism may be analogous to "anoikis," or induction of apoptosis in response to loss of cell contact, and may contribute to the anticarcinogenic effects of fish oil *in vivo*.

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Cancer of the colon is one of the major causes of mortality in the Western world (1). The most common route of disease progression is through development of adenomatous polyps, a small proportion of which develop into full-blown adenocarcinomas (2).The colonic crypts of healthy individuals have well-defined regions of cell proliferation, migration, and cell death (3). Increased rates of cell proliferation, associated with an enlargement of the proliferative compartment, have been observed in patients with adenomatous polyps (4), as well as other diseases known to predispose to colon cancer (5,6). The progression of colon cancer is thought to depend on the acquisition of mutations in genes controlling cell division and differentiation (7). A reduced rate of cell proliferation and an extension of the cell cycle time may therefore reduce the risk of mutation by allowing the repair of damaged DNA during S phase.

Epidemiological evidence suggests that diets rich in n-3 series polyunsaturated fatty acids (PUFA) found in marine fish oil are associated with a reduced risk of cancers (8,9) including colorectal carcinoma (10). Furthermore, dietary supplementation with n-3 PUFA has been found to reduce cell proliferation in the colonic crypts of patients who have previously undergone surgery for the removal of adenomas (11–13). In animal models, dietary supplementation with fish oil inhibits chemically induced colon carcinogenesis in rats (14,15) and mice (16), increases apoptosis in rats (17), and reduces colonic crypt cell proliferation in normal rats (17,18).

The effects of n-3 PUFA derived from marine oils on cell growth have previously been investigated using a variety of cultured tumor cell lines, including those derived from mammary (19,20) prostate (21), pancreatic (22,23), cervical (24), and colonic tissue (25). On the basis of this work two major theories have been proposed to account for the inhibition of tumor growth by n-3 PUFA. The first is that increased availability of n-3 PUFA modifies the lipoxygenase and cyclooxygenase pathways for eicosanoid synthesis so as to reduce the rate of cell proliferation (19,26). The second is that incorporation of n-3 PUFA into the membranes of cells increases their susceptibility to oxidative damage (27–29), and hence to programmed cell death (apoptosis). Since the rate of tumor growth is a function of the relative rates of mitosis and apoptosis (30), both theories provide plausible explanations for the inhibition of tumor growth by n-3 PUFA *in vivo*.

Apoptotic cell death in healthy colon tissue is well documented and is thought to be the means by which cell numbers are controlled within the crypt, and by which mature cells are lost from the epithelium into the colonic lumen (31). It has also been shown that reductions in the susceptibility to apoptosis play a role in the progression of colon cancer (32). In this study we explored the effects of the n-3 PUFA eicosapentaenoic acid (EPA) on the relative rates of proliferation and death of colon cancer cells, using the colonic adenocarcinoma cell line HT29. In preliminary studies using clonal survival assays we established that EPA was significantly more effective than either oleic or linoleic acid in reducing HT29 cell survival. The doses of EPA used in this study, 0–15 μg/mL,

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; EPA, eicosapentaenoic acid; FAK, focal adhesion kinase; PBS, phosphate-buffered saline; PUFA, polyunsaturated fatty acid(s).

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are equivalent to the 10–20 μg/mL found in the plasma of unsupplemented individuals and are well below the 120 μg/mL found in subjects consuming approximately 1.5 g of EPA per day (33). A particular aim was to examine systematically both adherent cells and those that had become detached and were freely floating in the media, treating them as two distinct populations of cells. Proliferation was quantified using both cell numbers and incorporation of tritiated thymidine into the DNA. Apoptosis was examined using acridine orange staining (34), gel electrophoresis (35), and flow cytometric analysis of annexin V binding to cell membranes with externalized phosphatidylserine, characteristic of early apoptotic cells (36). The effects on adherent cell number following blocking of the protease, caspase, known to be involved in apoptosis were also tested.

MATERIALS AND METHODS

Cell culture. The colon adenocarcinoma cell line, HT29, was obtained from the European Collection of Cell Cultures (Salisbury, United Kingdom). On receipt, the cells had already gone through 140 passages and were used in this study after a further 5 to 10 passages. HT29 cells were grown in Eagle's minimal essential medium (MEM) containing Earle's salts (Sigma Chemical Company, Poole, United Kingdom) supplemented with 5% fetal calf serum, 2 mM glutamine, 50 units/mL penicillin, and 50 μg/mL streptomycin (all obtained from Imperial Laboratories, Andover, United Kingdom). Cells were grown at 37°C, 5% vol/vol CO_2 in a humidified atmosphere and harvested using phosphate-buffered saline (PBS) containing 0.05% wt/vol trypsin and 0.02% wt/vol EDTA (Imperial Laboratories). Cells were then washed in medium to inactivate the trypsin before reseeding or analysis.

Treatment of cells with EPA. Cis-5,8,11,14,17-EPA (Sigma Chemical Company) was dissolved in ethanol to give a 25 mg/mL stock solution and stored under nitrogen in an amber vial at −20°C. This stock solution was further diluted in complete medium to supplement HT29 cells at levels of 5, 10 and 15 μg/mL, while maintaining ethanol concentrations at less than 0.1% vol/vol. Control cultures initially contained either the appropriate amount of ethanol or ethanol-free medium, but as no significant effect of this concentration of ethanol was detected subsequent control samples were grown in ethanol-free medium. The media were refreshed every 24 h. Floaters were obtained by gently tapping of the plates, removal of the supernatant, and one wash of the plate with PBS. Adherent cells were obtained by trypsinization, as described above.

Determination of cell growth rate. Cells were obtained from late log phase cultures, seeded in complete media onto 35-mm diameter plates (Imperial Laboratories) at a density of 5×10^4 cells per plate (5.2×10^3/cm^2) and allowed to settle for 24 h before treatment with the appropriate level of EPA. Floating and adherent cells were harvested and counted from three plates per treatment at 24-h intervals. A second set of identical plates was used to determine the incorporation of tritiated thymidine (see below).

Incorporation of ^{3}H-thymidine. The method used was a modification of that described by Mengeaud *et al.* (25). Sterile PBS (10 μL) containing ^{3}H-thymidine (37 kBq; Amersham, Little Chalfont, United Kingdom) was added to the medium in each 35-mm diameter plate and samples of medium (2 × 10 μL) were obtained immediately to determine the activity of the media. Plates were incubated at 37°C for 4 h to allow incorporation, washed twice in cold PBS, treated with 5% trichloroacetic acid for 10 min at 4°C, washed a further three times in absolute ethanol, and the cell layer was dissolved in 1 mL of 0.1 M NaOH. Samples of media (2 × 10 μL) and dissolved cells (2 × 400 μL) were each added to 10 mL of Quicksave A scintillant (Zinsser Analytic, Maidenhead, United Kingdom) and counted using an automatic scintillation counter (Packard, Reading, United Kingdom).

Light microscopy. The medium was removed from each 35-mm diameter dish of treated and untreated HT29 cells, and a coverslip placed over the cells. The samples were observed using a differential interference contrast light microscope and photographed using FP4 film (Ilford Ltd., London, United Kingdom) and a Photoautomat MPS 45 camera (Leitz Instruments Ltd., Luton, United Kingdom).

Acridine orange staining of apoptotic nuclei. HT29 cells were grown in 8 × 2 cm flaskettes (Nunc, Life Technologies, Paisley, United Kingdom) consisting of a microscope slide base, to which cells adhere, covered by a detachable plastic cover. Floating cells were obtained from triplicate 88-mm diameter tissue culture dishes, pooled, and resuspended in 200 μL PBS. Both adherent and floating cells were stained with 5 μg/mL acridine orange for 10 min and washed once with PBS. Adherent cells were mounted in DPX mounting fluid (BDH, Merk, Lutterworth, United Kingdom) and floating cells were observed while in solution. A cover slip was placed on the sample which was then viewed using a Nikon Optiphot fluorescence microscope (Nikon, Kingston-Upon-Thames, United Kingdom).

Gel electrophoresis of apoptotic DNA fragments. For gel electrophoresis and flow cytometry, HT29 cells were obtained from late log phase cultures and seeded in complete medium onto 88-mm diameter plates at a density of 8.5×10^5 cells per plate (1.4×10^4/cm^2). Cells were allowed to adhere to the plate and grow for a total of 48 h before treatment with EPA or control medium. Unless otherwise stated, floaters from three identical plates were pooled in order to obtain sufficient cells for analysis and adherent cells were obtained from a single plate. Both floating and adherent cell samples were taken and washed twice in PBS. After counting, samples containing 1×10^6 cells were transfered to Eppendorf tubes and resuspended in 10 mM EDTA, 50 mM Tris-HCl (pH 8.0) containing 0.5% wt/vol *N*-lauryl sarcosine and 0.5 mg/mL proteinase K (all obtained from Sigma). Samples were mixed gently and incubated at 50°C for 1 h. To each sample, 10 μL of 0.5 mg/mL ribonuclease A (Sigma) was added and samples were further incubated for 1 h. Samples were heated to 70°C, and 10 μL of the following buffer was added: 10 mM EDTA, 50 mM Tris-HCL (pH 8.0), containing 1% wt/vol low melting point agarose, 0.25% wt/vol bromophenol blue, and

40% wt/vol sucrose (all from Sigma). Samples were mixed and loaded into dry wells of a 2% agarose gel using siliconized pipette tips. The gel was run at 40 V using TBE running buffer (130 mM Tris, 75 mM boric acid, 2 mM EDTA) until the dye front had migrated approximately 3 cm. The gel was stained with 1 µg/mL ethidium bromide in H_2O for 30 min, after which the gel was observed under ultraviolet illumination and photographs were taken.

Flow cytometric cell cycle analysis. A modification of the whole cell method described by Nicoletti *et al.* (37) was used. Cells, grown as for gel electrophoresis, were washed twice in PBS containing 1 mM EDTA, and the concentration was adjusted to approximately 5×10^5 cells/mL. Samples were resuspended in 80% vol/vol ice-cold ethanol and fixed on ice for 30 min. After washing twice in PBS, cells were resuspended in 1 mL of staining solution, consisting of PBS containing 30 µg/mL ribonuclease A, 0.1% vol/vol Triton X-100, and 50 µg/mL propidium iodide (all Sigma). Cells were stained for 30 min at room temperature and analyzed using a flow cytometer designed and built in-house, which has been described elsewhere (38). Data were analyzed on logarithmic scales for forward angle and right angle light scatter, and on a linear scale for red fluorescence. Noncellular material was excluded from the analysis by gating on the forward angle and right angle light-scattering characteristics of either adherent or floating cells. Red fluorescence intensity was used for the determination of position within the cell cycle, using Phoenix Multicycle for Windows cell cycle analysis software (Coulter Electronics Ltd., Luton, United Kingdom). Data are plotted as cell number against DNA content as detected by level of red fluorescence. Cells with a DNA content ranging from 2n to 4n were designated as being in G0/G1, S, or G2/M phases of the cell cycle, as defined by the level of red fluorescence per cell, using the above software. Cell cycle distributions were compared using analysis of variance, and where the *F*-statistic was significant, Student's *t*-test was used to determine direction.

Flow cytometric analysis of annexin V binding to apoptotic cells. Externalization of phosphatidylserine and membrane integrity were quantified, by a modification of the method of van Engeland *et al.* (36), using the ApoDetect Annexin V-FITC kit (Zymed Laboratories Inc., San Francisco, CA). Adherent cells grown in 35-mm diameter plates were labeled prior to harvesting. After removal of media plates were washed in PBS, before addition of binding buffer (1 mL) and 50 µL Annexin V-FITC (10 µg/mL) and 50 µL (20 µg/mL) propidium iodide. After 10 min at room temperature, plates were washed twice in binding buffer and cells then harvested by scraping and then resuspended in binding buffer (5×10^5/mL) before analysis by flow cytometry. Cells were identified on the basis of their forward and right angle light-scattering characteristics. Red and green fluorescence were analyzed on a logarithmic scale. Live cells were defined as those showing no fluorescence and apoptotic cells those showing only green fluorescence. Cells exhibiting both green and red fluorescence were designated late apoptotic/necrotic and red cells were regarded as damaged (39).

Caspase inhibition. The effect of inhibiting caspase activity on cell number was undertaken using a 96-well plate assay. Cells were plated out at 1.5×10^4 per well and allowed to adhere for 24 h. Cells were then exposed to EPA (15 µg/mL) or the caspase inhibitor Z-DEVD-FMK [Calbiochem-Novabiochem (UK) Ltd., Nottingham, United Kingdom] at a final concentration of 75 µM, or EPA (15 µg/mL) plus Z-DEVD-FMK, for 48h. Cell number was compared by incubation for 3 h at the end of each experiment with 50 µM Neutral Red supravital dye (Sigma) in complete Dulbecco's modified Eagle's medium. Following fixation in 40% (vol/vol) formaldehyde (BDH)/10% (wt/vol) calcium chloride hexahydrate (Fisher Chemicals, United Kingdom) for 30 s, plates were washed three times in PBS, air dried and the neutral red was solubilized from cultured cells by addition of 200 µL per well ethanol (50%)/acetic acid (1%). Absorbancies were measured at 550 nm, in 16 wells for each treatment, using a Dynatech MR5000 plate reader (Dynex Technologies, Chantilly, Virginia). Results were calculated as a percentage of the absorbance for control cells.

RESULTS

Effect of EPA on the growth of HT29. Over a period of 8 d treatment with 5 µg/mL EPA caused a slight reduction in the rate of accumulation of adherent cells compared to controls between day 0 and day 1, after which the treated cells and controls grew at similar rates. Treatment at a level of 10 µg/mL EPA caused a similar reduction in adherent cell count, which became more marked in comparison to the controls after 4 d. Treatment with 15 µg/mL EPA caused adherent cell numbers to remain static throughout the experiment (Fig. 1A). In the same experiment the number of floating cells was similar in the control samples and those samples treated with EPA at a level of 5 µg/mL. However, in samples treated with 10 and 15 µg/mL EPA, there were significantly more floaters than in the controls, with the number of floating cells increasing with increased EPA supplementation (Fig. 1B). To represent the effect of EPA more clearly, floating cell numbers were expressed as a percentage of the total number of floating plus adherent cells (Fig. 1C). At the higher levels of EPA (10 and 15 µg/mL) there was a significantly greater proportion of floating cells, indicating that the rate at which cells were shed from the plate into the media had increased under these conditions.

Effect of EPA on the proliferation of HT29. Having established that EPA causes a reduction in the number of cells adhering to the plate, incorporation of ^{3}H-thymidine was expressed per adherent cell rather than per plate, in order to compensate for the reduction in cell number. Supplementation of the media with 0, 5, 10, or 15 µg/mL EPA did not significantly reduce the proliferation of HT29 cells (Fig. 2A). Indeed supplementation at the higher dose of EPA was associated with increased thymidine incorporation at each time point up to and including day 6 (Fig. 2B).

Microscopical analysis of cell morphology. Cells treated with 5 µg/mL EPA were similar in appearance to control

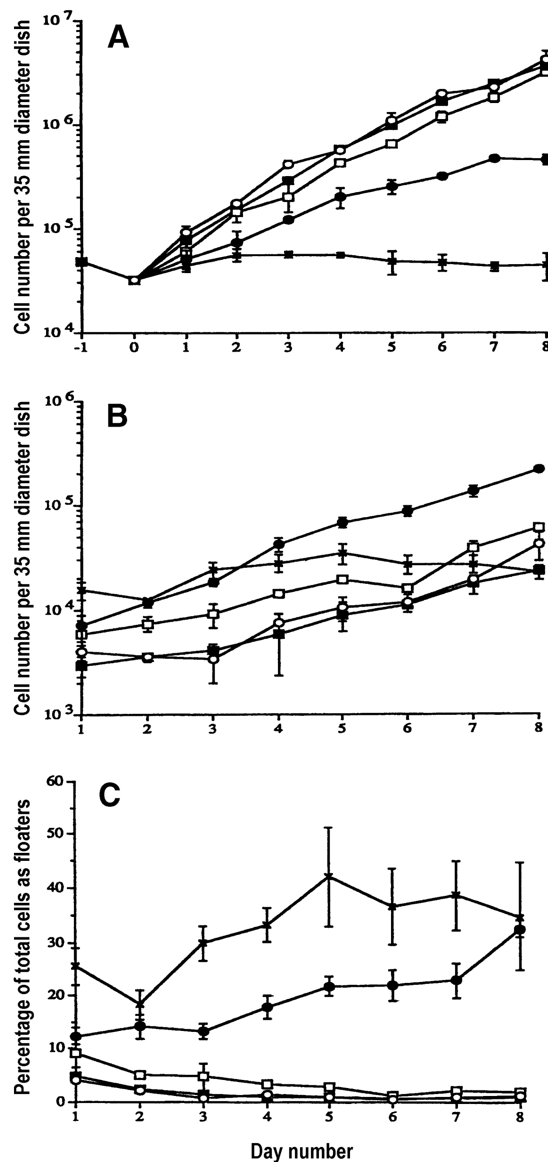

FIG. 1. The effect of supplementation with eicosapentaenoic acid (EPA) at various levels on the growth of HT29 colon adenocarcinoma cells. Complete media was supplemented with either 0 µg/mL (■), 0 µg/mL + ethanol (○), 5 µg/mL (□), 10 µg/mL (●) or 15 µg/mL (×). Growth of the cells was monitored over an 8-d period. Day −1 represents the cell seeding density, and day 0 shows the number of adherent cells after a 24-h cell adhesion period. Part A depicts the number of adherent cells, part B the number of floating cells and part C the percentage of total cells which were detached. Error bars represent the standard deviations of triplicate samples.

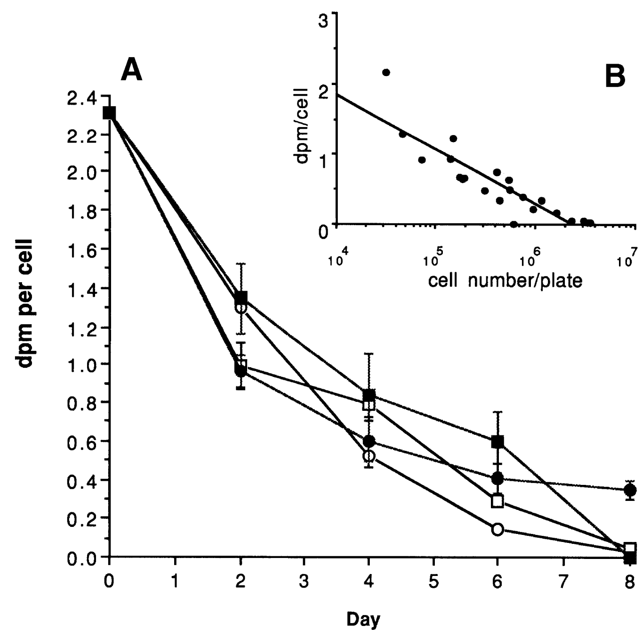

FIG. 2. The effect of supplementation with EPA on the proliferation of HT29 colon adenocarcinoma cells. Complete media was supplemented with either 0 (○), 5 (□), 10 (●), or 15 µg/mL (×). Cell proliferation was determined by measuring the incorporation of ^{3}H-thymidine into adherent cells over an 8-d period, as detailed in the Materials and Methods section. Error bars represent the standard deviation within the dpm values at each time point. Part B shows that proliferation is dependent on cell density, $R^2 = 0.787$. For abbreviation see Figure 1.

cells. At 10 µg/mL EPA, the cells became more granular in appearance, due the presence of large numbers of lipid droplets in their cytoplasm and colony morphology was less well defined. At 15 µg/mL EPA, there were discernible gaps between neighboring cells, and the remaining adherent cells had become more rounded.

No differences in acridine orange staining intensity between control adherent cells and those treated with EPA (15 µg/mL) were observed, and less than 1% of cells exhibited condensed chromatin characteristic of apoptosis. Many control detached cells contained condensed chromatin typical of cells entering mitosis, and some showed typical apoptotic features. In contrast, EPA-treated detached cells were generally smaller and less regularly shaped. Many showed typical apoptotic characteristics, and only a few the seemingly premitotic chromatin condensation of the control cells.

Gel electrophoresis. Samples were subjected to gel electrophoresis after 4 d growth. The results of gel electrophoresis carried out on floating and adherent cells treated with 10 and 15 µg/mL EPA are compared to controls in Figure 3. In adherent cells, treated or untreated, there was no DNA laddering, and intact genomic DNA was situated near the well at the top of the lanes. DNA laddering was, however, clearly evident in floating cells, irrespective of treatment.

Cell cycle analysis. Figure 4 shows the cell cycle distribution of adherent cells obtained from control samples, and those supplemented with 10 and 15 µg/mL EPA, over a period of 4 d. On day 2, cells treated with 10 and 15 µg/mL EPA had significantly more S phase cells than those in controls ($P < 0.05$ and $P < 0.01$, respectively), but no significant differences were seen between the two levels of EPA treatment. After 4 d, both treatments again led to significantly more cells in the S phase of the cell cycle when compared to control cells ($P < 0.01$ and $P < 0.01$, respectively), and differences were also seen in the numbers of cells in G2/M. There was no significant difference between control cells and those treated with 10 µg/mL EPA.

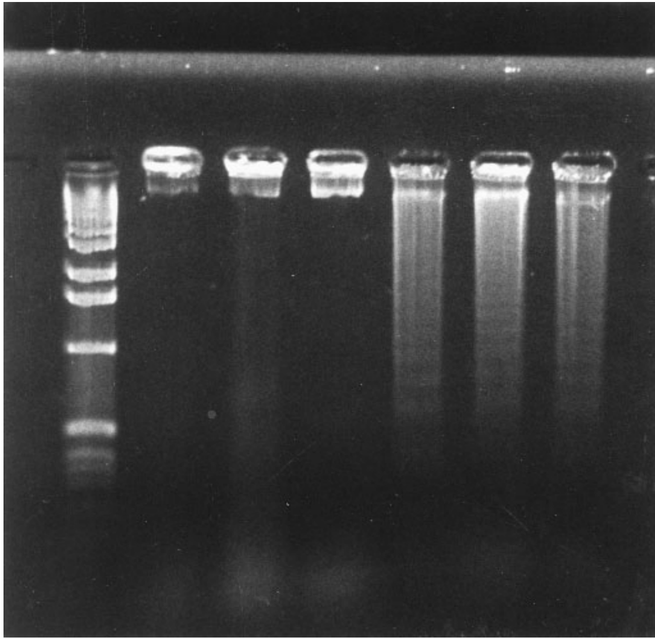

FIG. 3. DNA fragmentation in HT29 colon adenocarcinoma cells by agarose gel electrophoresis after growth in the presence of EPA. HT29 cells were grown in the presence of a range of concentrations of EPA for 4 d, the media being refreshed every 24 h. After this period, adherent and floating cells were harvested and analyzed for DNA fragmentation as detailed in the Materials and Methods section. Lane 1 represents a 1 Kb DNA ladder. Lanes 2, 3, and 4 represent adherent cells after treatment with 0, 10, and 15 μg/mL EPA, respectively. Lanes 5, 6, and 7 represent floating cells after treatment with 0, 10, and 15 μg/mL EPA, respectively. For abbreviation see Figure 1.

However, cells treated with 15 μg/mL EPA had significantly fewer cells in G2/M than both control cells ($P < 0.05$) and those treated with 10 μg/mL EPA ($P < 0.01$).

Cell cycle analysis on the floating cells was not possible because loss of fragmented DNA from apoptotic and necrotic cells means their position in the cell cycle is ambiguous. For example, detached cells with a DNA content equal to that of adherent S phase cells could not be distinguished from G2/M cells with reduced DNA content. Similarly, cells with a DNA content equal to that of adherent G0/G1 cells could be G0/G1 cells, or S, G2, or M cells that had lost varying amounts of DNA. The DNA profiles of floating cells treated for 4 d with control medium show that these cells contain considerably more DNA in the sub G0 peak, and few cells are present in the G1 and G2 peaks, compared to those exposed to medium containing 15 μg/mL EPA (Fig. 5), although both cell populations have been left for the same 24 h period.

Detection of early apoptotic cells by annexin-V labeling. The effects of EPA supplementation on the incidence of apoptosis (fluorescent green cells) in adherent HD29 cells, determined by the extent of binding of annexin V, were as follows: 0 μg/mL EPA, 1.8 ± 0.8; 15 μg/mL EPA, 1.6 ± 0.4; and 400 μM indomethacin (positive control), 27.4 ± 4.5. There were no significant differences between the numbers of apoptotic adherent cells, i.e. those stained green but not red, under control conditions compared to those treated with 15 μg/mL EPA. Treatment with 400 μM indomethacin, which is a well-documented inducer of apoptosis in HT29 cells (40,41), resulted in high numbers of apoptotic adherent cells.

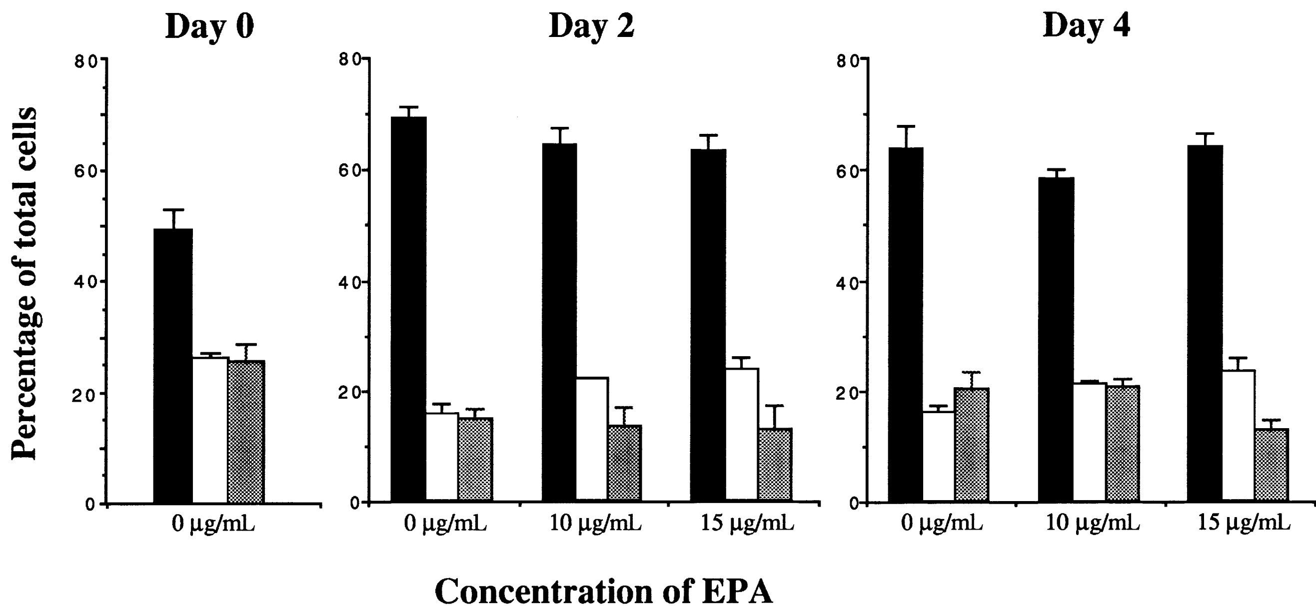

FIG. 4. The effect of supplementation with EPA at various concentrations on the cell cycle distribution of adherent HT29 colon adenocarcinoma cells over 4 d. HT29 cells were grown in complete media supplemented with either 0, 10, or 15 μg/mL EPA. After 2 and 4 d treatment, adherent cells were harvested and their position within the cell cycle determined by staining cellular DNA with ethidium bromide and quantifying red fluorescence by flow cytometry (see Materials and Methods section). ■ represents cells in G0/G1, □ cells in S, and ▩ cells in G2/M of the cell cycle. Error bars represent the standard deviations of triplicate samples. For abbreviation see Figure 1.

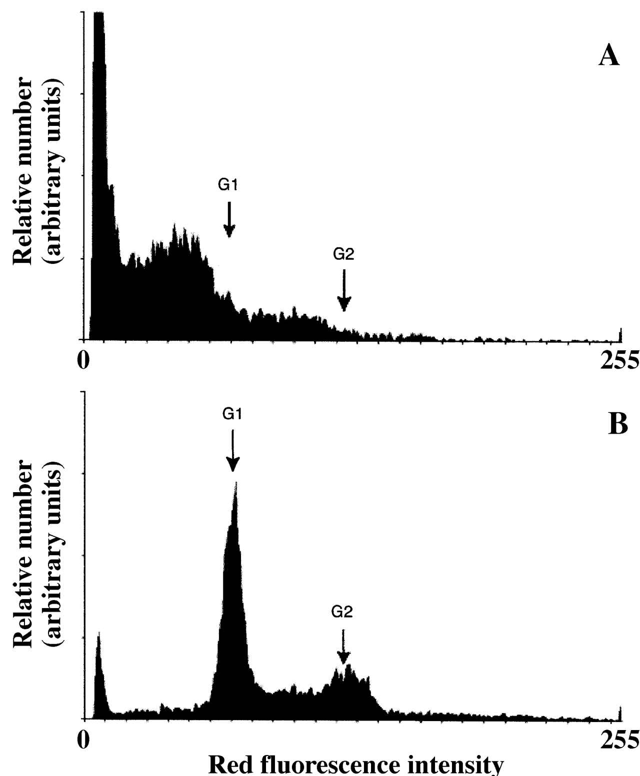

FIG. 5. The effect of EPA supplementation on the DNA content of nonadherent HT29 colon adenocarcinoma cells. HT29 cells were grown for 4 d in either complete media (A) or media supplemented with 15 μg/mL EPA (B), the media being refreshed every 24 h. Nonadherent cells were harvested and their DNA content determined by staining cellular DNA with ethidium bromide and quantifying red fluorescence by flow cytometry (see Materials and Methods section). In both panels, the position of cells with a DNA content corresponding to G1 and G2 are indicated. The data represent only those cells collected during the previous 24 h. For abbreviation see Figure 1.

Reduction in cell loss by caspase inhibition. The exposure of cells grown in 96-well plates to 15 μg/mL EPA reduced cell number to 52.7 ± 4.4% (mean ± SD) of control values. The addition of the caspase inhibitor Z-DEVD-FMK (75 μM) alone had no effect on cell number (104.1 ± 8.8%) but in combination with EPA cell number returned to 85.5 ± 3.4% of that of control cells.

DISCUSSION

When investigating the effects of exogenous compounds on the cytokinetics of adherent cell lines a reduction in the adherent cell number is commonly assumed to indicate a reduction in the rate of mitosis (19,40,41). However, it is theoretically possible for the rate of proliferation to remain constant, while a reduction in the number of adherent cells occurs owing to an increase in the rate at which the cells are shed from the plate into the surrounding medium. Previous studies on the effect of PUFA (20,22,25) and other compounds have not addressed this issue. The present study concentrates on the effects of EPA, as preliminary studies from our group established that EPA (10 μg/mL) reduced clonal survival of HT29 cells by more than 99% whereas cell survival was virtually 100% in the presence of either linoleic or oleic acid at the same concentration (42). In the present study we have shown that as the numbers of adherent cells were reduced on treatment with higher levels of EPA, the numbers of floating cells increased in comparison to controls. This suggests that treatment with EPA increased the rate at which adherent cells were shed into the medium without reducing the rate of mitosis.

In order to examine more closely the proliferation rate of adherent cells we determined ^{3}H-thymidine incorporation in treated and untreated cultures. Supplementation with EPA at 5, 10, and 15 μg/mL did not reduce the proliferation of HT29 cells compared to controls. In fact there was an apparent increase in proliferation at the higher dose of EPA, which was probably due to the lower cell density in these samples. This reinforces the view that the reduced adherent cell numbers seen on supplementation with the higher levels of EPA were due to an increase in the rate at which cells were shed from the substrate, rather than reduced cell division. This cell loss could have been due to increased cell death, either by apoptosis or necrosis, or to changes in the adherence properties of the cells, leading to detachment from the substrate.

Compounds that modulate cell growth commonly exert their effect at a particular stage of the cell cycle. This has been shown to be true of EPA acting on the promyelocytic leukemic cell line HL-60 (43) and on the pancreatic cancer cell line MIA PaCa-2 (44), where arrest occurs in G1/S and S/G2, respectively. Our results showed that after treatment with 10 and 15 μg/mL EPA there was an accumulation of HT29 cells in the S phase of the cell cycle, which could indicate blockade of the cell cycle in either late S phase or early G2. It is more common for chemotherapeutic agents to cause cell cycle arrest in G1, where damage to DNA can be repaired before it is replicated during the S phase. This G1 arrest is usually controlled *via* a p53 dependent pathway (45), but it is probably of little relevance to the present study because we have no evidence that DNA damage is induced by EPA, and in any case HT29 lacks wild type p53. It may be that EPA-mediated arrest occurs in late S or early G2/M *via* another control pathway (46). Unlike a cell cycle arrest in G1, the arrest of cells in S/G2 will not cause a reduction in the level of ^{3}H-thymidine incorporation into individual cells. If the cells are lost from the substrate at, or shortly after, the point of arrest there will be no apparent reduction in the amount of ^{3}H-thymidine incorporated per remaining adherent cell, as was observed in this study.

A well-documented characteristic of apoptosis is the internucleosomal cleavage of DNA into fragments 180 bp long, and multiples thereof. When subjected to agarose gel electrophoresis, such apoptotic DNA forms a discreet ladder (35). Our results confirm previous reports (47,48) that adherent cells, treated or untreated, show no DNA laddering, whereas floating cells do, regardless of treatment. Previous authors have tended to assume that floating cells are uniformly apoptotic and that the degree of apoptosis can be deduced directly

from the rate at which cells detach from their substrate and become floaters (49). However, this assumption may be flawed because gel electrophoresis only confirms that some of the floating cells contain fragmented DNA; there is no indication of the proportion of the floating cell population undergoing apoptosis. In addition, detection of apoptosis by DNA laddering in floating cells does not demonstrate whether the cells are detaching because they are apoptotic or are becoming apoptotic because they have detached.

The loss of fragmented DNA, producing cells with a DNA content of <2n (i.e., sub-G0), is now an accepted feature of apoptotic cells. When studying substrate-growing cell lines, we observed two distinct populations; those which were intact and adhering to the substrate, and those which had become, or were about to become, detached. Some previous studies, using flow cytometry, have examined only the adherent cells (49,50), others have examined the total cell population (41,43,51), but few have examined the characteristics of adherent cells and floating cells separately (52,53). In using morphological criteria, apoptotic adherent cells were rarely observed. Given such small numbers, the process of removing background debris and aggregates from the calculation for cell cycle analysis of adherent cells effectively excluded cells with a sub-G0 DNA content. It was not possible therefore to give a value for sub-G0 cells in the adherent cell populations using this flow cytometric technique. The absence of any characteristic features of apoptosis in adherent cells suggests that detachment of floaters, if associated with apoptosis, occurs at an early stage of the process.

While not directly affecting the rate of DNA synthesis of HT29 cells, measured using incorporation of ^{3}H thymidine, EPA did influence cell cycle distribution and increased the rate at which adherent cells became detached from the substrate. Although this detachment may be associated with the process of apoptosis, the flow cytometric data suggests that it occurred before any change in membrane asymmetry or the cleavage of DNA. The binding of annexin V to externalized phosphatidylserine is considered to be an earlier event in the apoptotic process than the fragmentation of DNA. However, treatment with EPA at 15 μg/mL did not lead to any increase in annexin V binding in adherent cells, reinforcing the view that treatment with EPA did not cause loss of cells from the substrate *via* the classical apoptotic process. Significant numbers of floating cells collected under control conditions contained sub-G0 quantities of DNA, implying that these cells are apoptotic, while the population of floating cells, collected over the final 24 h period of EPA treatment (4 d), contained virtually no sub-G0 cells. This suggests that the nonadherent cells found in response to EPA exposure may be detaching by a different mechanisms or at different stages in the cell cycle. For example, cell detachment following blocking in late S phase will mean that cells detach with 4n DNA content which is then lost from the cell to give sub-G2 DNA content, but by the time DNA levels are at a sub-G0 level the cell membrane is no longer intact and thus these cells will be excluded from the measurements. In contrast, the nonadherent cells found in control conditions may become detached at G1 and thus loss of DNA during the process of apoptosis will lead to a detectable sub-G0 peak. The difference in results cannot be explained by a delayed effect of EPA as these cells have been continuously exposed to EPA for the previous 72 h.

We propose that EPA supplementation leads to changes in the adherence properties of cells, causing them to be shed from the substrate prior to identifiable apoptosis, a process which may be analogous to "anoikis" (induction of apoptosis as a result of loss of cell contact) typical of exfoliating epithelial cells (54,55). In this context it is interesting to note that dietary supplementation with fish oil has recently been reported to increase differentiation in the colonic epithelium of rats (56) and to cause reduced expression of cell adhesion molecules in human monocytes (57). In normal mucosa colonocytes cease to divide in the upper third of the crypt and rapidly undergo differentiation. In tumor-bearing colorectal mucosa, and apparently even in healthy subjects at risk of neoplasia, there is increased mitosis in the colorectal mucosa (58). If some general spatio-temporal delay in differentiation occurs within the crypt before or during the onset of neoplasia, increasing differentiation induced by fish oil may contribute to its anticarcinogenic effects in animal models.

The question remains as to whether the loss of cell adhesion induced by EPA should be regarded as an early stage in apoptosis or as part of a distinct differentiation pathway ending in cell death, which occurs as a secondary consequence of detachment. The addition of the caspase inhibitor Z-DEVD-FMK to the medium suppressed the reduction in adherent cell number seen in response to EPA. This inhibitor blocks caspase 3 (CPP32) (59), which is a key enzyme activated during the early stages of apoptosis, and responsible for the cleavage of several substrates including poly(ADP-ribose) polymerase, β-catenin, and focal adhesion kinase (FAK) (60,61,63). The latter is intimately involved in cell signaling events necessary for the maintenance of cell adhesion. Van de Water *et al.* (63) have recently reported that caspase-mediated FAK cleavage is a critical step preceding detachment of renal epithelial cells undergoing anoikis. Thus caspase-mediated loss of cell adhesion may be an early stage in the programmed cell death of adherent cells, which, although showing no classical features of apoptosis, are already committed to self-destruction. The issue of whether EPA-mediated cell detachment is part of a single unified pathway of apoptosis in these cells, or alternatively, a separate form of programmed cell death, which should be termed "anoikis," is outside the scope of the present study, but it certainly deserves further attention.

To what extent are the present findings of relevance to the effect of fish oil on the intact intestinal mucosa *in vivo*? As mentioned earlier, substances or conditions which favor differentiation and apoptosis, or suppress mitosis, of tumor cells *in vitro* may be anticarcinogenic *in vivo*. On this principle, the short-chain fatty acid butyrate, which promotes differentiation of many tumor cell lines including HT29, has been proposed as a possible anticarcinogen which can be derived from the human diet (64). If the increased exfoliation of HT29 cells from

adherent cultures in response to EPA described in this study is an outcome of increased terminal differentiation, the same mechanisms might also favor differentiation and suppression of mitosis within the intact crypt, and hence contribute to the anticarcinogenic effects of fish oil observed in animal models. It may therefore be important to search for evidence for effects of n-3 PUFA on markers of cell differentiation in the intestine and other rapidly proliferating tissues *in vivo*.

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Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Plasma Membrane Fluidity of Aortic Endothelial Cells

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ABSTRACT: We investigated the relative effects of n-3 eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) on the plasma membrane fluidity of endothelial cells (EC) cultured from the thoracic aorta by determining fluorescence polarization of 1,6-diphenyl-1,3,5-hexatriene (DPH) and its cationic derivative trimethylamino-DPH (TMA-DPH). Fluidity assessed by TMA-DPH demonstrated no significant differences in plasma membranes of vehicle (dimethyl sulfoxide; DMSO)-, EPA-, and DHA-treated EC. Plasma membrane fluidity assessed by DPH polarization, however, was significantly higher in the order of DHA > EPA > DMSO. Total cholesterol content decreased significantly by 28.4 and 15.9% in the plasma membranes of DHA- and EPA-treated cells, respectively. Total phospholipid content remained unaltered in the plasma membranes of the three groups of cells; however, the molar ratio of total cholesterol to phospholipid decreased significantly only in the membranes of DHA-treated EC. The unsaturation index in the plasma membranes of EPA- and DHA-treated cells increased by 35.7 and 64.3%, respectively, compared with that in the plasma membranes of control cells. The activities of catalase and glutathione peroxidase in the whole-cell homogenates, and levels of lipid peroxides in either the whole-cell homogenates or in plasma membrane fractions were not altered in EPA- or DHA-treated EC. These results indicate that the influence of DHA is greater than that of EPA in increasing plasma membrane fluidity of vascular EC. We speculate that the greater effect of DHA compared to EPA is due to its greater ability to decrease membrane cholesterol content or the cholesterol/phospholipid molar ratio, or both, and also to its greater ability in elevating the unsaturation index in the plasma membranes of EC.

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Although regular consumption of fish oil is associated with a reduced risk of coronary heart disease (1,2), the association is probably of a multifactorial nature, and many of the underlying mechanisms at the molecular level for polyunsaturated fatty acids (PUFA) are not clearly understood (3). Fatty acids cause endothelium-dependent vasodilation, probably by changing the fluidity of endothelial membranes (4). Several other lines of evidence indicate that membrane fluidity may modulate endothelial cell (EC) membrane-dependent functions such as receptor–ligand interaction (5), membrane transport (6) and membrane-bound adenylate cyclase activity (7). Therefore, it is likely that some significant physiological responses would depend on the alterations of physical properties occurring at the cellular bilayer membrane level. Repression of blood pressure by eicosapentaenoic acid (EPA, 20:4n-3) (8,9) and docosahexaenoic acid (DHA, 22:6n-3) (10), and alterations of β-adrenergic responsiveness by DHA (11) have also been tentatively attributed to their influences on membrane fluidity.

Studies with purified components of fish oil, EPA and DHA have shown differences in their effects on plasma lipid profiles. EPA has hypotriglyceridemic and hypocholesterolemic properties (12), and DHA has no effect on plasma triglycerides (13). These two fatty acids have also been described as differing in activity at the cellular level. Bates *et al.* (14) reported that treatment of neutrophils with DHA, but not EPA, increases neutrophil-mediated endothelial detachment. Likewise, DHA has been shown to be less effective than EPA in inhibiting vascular smooth muscle proliferation (15), a phenomenon thought to be involved in the genesis of atherosclerosis. Khalfoun *et al.* (16) suggested that DHA is a more potent inhibitor of lymphocyte adhesion to EC than is EPA. In addition, in human skin fibroblasts, DHA but not EPA has been shown to increase membrane fluidity and membrane-associated enzyme activities, including 5′-nucleotidase and adenylate cyclase (17). Previously we showed that a decrease in the membrane DHA/AA (arachidonic acid, 20:4n-3) molar ratio with age is correlated with the deterioration of platelet membrane fluidity, but EPA/AA does not have a significant relation with membrane fluidity (18). Whether similar differential effects of EPA and DHA can be exerted in endothelial cell membrane fluidity has, however, remains obscure. DHA contains one more double bond per molecule than does EPA. Thus, it is likely that DHA's high content in the membrane bilayer has a specific role in membrane dynamics. Of vital im-

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Abbreviations: AA, arachidonic acid; CAT, catalase; DOW, double-distilled water; DHA, docosahexaenoic acid; DMSO, dimethyl sulfoxide; DPA, docosapentaenoic acid; DPH, 1,6-diphenyl-1,3,5-hexatriene; EC, endothelial cells; EPA, eicosapentaenoic acid; GPx, glutathione peroxidase; HMB-CoA, hydroxymethylglutaryl-CoA; LA, linoleic acid; LLN, linolenic acid; LPO, lipid peroxide products; OA, oleic acid; PM, plasma membrane(s); PUFA, polyunsaturated fatty acid(s); TBARS, thiobarbituric acid-reactive substances; TMA-DPH, 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5-hexatriene; USI, unsaturation index.

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portance to the maintenance of EC integrity is the preservation of membrane function and structural properties, such as plasma membrane (PM) fluidity.

Optimal membrane function requires a fluid state of the membrane (19), and this fluidity is largely dependent on lipid composition, such as the extent of membrane unsaturation, cholesterol levels and the cholesterol/phospholipid ratio (20). Nonetheless, it should be kept in mind that a high level of PUFA would readily increase PM susceptibility to peroxidative attacks (21). Peroxidation of membrane PUFA results in the formation of lipid peroxides including malondialdehyde and aldehyde breakdown products (22). Lipid peroxidation, which is increasingly attributed to a decrease in membrane fluidity that accompanies aging (18,23), was demonstrated in *in vitro* oxidation (24) and also has been shown to modulate the activity of membrane-bound enzymes (25). The lipid peroxidation-related exacerbation of membrane functions, however, depends on the imbalance between scavenging and production of free radicals. Thus, we were aware of the potential for oxidative antioxidation of the EC during incubation with EPA and DHA. With respect to the effects of EPA and DHA on plasma membrane profiles of cholesterol, phospholipid, fatty acid unsaturation, and lipid peroxide, we investigated the relative influence of these PUFA on the plasma membrane fluidity of EC isolated from rat thoracic aortas.

MATERIALS AND METHODS

Cell culture of EC from rat thoracic aortas. All animal experiments were performed in accordance with the Guidelines for Animal Experimentation of Shimane Medical University, compiled from the Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science. Thoracic aortas of young (5-wk-old) female Wistar rats were prepared, and the primary cultures of EC were established as described (26). After growing to confluency, the cells were harvested with 0.05% trypsin and 0.01% EDTA and passaged. The EC were characterized by immunoperoxidase staining using Factor VIII-related antigen (Biomeda Histo Scan Kit, Cosmo Bio Co., Ltd., Tokyo, Japan) and by their uptake of acetylated low density lipoprotein labeled with the fluorescent probe 1,1′-dioctadecyl-1-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate (Biomedical Technologies, Inc., Stoughton, MA). The EC at the second passage were used in this study.

Fatty acid enrichment of EC. Cells were grown in medium to confluence in 95-mm collagen plates (Celltight C-1, Sumitomo Bakelite Co. Osaka, Japan). Highly purified ethyl ester of all-*cis*-5,8,11,14,17-EPA (Mochida Pharmaceutical Co., Ltd., Tokyo, Japan) and all-*cis*-4,7,10,13,16,19-DHA (Sagami Chemical Research Center, Sagamihara, Japan) were dissolved in dimethyl sulfoxide (DMSO), each at a final concentration of 5 µg fatty acid/mL culture medium; the controls, designated as "DMSO," were cultured to the same concentration as that of the vehicle. DMSO was used at a concentration of 0.02%. The medium was further renewed twice at 24-h intervals. After treatment of the cells with PUFA or vehicle for 72 h, the culture medium was removed and the cell layers were washed with Dulbecco's PBS(+) (phosphate-buffered saline). The trypsinized cells, after several washes in excess 25 mM Tris-HCl buffer (pH 7.4) containing 137 mM NaCl, 5.4 mM KCl, and 11 mM glucose, were used to prepare plasma membranes.

Preparation of PM. PM were isolated as previously described (27) with a slight modification. Twenty collagen culture plates (95-mm diameter) were pooled to obtain sufficient PM. After trypsinizing and centrifuging, 1 mL of chilled 0.1 M sucrose in 10 mM Tris (pH 7.4 at 4°C) was added to the cell pellets. The cells were lysed by five freeze-thaw cycles, then by a bath sonicator at ice-cold temperatures; the lysates were subjected to a microtip Branson sonifier (Cell disruptor 200; Branson Sonic Power Co., Danbury, CT) for 30 to 60 s. After centrifugation at 1,000 × *g* for 10 min at 4°C, the supernatant was transferred to Eppendorf centrifuge tubes. The remaining pellet was rehomogenized in 400 µL of the above Tris-sucrose buffer, and the supernatant was obtained as above. The rehomogenizing step was repeated one further time, then all the supernatants were combined and centrifuged at 9,000 × *g* for 10 min. The supernatant was further centrifuged at 40,000 × *g* for 30 min and then discarded, and the remaining membrane-rich pellet was vigorously suspended in the Tris-sucrose buffer in a mixer. The protein was determined by the method of Lowry *et al*. (28). Purity of the PM fraction was confirmed by measuring the activity of 5′-nucleotidase, with a Sigma Chemical Co. diagnostic kit (St. Louis, MO). The PM fraction was enriched ~sixfold over the initial crude homogenate.

*Measurement of membrane fluidity***.** Fluorescence probes, 1,6-diphenyl-1,3,5-hexatriene (DPH; Sigma Chemical Co.) and 1-(4-trimethylammonium-phenyl)-6-phenyl-1,3,5,-hexatriene (TMA-DPH; Sigma Chemical Co.) were used as an index of rotational mobility in PM. DPH and TMA-DPH labeling of PM and the measurements of steady-state fluorescence polarization were carried out by the methods of Shinitzky and Barenholz (29) and Kuhry *et al*. (30), respectively. A quantity of 100 µL PM suspension containing 100 µg protein was added to 1.4 mL of Tris-HCl buffer (25 mM Tris-HCl buffer, containing 137 mM NaCl, 5.4 mM KCl, and 11 mM glucose, pH 7.4) with a final probe concentration at 1 µM. This dispersion was incubated at 37°C for 60 min for DPH and 30 min for TMA-DPH. Fluorescence intensity was measured with a Hitachi 850 spectrofluorometer (Hitachi Ltd., Tokyo, Japan) equipped with rotating polarizer filters and a thermostatic cell holder set at 37°C. The effect of light scattering on intensity values was minimized with a 390-nm cutoff filter and by subtracting the values of unlabeled preparations. Excitation wavelengths of 360 and 365 nm, and emission wavelengths of 430 and 435 nm were used for DPH and TMA-DPH, respectively. The slit width was 8 nm for excitation and 14 nm for emission for each of the probes. Fluorescence polarization was calculated as $P = (I_{VV} - I_{VH})/(I_{VV} + 2\,I_{VH})$ (29,30). I_{VV} and I_{VH} are the intensities measured parallel and perpendicular to the vertical axis of the excitation beam. Plasma membrane fluidity was expressed as 1/polarization (1/*P*).

Fluorescence lifetime measurement of DPH. Fluorescence lifetime of embedded DPH was measured from the normalized total fluorescence intensity decay with a single photon-counting fluorometer (Horiba Naes-1100 time-resolved spectrofluorometer, Horiba Ltd., Kyoto, Japan) equipped with a computer program that enables it to analyze decay curves of one to three components. The samples were excited with a free-running spark gap-type nanosecond flash lamp. The light of the hydrogen lamp was first passed through a glass filter 1 plate-mesh with a 360 nm band pass for excitation (Horiba). The emitted fluorescence was filtered through a combination of two glass prisms, L-380 and UV 390 (Horiba).

Lipid peroxide product (LPO) analysis. LPO were assessed by the thiobarbituric acid-reactive substances (TBARS) assay of Ohkawa *et al*. (31). Briefly, 200 µL of 8.1% sodium dodecyl sulfate, 3.0 mL of 0.4% thiobarbituric acid in 20% acetic acid (pH 3.5), and 700 µL of double-distilled water (DDW) were added to 100 µL of PM suspension or whole cell homogenate containing 100 µg protein; the mixture was then incubated at 95°C for 1 h. After cooling in tap water, 1.0 mL of DDW and 4.0 mL *n*-butanol/pyridine (15:1, vol/vol) were added, and the mixture was shaken vigorously for 20 min. After centrifugation at 3,000 rpm for 10 min, the fluorescence intensity of the upper organic layer was determined with a Hitachi 850 spectrofluorometer. The excitation and emission wavelengths were 515 and 553 nm, respectively. Malondialdehyde levels were calculated relative to a standard preparation of 1,1,3,3-tetraethoxypropane.

Enzyme activity. Cell homogenates were used to measure activities of catalase (CAT; EC 1.11.1.6) and glutathione peroxidase (GPx; EC 1.11.1.0). The activity of CAT was measured by exploiting its peroxidation function at 20°C according to the procedures of Johansson and Borg (32) and Wheeler *et al*. (33), as previously described (34) with one modification of total assay volume of 1.0 mL. CAT activity was determined by linear least-squares regression of the absorbance of formaldehyde standards. GPx was assayed as described previously (34) with one modification of total assay volume of 1.0 ml. Units of enzyme activity were calculated as µmol of NADPH oxidized per min using a millimolar extinction coefficient of 6.22 mM^{-1} for NADPH.

Fatty acid analysis. Fatty acid profiles of EC plasma membranes were determined by the one-step analysis procedure of Lepage and Roy (35) as described previously (9,10), using capillary gas chromatography of the corresponding methyl esters prepared by transesterification with acetyl chloride.

Total cholesterol and phospholipid analysis. To determine the PM contents of cholesterol and phospholipid, endothelial PM total lipids were extracted with chloroform/methanol (2:1, vol/vol) by the method of Folch *et al*. (36). The extract was passed through glass Pasteur pipettes filled with glass wool and divided into two aliquots, one for total cholesterol estimation and the other for total membrane phospholipid estimation. The extract was dried extensively with a stream of N_2 gas. Total cholesterol and phospholipid were analyzed with commercially available analytical kits (Cholesterol E-test and Phospholipid B-test, Wako Pure Chemical Industries Ltd., Osaka, Japan, respectively).

Statistical analysis. Results are expressed as means ± SE. Data were subjected to analysis of variance followed by Fisher's partial least-squares differences test for *post hoc* comparison and regression analysis with the computer program Stat View® 4.01; MindVision Software (Abacus Concepts, Inc., Berkeley, CA). A level of $P < 0.05$ was considered statistically significant.

RESULTS

Cellular cholesterol-phospholipid composition. Figure 1A shows that PM cholesterol content of DHA-treated and EPA-treated EC decreased by 28.4 and 15.9%, respectively, compared with that in the PM of DMSO-treated control cells.

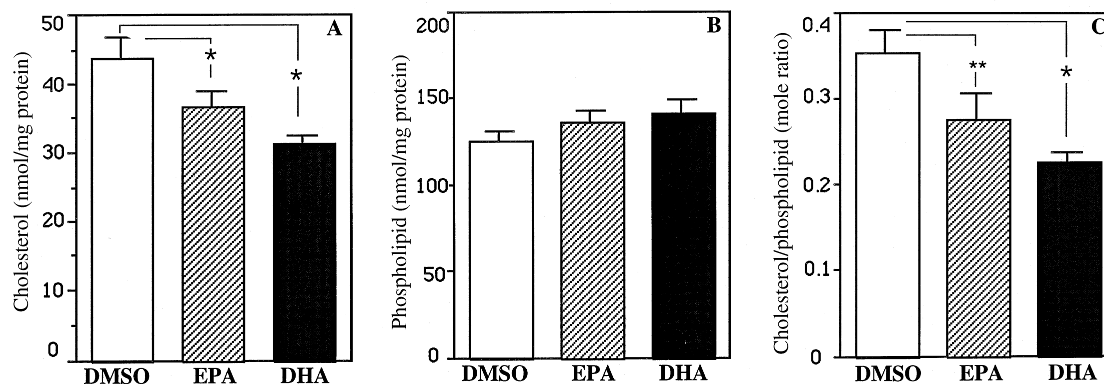

FIG. 1. Effect of eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) on (A) cholesterol, (B) phospholipid, and (C) cholesterol/phospholipid molar ratios of endothelial cell (EC) plasma membranes (PM). Cells were grown in 10% fetal bovine serum to confluence in 95-mm plastic dishes (Falcon, Oxnard, CA). Highly purified ethyl esters of EPA and DHA were dissolved in dimethyl sulfoxide (DMSO) as a vehicle. All media containing supplemental DMSO, EPA, and DHA were sterilized by passage through a low-binding 22-µm Millipore filter (Milford, MA). After treatment with polyunsaturated fatty acid or vehicle alone for 72 h, the endothelial cells were used for plasma membrane isolation and total cholesterol and phospholipid estimation. Results are mean ± SE (bars) values from four independent experiments, each with at least duplicate determinations. *$P < 0.05$. **$P < 0.05 \sim 0.10$.

TABLE 1
Effect of EPA and DHA on the Fatty Acid Profile of EC Plasma Membrane[a]

	PA	SA	OA	LA	LLN	AA	EPA	DPA	DHA	USI	EPA/AA	DHA/AA
DMSO	235^{a} ±13.8	227^{a} ±18.0	333^{a} ±12.0	28.0^{a} ±2.70	8.30^{a} ±1.20	32.8^{a} ±2.70	12.0^{a} ±0.90	21.0^{a} ±1.70	34.0^{a} ±1.95	0.70^{a} ±0.02	0.40^{a} ±12.0	1.10^{a} ±0.10
EPA	165^{b} ±11.3	186^{b} ±14.5	580^{b} 47.0	43.5^{b} ±3.70	13.0^{b} ±1.40	16.5^{b} ±1.80	41.5^{b} ±3.70	40.0^{b} ±3.30	44.5^{b} ±2.45	0.95^{b} ±0.02	2.70^{b} ±0.30	2.90^{b} ±0.30
DHA	190^{b} ±11.4	130^{c} ±12.0	357^{a} ±27.8	35.0^{c} ±2.60	14.0^{b} ±1.20	19.0^{b} ±1.30	27.9^{c} ±2.60	33.0^{b} ±2.80	87.0^{c} ±5.30	1.15^{c} ±0.03	1.55^{c} ±0.20	4.80^{c} ±0.50

[a]Values are means ±SE (nmole/mg protein) of 9 or 10 independent experiments. EC, endothelial cell; PA, palmitic acid (16:0); SA, stearic acid (18:0); OA, oleic acid (18:1); LA, linoleic acid (18:2); LLN, linolenic acid (18:3); AA, arachidonic acid (20:4); EPA, eicosapentaenoic acid (20:5); DPA, docosapentaenoic acid (22:5); DHA, docosahexaenoic acid (22:6); USI, unsaturation index. USI was calculated as [∑ (mole% of each polyunsaturated fatty acid × number of double bonds)/100)]. Values in the same column for a given fatty acid sharing the same lowercase roman superscripts are not significantly different at $P < 0.05$.

Total phospholipid content was found to be unaltered in the PM of EPA- and DHA-enriched cells (Fig. 1B). The PM cholesterol/phospholipid molar ratio decreased by 36.3% in the DHA-treated EC and by 21.5% in the EPA-treated EC compared with that in control cells. However, it reached significance only in the PM of DHA-enriched endothelial cells (Fig. 1C).

Effect of EPA and DHA on PM fatty acid composition. As shown in Table 1, a highly significant increase in both EPA and DHA content occurred in the PM of both EPA- and DHA-enriched EC compared with the DMSO-treated cells. The PM of EPA-enriched EC showed a 246% increase in EPA and those of DHA-enriched cells showed a 155% increase in DHA content compared with the increase in the PM of control cells. Both docosapentaenoic acid (DPA) and DHA increased ($P < 0.05$) in the PM of EPA-enriched cells; both EPA and DPA increased ($P < 0.05$) in the PM of DHA-enriched cells compared with the PM of control EC. AA decreased ($P < 0.05$) in the PM of both the EPA- and the DHA-enriched EC. Oleic acid (OA, 18:1) increased only in the EPA-treated PM. Linoleic acid (LA, 18:2) and linolenic acid (LLN, 18:3) increased in both the EPA- and DHA-treated PM. Palmitic and stearic acids decreased in the PM on EPA or DHA treatment. Stearic acid decreased by ~43% in the DHA-treated PM and by 18% in the PM of EPA-treated EC. Compared with the PM of DMSO-treated cells, both EPA- and DHA-enriched EC showed a significant increase in the total unsaturation index (USI), a measure of the extent of double bonds; however, the latter showed a 29% higher increase in membrane unsaturation compared with that in the PM of EPA-enriched EC.

Effect of EPA and DHA on lipid peroxide levels and antioxidative enzymes. Lipid peroxide (TBARS) values of EC PM did not change significantly on either EPA or DHA treatment as compared with those in the PM of DMSO-treated control cells (Table 2). Whole cell lipid peroxide values and catalase or glutathione peroxidase activities also did not change to a significant extent in either of the treated cells.

*Effect of EPA and DHA on EC PM fluidit*y. The term fluidity is inversely related to fluorescence polarization; thus, the higher the polarization, the lower the fluidity, and vice versa. DPH-determined membrane fluidity increased significantly in the PM of both EPA- and DHA-enriched EC compared with that in the PM of control EC; however, DHA showed a higher increase in PM fluidity relative to that of EPA compared with the fluidity of control cell PM (Fig. 2A). The PM fluidity (determined by the measurement of TMA-DPH polarization) of both EPA- and DHA-enriched cells did not change compared with that of the PM of DMSO-treated control EC (Fig. 2B).

Effect of EPA and DHA on the rotational relaxation time of DPH embedded in PM. The purpose of the measurement of the DPH fluorescence lifetime was to evaluate the

TABLE 2
Effect of EPA and DHA on Catalase and Glutathione Peroxidase Activities in Whole Cell Homogenates and on Lipid Peroxides Either in the Whole Cell Homogenates or Plasma Membrane Fractions of Rat Aortic EC[a]

	Catalase (mU[b])	Glutathione peroxidase (mU[c])	Lipid peroxide (nmol/mg protein)	
	Whole cell homogenate		Whole cell homogenate	Plasma membrane fraction
DMSO	25.30 ± 2.70	30.65 ± 1.50	8.25 ± 1.45	16.40 ± 1.60
EPA	24.70 ± 1.60	33.00 ± 2.00	8.90 ± 0.85	20.00 ± 2.20
DHA	29.50 ± 1.75	36.70 ± 5.40	11.75 ± 2.05	21.00 ± 1.65

[a]Results are mean ± SE of four independent experiments each with at least duplicate determinations.
[b]One unit is defined as μmol of methanol converted to formaldehyde equivalent to μmol H_2O_2 oxidized/min.
[c]One unit is defined as μmol of NADPH oxidized/min. For abbreviations see Table 1.

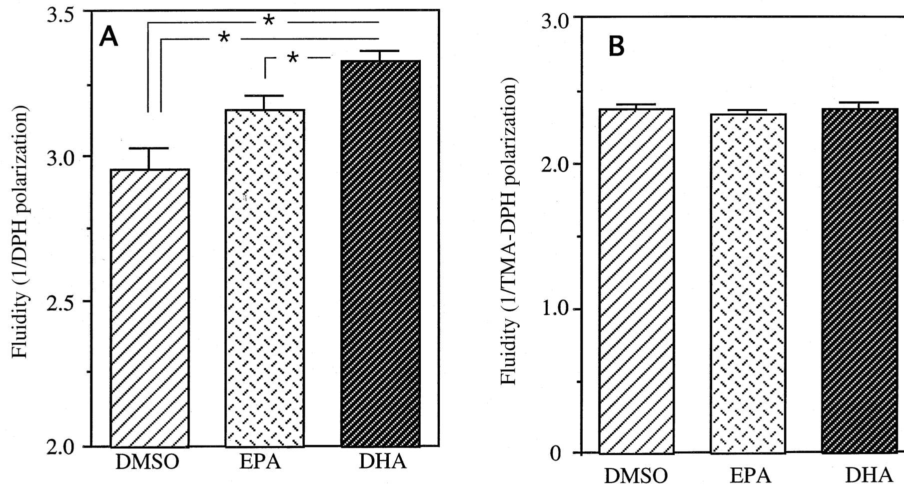

FIG. 2. Effect of EPA and DHA on the whole EC PM fluidity determined by (A) nonpolar 1,6-diphenyl-1,3,5-hexatriene (DPH) probe and (B) polar derivative of DPH, trimethylamino-1,6-diphenyl-1,3,5-hexatriene (TMA)-DPH. PM (100 μg) were incubated with either DPH or TMA-DPH, and membrane fluidity was expressed as 1/polarization. The PM of DHA-treated EC had the highest fluidity compared with the PM of EPA-treated EC or control EC. TMA-DPH-determined membrane fluidity was found not to change either upon EPA or DHA treatment as compared with that in the PM of control EC. Results are mean ± SE (bars) values from six independent experiments each with at least quadruplicate determinations. *$P < 0.05$. For abbreviations see Figure 1.

rotational relaxation time (ρ) using the Perrin equation: $r_o/r_s = 1 + (3\tau)/\rho$, as previously described, where r_o is the limiting anisotropy of DPH [i.e., the ansiotropy in the absence of rotational motion is 0.362 (29)]; r_s is the steady-state anisotropy calculated from the measured polarization (P) as $r_s = (2P)/(3 - P)$ (26); τ is the excited-state lifetime of DPH; and ρ is the time taken by the DPH probe molecule within the membrane to rotate through an angle θ such that $\cos\theta = e^{-1}$, which reflects the rotational motion of the fluorescent probe incorporated into the lipid bilayer membranes (37,38). Unlike other fluidity parameters such as microviscosity, ρ has distinct advantages because it quantifies both the rate and range of rotational motion of the probe and does not depend on calibration curves derived from reference solvents. The fluorescence decay data for lifetime measurements are best fitted by two-component analysis, as indicated by least χ^2 (chi square) values. Thus, average fluorescence lifetime (39) was used in the calculation of ρ. The rotational relaxation time (ρ) of the embedded DPH probe changed significantly on either EPA or DHA treatment. The ρ of DPH was 63.0 ± 1.3 ns in the PM of DMSO-treated controls; 57.6 ± 1.70 ns in those of EPA-enriched EC; and 48.6 ± 0.95 in those of DHA-enriched EC.

Correlation of EC PM fluidity with its fatty acid and cholesterol-phospholipid composition. As shown in Table 3, a highly significant positive correlation of PM fluidity was observed only with DHA, whereas its correlation with EPA, although not significant, was positive. Likewise, DHA/AA correlated significantly with membrane fluidity while EPA/AA did not. All other PUFA, except AA, correlated positively; however, their correlation coefficients were insignificant. AA was negatively correlated with PM fluidity. Stearic acid also was negatively correlated with PM fluidity, while the correlation with palmitic acid was not significant. Finally, PM fluidity was directly correlated with the USI. Cholesterol and cholesterol/phospholipid molar ratios were negatively correlated with membrane fluidity, and phospholipid had no correlation with PM fluidity.

DISCUSSION

The present study was designed to evaluate the differential effects of EPA and DHA on the membrane fluidity in PM of EC. The causal affectors of these differential effects were described in terms of their influence on PM total cholesterol, phospholipid, fatty acid unsaturation, and lipid peroxide profiles. Our results have confirmed that the effect of DHA ex-

TABLE 3
Correlation Coefficients Between Plasma Membrane Fluidity (1/*P*) and Lipid Profile of EC[a]

PA	SA	OA	LA	LLN	AA	EPA	DPA	DHA	EPA/AA	DHA/AA	USI	Cho	PL	Cho/PL
−0.44	−0.76	+0.03	+0.48	+0.16	−0.60	+0.44	+0.48	+0.75	+0.35	+0.77	+0.87	−0.72	+0.27	−0.61
(0.10)	(0.0009)	(0.90)	(0.070)	(0.58)	(0.014)	(0.10)	(0.070)	(0.001)	(0.20)	(0.0008)	(0.001)	(0.002)	(0.32)	(0.015)

[a]Correlation coefficients were calculated by linear regression analysis. Figures in parentheses represent the levels of significance. *P*, 1,6-diphenyl-1,3,5-hexatriene-polarization; Cho, cholesterol; PL, phospholipid. For other abbreviations see Table 1.

ceeds that of EPA in human skin fibroblasts (17), demonstrating that despite being members of the same n-3 family, EPA and DHA have different effects on membrane structures in EC. Our results are qualitatively consistent with those found with cardiomyocytes in which the incubation of EC with EPA displays a high content of EPA and DPA (20:5n-3) (11). Also, incubation of EC with DHA induces a significant retroconversion to DPA and EPA. Fatty acid composition of both EPA- and DHA-enriched EC revealed a significant decrease in the content of AA with concomitant increases in both the EPA/AA and DHA/AA molar ratios. The decrease in AA content is in agreement with other studies (40) and also consistent with our previous studies where significant increases of both the EPA/AA and DHA/AA ratios were found in rat caudal artery after chronic administration of EPA and DHA (10). The PM of DHA-enriched EC showed a larger increase in membrane fluidity and a shorter rotational relaxation time of DPH than did those of EPA-enriched cells. This assumption is in accordance with the work of Stubbs *et al*. (41), which showed a definite increase in the rate of motions (hence shorter rotational relaxation times) with the number of double bonds. This indicates that the lipid constituents of the PM of DHA-enriched EC in the present study probably experienced a greater motional freedom than those in the PM of either EPA- or DMSO-treated cells. Our speculation is also consistent with some previous investigations (42) demonstrating that rotational relaxation time increases with membrane rigidification.

Other indicators of membrane fluidity that were also altered to a greater extent in the PM of DHA-enriched EC compared to EPA-enriched EC included stearic acid, the USI, cholesterol content, and the cholesterol/phospholipid molar ratio. Membrane fluidity of EC exposed to stearic acid decreased as compared with that of the EC exposed to OA, LA, or LLN (43). Although the mechanism of the stearic acid-induced decrease in membrane fluidity is not direct (44), the increased membrane content of saturated fatty acid is associated with decreased membrane fluidity (45). Thus, our detection of a greater decrease ($P < 0.05$) in stearic acid suggests a partial contribution to a greater fluidity in the PM of DHA-enriched EC compared with PM of EPA-enriched EC. The negative correlation of AA with PM fluidity implies membrane rigidity. This observation is consistent with our previous study with platelet membrane (18). AA is presumed to produce endoperoxides not only through cyclooxygenase pathways but also by autoperoxidation (46). These endoperoxides may decrease membrane fluidity by peroxidizing membrane lipids. The reason for the absence of any direct correlation of plasma membrane fluidity with its EPA content is not clearly understood, yet it can be explained as follows: in the absence of EPA and DHA being added to the culture, these two PUFA represent 0.95 and 2.7% of the total fatty acid determined, respectively. Upon EPA enrichment these percentages rose to 2.7 and 2.9%; and with the addition of DHA these values were 2.3 and 7.1%, respectively. Thus, the maximal accumulation of EPA was found to be the basal level of DHA. Our speculation that EPA might exert its fluidizing effects indirectly through its retroconversion product, DHA, is lent further credence from the increasing support to the conception that DHA is primarily a structural fatty acid (47,48). From the physicochemical point of view, it could also be surmised that the lower capability of EPA than that of DHA in increasing membrane fluidity is related to the qualitative differences between these two fatty acids. For example, the differences in the *in situ* three-dimensional structure in the bilayer leaflet (cross-sectional area per fatty chain and motional freedom along the long axis of the acyl chain between EPA and DHA) might be involved in their differential effect on membrane fluidity. Also, in accordance with our previous studies with platelets, the correlation of endothelial PM EPA/AA with membrane fluidity was not significant, whereas the correlation with DHA/AA was highly significant (18). Moreover, the results of an increased USI in the PM of DHA-enriched cells over that of the PM of EPA-enriched cells led to the premise that the higher USI most likely accounted for the higher increase in membrane fluidity upon DHA enrichment.

We observed a greater decrease in total cholesterol content in the PM of DHA-enriched cells than in the PM of the EPA-enriched EC. The reason(s) for this greater decrease are not clearly understood; however, a higher increase (64 vs. 36% as compared to that of the control cells) in unsaturation in the DHA-enriched EC might be involved in such a greater decrease in the cholesterol level. It has been assumed that the higher the extent of unsaturation, the greater the inhibition of the rate-limiting enzyme of cholesterol biosynthesis, hydroxymethylglutaryl (HMG)-CoA reductase (49), and hence, the lower the cholesterol synthesis. Thus it is conceivable that the longer PUFA, DHA, may thus have a somewhat stronger hypocholesterolemic effect than that of EPA. Recently, a decrease in endogenous cholesterol biosynthesis by sivastatin—a HMG-CoA reductase inhibitor—has also been ascribed to the enhancement of membrane fluidity (50). That cholesterol has significant effects on membrane structure (51) and fluidity (52) has been provided by investigations carried out under various methodologies, including fluorescence polarization (53). Thus the higher increase in membrane fluidity upon DHA enrichment over that of EPA is, in part, due to the greater decrease in membrane cholesterol content along with a significant decrease in the molar ratio of cholesterol/phospholipid (20) only in the plasma membranes of DHA-enriched cells. This observation is consistent with other membrane systems including platelets (54,55) and synaptosomal membranes (56).

PUFA increase membrane fluidity; conversely, they increase susceptibility to oxidation and, as a result, may increase lipid peroxide concentrations in the cellular systems (21). In this context, PM fluidity should decrease because lipid peroxidation rigidifies the membrane by extensive cross-linking of the membrane constituents (57). Under the present experimental condition, although both antioxidative defenses (CAT and GPx activity) and oxidative potential (LPO level) increased quantitatively, the values did not reach significance on either

EPA or DHA treatment. Thus these data indicate that the dose (5 μg/mL medium) used did not elicit oxidative insults and failed to deteriorate, rather ameliorated, membrane fluidity. Regarding antioxidative-oxidative potential, however, our results contrast with those of Vankatraman *et al.* (58) and L'Abbé *et al*. (59), where feeding of fish oil increased both CAT and GPx activity along with lipid peroxide concentration. The reason for this discrepancy is not clear; however, differences in *in vivo* and *in vitro* conditions may be responsible, and this deserves further clarification.

Our results with the TMA-DPH fluoroprobe appear to conflict with those of Brown and Subbaiah (17), who found an increase in membrane fluidity in DHA-enriched fibroblasts but no effect of EPA on membrane fluidity. These discrepancies may relate to the differences in cell types, in the extent of membrane perturbations conferred by EPA or DHA, or both, and in techniques employed to determine membrane fluidity. Our experiments with EC suggest, however, that both EPA and DHA probably failed to generate perturbations at the water-lipid interfacial region (as probed by TMA-DPH) of the EC bilayer membrane. Conceivably therefore these two fatty acids failed to alter the bulk fluidity of the fatty acyl chains at or in the polar vicinity of the phospholipid head groups.

In conclusion, EPA and DHA have differential effects on EC PM fluidity. Three major factors that play significant roles in the determination of the higher fluidity of DHA over that of EPA in the PM of EC are lower contents of stearic acid and cholesterol, lower cholesterol/phospholipid molar ratios, and a higher fatty acid USI. Thus, we predict that the fluidity-dependent functions of EC would be influenced to a greater extent by DHA than that by EPA.

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Effect of Peanut Oil and Randomized Peanut Oil on Cholesterol and Oleic Acid Absorption, Transport, and Distribution in the Lymph of the Rat

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ABSTRACT: Peanut oil was shown to be atherogenic in cholesterol-fed rats, rabbits, and monkeys. However, after randomization, a process in which the fatty acids in peanut oil are randomly rearranged, its atherogenicity was significantly reduced in cholesterol-fed rabbits and monkeys. The mechanism for this effect remains unknown. This study was designed to investigate whether the absorption, transport and distribution of dietary cholesterol and oleic acid in the lymph were altered in the presence of peanut oil or randomized peanut oil. Previous investigators collected lymph through the mesenteric duct for 6 h and analyzed lymph for cholesterol. In the present study, lymph fluids were collected at timed intervals for up to 8 h and then at 24 h *via* the thoracic duct. Cholesterol and oleic acid (fatty acid) were estimated not only in the whole lymph but also in lymph lipoprotein fractions and in major lipid fractions. A 24-h lymph collection will enhance accuracy as short-term fluctuations in lipid absorption will not affect the results. Thoracic duct lymph collection is quantitative compared to mesenteric duct lymph collection, which provides only a fraction of the total lymph. Rats were given a lipid emulsion containing either peanut oil or randomized peanut oil. The emulsion also contained cholesterol, oleic acid, and sodium taurocholate in saline and was given through a duodenal catheter. Results show that absorption, transport, and distribution of cholesterol and oleic acid in the lymph fluids were similar in both dietary groups. These results suggest that the atherogenicity of peanut oil may be due to other events taking place subsequent to the release of cholesterol-containing chylomicrons and very low density lipoprotein by the small intestinal epithelial cells into the blood or may be due to the triglyceride structure itself.

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Peanut oil is atherogenic in rats (1), rabbits (2,3), and monkeys (4,5). However, when peanut oil is randomized, it is less atherogenic than native peanut oil in monkeys (5) and rabbits (6). The mechanism for this effect is not known. Randomization of peanut oil is carried out by pancreatic lipase (7). Several attempts were made to determine if any of the chemical changes during randomization were responsible for the decrease in atherogenic response. The rate of lipolysis was not different between peanut oil and randomized peanut oil using either pancreatic lipase or milk protein lipase (8), suggesting that lipolysis was not related to atherogenicity. Analysis of the triglyceride structure of native and randomized peanut oils showed that the native oil had more triglycerides with linoleic acid (18:2n-6) in the *sn*-2 position and saturated fatty acids in the *sn*-1 and *sn*-3 positions than the randomized peanut oil (9). In lymph-fistulated rats (10), the amounts of triglyceride, cholesterol, and phospholipid in the lymph were similar, as was the absorption of dietary cholesterol in both peanut oil and randomized peanut oil groups. From these results, it was concluded that differences in atherogenicity between peanut oil and randomized peanut oil are not due to differences in the amount of cholesterol transported into the lymph, but resulted from events subsequent to the release of cholesterol-containing chylomicrons and very low density lipoproteins (VLDL) by the small intestinal epithelial cells. In healthy individuals, substituting randomized butter for natural butter reduced serum cholesterol and triglycerides (11). Infants fed randomized lard had a 50% lower efficiency of absorption of stearic acid compared to consumption of regular lard (12). The rearrangements might have influenced the absorption of cholesterol and subsequently its entry into chylomicrons and lipoprotein fractions. The effect of peanut oil and randomized peanut oil on the main route of cholesterol transport, particularly *via* chlylomicrons, VLDL, low density lipoproteins (LDL), and high density lipoproteins (HDL) and its distribution in the major lipid classes, e.g., phospholipids (PL), mono- (MG), di- (DG), and triglycerides (TG), free fatty acids (FFA), and cholesterol ester (CE) has not been examined. In the present study, lymphatic transport and distribution of cholesterol and fatty acids in the chylomicron and lipoprotein fractions and their distribution in major lipid classes are compared in rats given peanut oil or randomized peanut oil.

MATERIALS AND METHODS

Animals and diets. Male Wistar rats (Harlan Sprague Dawley, Indianapolis, IN), weighing 200–250 g, were divided ran-

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Abbreviations: CE, cholesterol ester; DG, diglyceride; FAME, fatty acid methyl ester; FFA, free fatty acid; HDL, high density lipoprotein; LDL, low density lipoprotein; MG, monoglyceride; PL, phospholipid; TLC, thin-layer chromatography; VLDL, very low density lipoprotein.

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domly into two groups (10 rats/group) of comparable weights and maintained at 23°C in an animal room with a 12-h light/dark cycle. They were individually housed in suspended stainless-steel mesh cages. Rats were fed a commercial diet (Purina Rodent Chow 5002; Purina Mills Inc., St. Louis, MO) and distilled water *ad libitum* for 1 wk prior to surgical procedures and treatment with lipid emulsions. This study was conducted in accordance with the *Guide for the Care Use of Laboratory Animals,* Institute of Animal Resources, National Research Council.

Thoracic duct cannulation and lymph collection. After 1 wk on a standard diet, the animals were anesthetized with pentobarbital (50 µg/kg of body weight), and the thoracic duct was cannulated with PE-50 tubing (Clay Adams, Parsippany, NJ) according to the method of Bollman *et al.* (13). A second indwelling infusion catheter, PE-60 (Clay Adams), was placed in the duodenum at the pylorus for constant infusion of glucose–normal saline at 3 mL/h (glucose, 50 g/L; NaCl, 9 g/L) and for the administration of the test lipid emulsions. The test lipid emulsion was prepared and administered as described by Vahouny *et al.* (14) as follows: 0.5 mL stock I (150 mg cholesterol and 870 mg oleic acid in 3 mL of ethyl ether), 0.5 mL stock II (150 mg albumin and 834 mg sodium taurocholate in 3 mL saline), and 1.0 mL saline were mixed in a grinding vessel (Thomas Scientific, Swedesboro, NJ) and homogenized and the ether was evaporated under nitrogen. This process was repeated until no smell of ether was detected in the emulsion. The albumin was included in the preparation of the emulsion to provide a more physiological mixture and to aid in complete dispersion of the water-insoluble ingredients (15). Bovine serum albumin fraction V (fatty acid-poor), oleic acid (>99% pure), sodium taurocholate, and cholesterol were obtained from Sigma Chemical Co. (St. Louis, MO). Peanut oil (Planters, East Hanover, NJ) was purchased locally, and randomized peanut oil was obtained from Standard Brands, Inc. (Wilton, CT). The composition of the test lipid emulsions was as follows: albumin, 25; sodium taurocholate, 139; cholesterol, 25; oleic acid, 145; peanut oil or randomized peanut oil, 170 mg/1.5 mL lipid emulsion. The fatty acid composition of the two oils is given in Table 1. Animals were placed in restraining cages and allowed free access to glucose–normal saline. Lymph was collected from each rat from the time they were cannulated until they were administered the lipid meal. Amounts of cholesterol and oleic acid were estimated in the lymph. The average value for cholesterol was 7.4 ± 3.0 mg/dL and for oleic acid was 19.1 ± 4.2 mg/dL. These amounts were deducted from the values of cholesterol and oleic acid after the lipid emulsion was administered. After a constant infusion of glucose–normal saline overnight, 1.5 mL of the lipid emulsion was administered through the duodenal catheter, and then the constant infusion of glucose–normal saline was reestablished. Lymph samples were collected every 2 h for the first 8 h following the lipid administration, and then again at 24 h.

Separation of chylomicron and lipoproteins. Aliquots of 2- and 8–24-h lymph samples were pooled for each animal to obtain a 24-h collection. Portions of the 24-h lymph specimens were ultracentrifuged on density gradient by the method of Havel *et al.* (16) to separate chylomicrons ($d < 1.006$ kg/L; 3×10^6 g average minimum); VLDL ($d < 1.006$ kg/L; 1×10^8 g average minimum); LDL ($d < 1.063$ kg/L; 1.3×10^8 g average minimum); and HDL ($d < 1.063$ to 1.21 kg/L; 1.7×10^8 g average minimum). Appropriate fractions of chylomicron and lipoproteins were collected, and aliquots of 50 to 100 µL of each lipoprotein fraction were taken for fatty acid and cholesterol analysis by the method of Liebich *et al.* (17) as described below.

TABLE 1
Fatty Acid Composition of Oils (mg/100 mg total fatty acids)[a]

Fatty acid	Peanut oil	Randomized peanut oil
14:0	0.03	0.04
16:0	10.06	11.42
16:1	0.8	0.07
18:0	2.47	3.08
18:1	48.70	49.39
18:2	31.02	25.55
18:3	0.14	0.09
20:0	1.32	1.52
20:1	1.53	1.39
20:2	0.13	0.09
20:4	3.38	3.46
22:0	0.12	0.17
22:3	0.15	0.08
22:6	0.08	0.51
24:0	0.22	0.26

[a]Fatty acid analysis was performed as described in the Materials and Methods section.

Separation of lipids on thin-layer chromatography (TLC). Aliquots of 1.0 mL of 24-h lymph were extracted using chloroform/methanol (2:1, vol/vol) according to the method of Folch *et al.* (18). The chloroform/methanol extracts were evaporated under nitrogen and lipids were solubilized in hexane and separated into major lipid classes (19) by silica gel TLC (Analab, Norwalk, CT) using hexane/diethyl ether/acetic acid (80:16:2, by vol) as solvent. The separated lipid fractions were stained with iodine, which was placed in the bottom of the TLC tank. Areas on each plate co-migrating with authentic PL, MG/DG, TG, FFA, CE, and free cholesterol standards were individually scraped from the TLC plates and transferred to tubes.

Fatty acid analysis. Aliquots of chylomicron and lipoprotein fractions obtained by ultracentrifugation and fractions containing PL, MG/DG, FFA, TG, and CE separated and isolated on TLC plates were analyzed for fatty acids and cholesterol by the method of Liebich *et al.* (17). The fractions were extracted with chloroform/methanol (2:1) (18). The lipid-soluble fractions were then converted to fatty acid methyl esters (FAME) by the method of Liebich *et al.* (17) as described below. Lymph samples were heated at 100°C for 1 h with methanol, toluene, and acetyl chloride. The mixtures were partitioned with 6% potassium carbonate, and the upper toluene layer was decanted, heated with trifluoroacetic anhydride, dried, and dissolved in isooctane. Derivatized FAME

and cholesterol (as trifluoroacetate) were quantitated on a gas chromatograph (Hewlett-Packard, Palo Alto, CA) fitted with a capillary column optimized to separate FAME and cholesterol. Methyl tricosanoate (23:0) was used as an internal standard. The column was 30 m long (i.d. 0.25 mm) and coated with DB-23, cyanopropyl/methylpolysiloxane (J&W Scientific, Folsom, CA). Peanut oil and randomized peanut oil were analyzed by gas chromatography for fatty acid composition as described above.

Statistical analysis. Means and standard errors were calculated, and significant differences between groups were assessed by a Kruskal-Wallis test followed by Dunnett's procedure for pair-wise comparisons (20). Results were considered statistically significant at $P < 0.05$.

RESULTS

Lymph flow rate. The average lymph flow rate in the peanut oil group was 5.2 ± 1.6 mL/h and was 7.1 ± 2.0 mL/h in the randomized peanut oil group. There were no significant differences between the two groups.

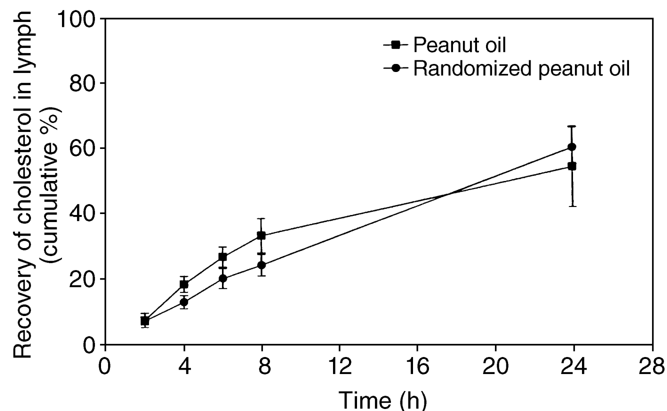

FIG. 1. Percentage recovery of cholesterol in lymph of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group.

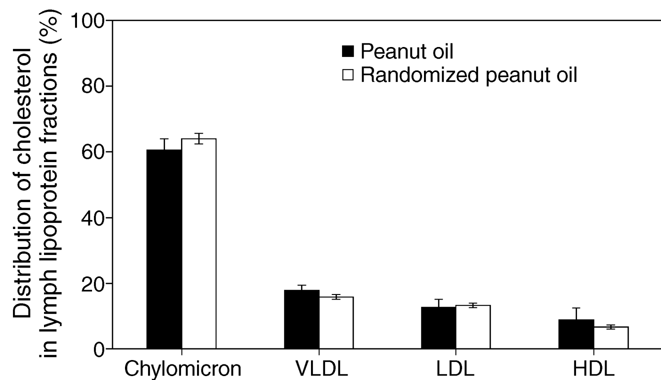

FIG. 2. Percentage distribution of cholesterol in lymph lipoprotein fractions (VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein) of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group.

Cholesterol absorption and recovery. The cumulative percentage absorption of total cholesterol into the thoracic duct of rats that received either peanut oil or randomized peanut oil is shown in Figure 1. Cumulative percentage of absorption of cholesterol during 24 h continued to increase in both groups. During this period 55–60% of the infused cholesterol was re-

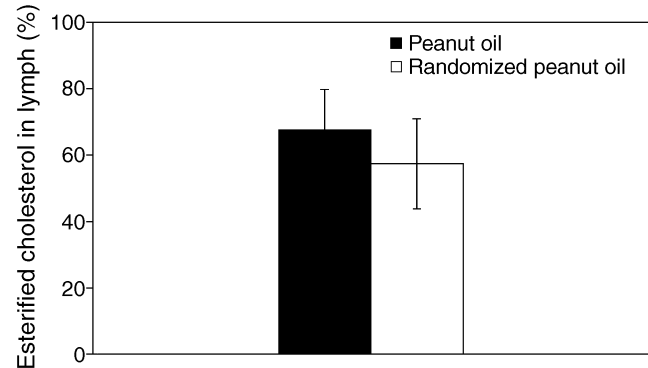

FIG. 3. Percentage of esterified cholesterol in the lymph of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group.

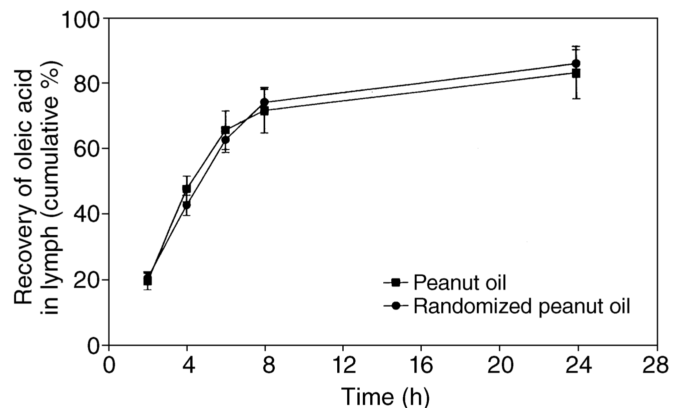

FIG. 4. Percentage recovery of oleic acid in the lymph of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group.

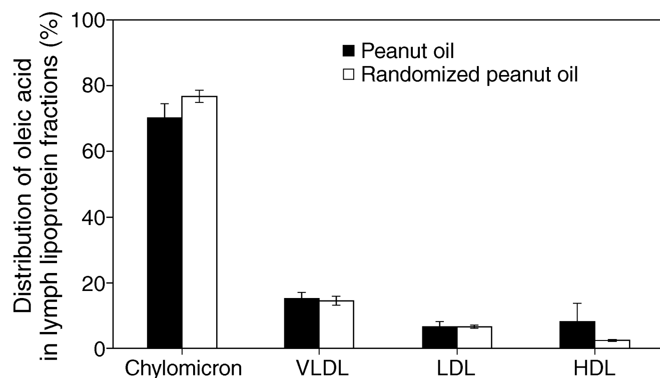

FIG. 5. Percentage distribution of oleic acid in lymph lipoprotein fractions of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group. See Figure 2 for abbreviations.

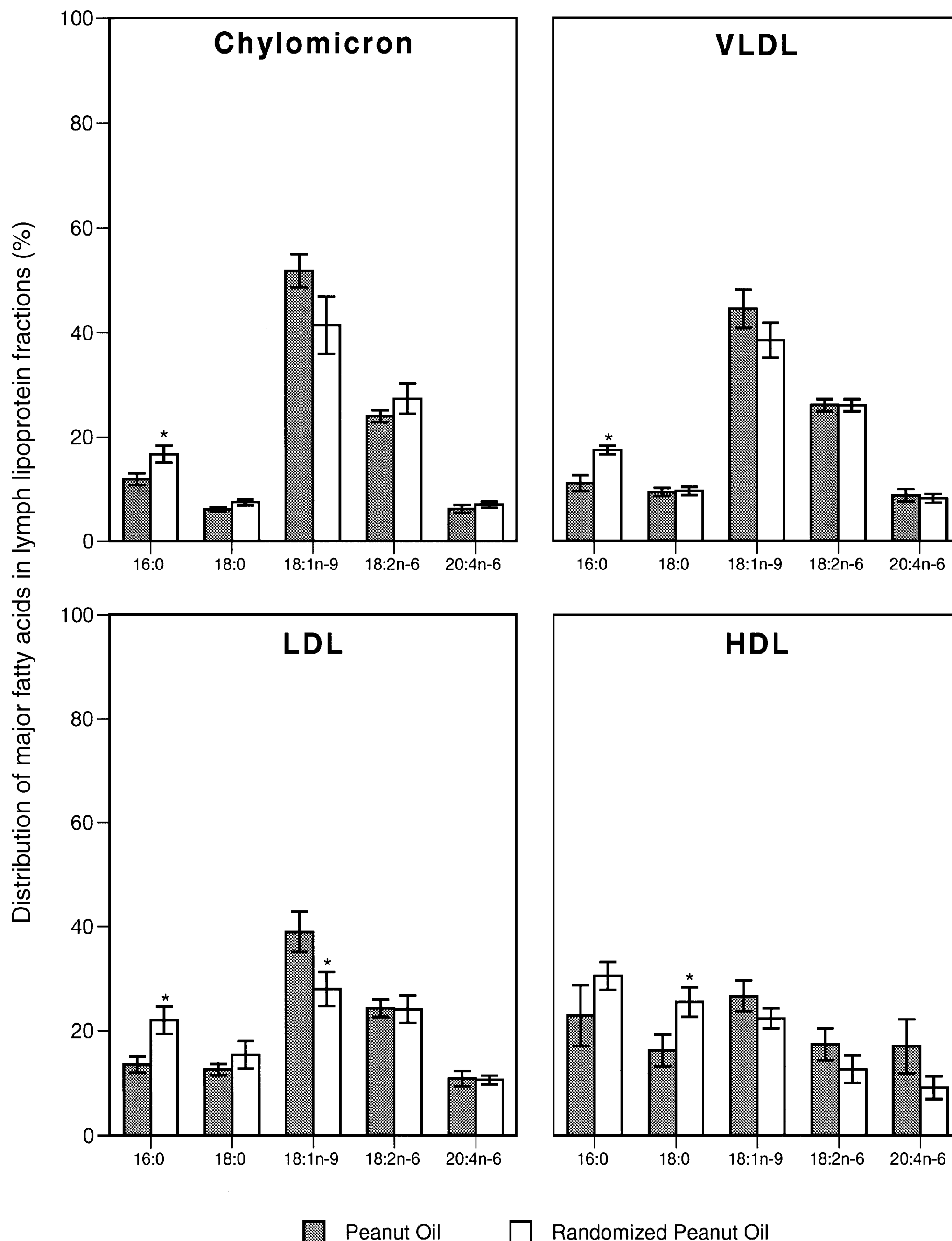

FIG. 6. Percentage distribution of major fatty acids in lipoprotein fractions of rats that received either peanut oil or randomized peanut oil. Mean ± SEM; $n = 10$ rats/group. *Significantly different from peanut oil group, $P < 0.05$. See Figure 2 for abbreviations.

covered in the lymph of both groups. There were no significant differences either in cumulative absorption of cholesterol or total recovery of cholesterol between the two groups.

The percentage distribution of total cholesterol in lymph lipoprotein fractions is shown in Figure 2. About 60–63% of the absorbed cholesterol was associated with the chylomicron fraction ($d < 1.006$ kg/L); the remaining 37–40% was distributed in the VLDL, LDL, and HDL fractions—about 15–17% in the VLDL, 12–13% in the LDL, and 6–8% in the HDL fractions. There were no significant differences between the two groups.

The percentage of esterified cholesterol in the lymph of rats that received either peanut oil or randomized peanut oil is shown in Figure 3; 67.4 ± 12.3% of cholesterol was esterified in the group that received peanut oil, whereas 57.2 ± 13.5% of cholesterol was esterified in the group that received randomized peanut oil. However, there were no significant differences between the groups.

Oleic acid absorption and recovery. The cumulative percentage absorption of oleic acid (18:1n-9) in the lymph of rats that received either peanut oil or randomized peanut oil is shown in Figure 4. Cumulative absorption of 18:1n-9 increased rapidly until 8 h. From 8–24 h, absorption slowed. In both groups, about 82–85% of the infused 18:1n-9 was recovered in the lymph over a 24-h period. There were no significant differences between the two groups.

Distribution of fatty acids in lipoprotein fractions. The percentage recovery of 18:1n-9 in lymph lipoprotein fractions is shown in Figure 5. In both groups, about 69–76% of the absorbed 18:1n-9 was recovered in the chylomicron fraction. The remaining 24–31% was distributed as follows: 14–15% in VLDL, 6% in LDL, and 7% in the HDL fractions. There were no significant differences between the two groups.

The percentage distribution of major fatty acids in lymph lipoprotein fractions is shown in Figure 6. The major fatty acids isolated from both groups were palmitic (16:0), stearic (18:0), oleic (18:1n-9), linoleic (18:2n-6), and arachidonic (20:4n-6) acids. The percentage of 16:0 in the chylomicron, VLDL, and LDL fractions was significantly higher in rats fed randomized peanut oil compared to the peanut oil-fed group. The LDL fraction also showed significant reduction in the percentage of 18:1n-9 in the randomized peanut oil group compared to the peanut oil-fed group. In the HDL fraction, only 18:0 is significantly higher in the randomized peanut oil group compared to the peanut oil group.

The percentage of total fatty acids estimated in each lipid fraction is shown in Figure 7. About 62–71% of the total fatty acids was recovered in the TG fraction, 13–19% in the PL fraction, 7–9% in the FFA fraction, 3–4% in the MG/DG fractions, and 2–3% in the cholesterol and CE fractions between the two groups. There were no significant differences in the percentage of total fatty acids distributed in the lipid fractions. The percentage of major fatty acids distributed in lipid fractions is shown in Figure 8. Major fatty acids isolated from both groups were 16:0, 18:0, 18:n-9, 18:2n-6, and 20:4n-6. There were no significant differences in the distribution of major fatty acids between the groups.

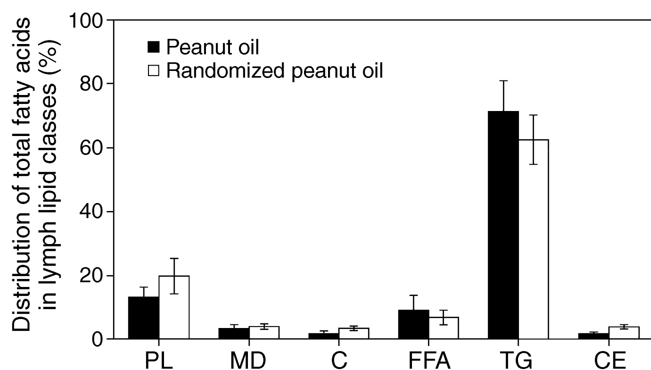

FIG. 7. Percentage distribution of total fatty acids in each lipid fractions (PL, phospholipid; MD, mono- and diglycerides; C, cholesterol; FFA, free fatty acid; TG, triglyceride; CE, cholesterol ester) of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; *n* = 10 rats/group.

DISCUSSION

The present studies were conducted to evaluate whether peanut oil and randomized peanut oil affect absorption, transport, or distribution of cholesterol and 18:1n-9 in the lymph of rats. The lipid emulsion that was used contained cholesterol, oleic acid, sodium taurocholate (bile acid), and albumin, all of which form micelles in the lumen of the small intestine. Earlier studies by Vahouny *et al.* (15) found this composition of emulsion to be the ideal mixture that produced maximal sterol absorption. The sterols in the emulsion are taken up by the enterocytes, undergo obligatory esterification, and are incorporated into the nonpolar core of chylomicrons, which then enter the lymph (15). Our results show that in the presence of either oil, cholesterol and 18:1n-9 were absorbed equally into the lymph. There were no significant differences in the distribution of cholesterol and 18:1n-9 in various fractions of lymph lipoprotein. Figure 3 indicates that cholesterol was esterified equally in both groups. However, there were some statistically significant differences in the fatty acid composition of some of the lipoprotein fractions. With randomized peanut oil infusion, palmitic acid in the chylomicron, VLDL, and LDL fractions was significantly increased, while 18:0 was significantly increased in the HDL fraction and 18:1n-9 was significantly decreased in the LDL fraction compared to peanut oil. We do not know how important these statistically significant increases may be in relation to atherogenicity. The observed significant increase in palmitic acid observed in Figure 6 was due to the rearrangement of palmitic acid in the randomized peanut oil. In native peanut oil, palmitic acid was present in a high amount and equally distributed between *sn*-1 and *sn*-3 positions, while a low amount was present in *sn*-2 position of the triacylglycerol molecule (10). Once the native peanut oil was randomized, palmitic acid in the *sn*-2 position of the triacylglycerol molecule increased and resulted in high amounts of palmitic acid as observed in lipoprotein fractions. As shown in Figures 7 and 8 there were no significant differences in either the total fatty acid distribution or amount of major individual fatty acid distribution in the lipid fractions (PL, MG/DG, FFA, TG, or CE) of rats administered peanut oil or randomized peanut oil.

The results of this study show that there were no significant differences between peanut oil and randomized peanut oil on cholesterol and 18:1n-9 absorption, distribution, and transport in the lymph. Tso *et al.* (10) also reported that TG, cholesterol, and PL outputs were similar in both dietary groups. The recovery of cholesterol from the intestinal lumen and mucosa showed that absorption and transport also were similar in both groups. In their study, analysis of the fatty acid of both lymph and intestinal mucosal lipids failed to reveal any differences between the two groups. Based on this study and others (8–10), it can be concluded that the atherogenicity of peanut oil may be due to events taking place subsequent to the entry into the plasma of the cholesterol-containing chylomicrons or VLDL. Recently, lectins present in peanut oil have been implicated in the atherogenic effect. Kritchevsky *et al.* (21,22) showed that the randomization process could destroy lectins

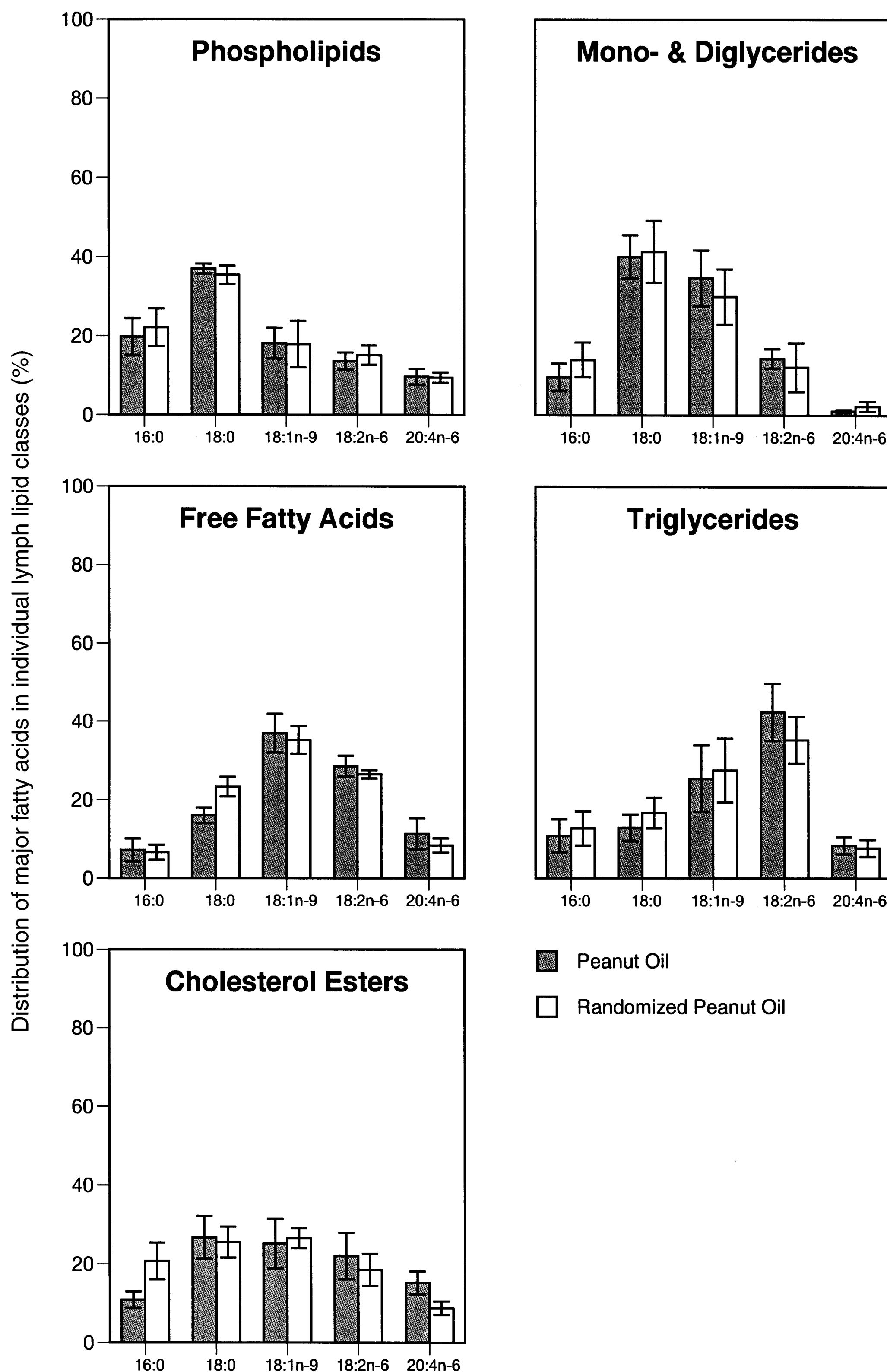

FIG. 8. Percentage distribution of major fatty acids in each lipid fractions of rats that received either peanut oil or randomized peanut oil. Values are mean ± SEM; n = 10 rats/group. See Figure 7 for abbreviations.

and thus prevent atherogenicity. In their study, they reported that randomized peanut oil had 36% less lectin than peanut oil, suggesting that reducing the lectin content of peanut oil reduces its atherogenicity (22). It is also possible that randomized peanut lectins inhibit recruitment of macrophages, which are the major cell type occurring in aortic fatty streaks (23).

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Dietary Phospholipid Alters Biliary Lipid Composition in Formula-Fed Piglets

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ABSTRACT: Plasma cholesterol, arachidonic acid (AA, 20:4n-6), and docosahexaenoic acid (DHA, 22:6n-3) are higher in breast-fed infants than in infants fed formula without cholesterol, AA, or DHA. This study investigated differences in plasma, hepatic, and bile lipids and phospholipid fatty acids, and expression of hepatic proteins involved in sterol metabolism that result from feeding formula with cholesterol with egg phospholipid to provide AA and DHA. For this study, three groups of piglets were evaluated: piglets fed formula with 0.65 mmol/L cholesterol, the same formula with 0.8% AA and 0.2% DHA from egg phospholipid, and piglets fed sow milk. Piglets fed the formula with phospholipid AA and DHA had higher plasma high density lipoprotein, but not apoprotein (apo) B cholesterol or triglyceride; higher bile acid and phospholipid concentrations in bile; and higher liver and bile phospholipid AA and DHA than piglets fed formula without AA and DHA ($P <$ 0.05). Hydroxy methylglutaryl (HMG)-CoA reductase and 7-α-hydroxylase, the rate-limiting enzymes of cholesterol and bile acid synthesis, respectively, and low density lipoprotein receptor mRNA levels were not different between piglets fed formula without and with phospholipid AA and DHA, but HMG-CoA reductase and 7α-hydroxylase mRNA were higher, and plasma apo B containing lipoprotein cholesterol was lower in all piglets fed formula than in piglets fed milk. These studies show that supplementing formula with AA and DHA from egg phospholipid alters bile metabolism by increasing the bile AA and DHA, and bile acid and phospholipid.

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Human milk provides small amounts of arachidonic acid (20:4n-6, AA) and docosahexaenoic acid (22:6n-3, DHA) (1,2), and as a result blood lipid concentrations of AA and DHA are higher in infants fed human milk than in infants fed formula without AA and DHA (3–6). Plasma concentrations of cholesterol, particularly low density lipoprotein (LDL) cholesterol, are also higher in infants fed milk than infants fed formula (7), but whether this is due to the presence of cholesterol in milk but not formula is not clear. Much of the current interest with respect to AA and DHA in infant lipid nutrition has focused on the important role of AA and DHA in growth, and brain and visual function (3–6,8–10). The n-6 and n-3 polyunsaturated fatty acids, however, are involved in numerous important metabolic processes, including eicosanoid synthesis, gene expression, and hormonal signaling and are known to influence triglyceride and lipoprotein metabolism (11,12). AA and DHA can be synthesized in the liver from the precursors, linoleic acid (18:2n-6) and α-linolenic acid (18:3n-3), respectively (13), or provided in the diet as preformed dietary AA and DHA. In the liver, AA and DHA can be incorporated into membrane phospholipids (PL), or secreted in lipoprotein or bile PL. Several studies have shown that dietary long-chain polyunsaturated fatty acids influence bile acid metabolism and bile flow (14–19). Diet-related differences in bile acid and hepatic lipid metabolism between breast-fed and formula-fed baboons (20,21) and piglets (22,23) have been reported. Longer-term studies have also shown altered cholesterol and bile acid metabolism in older baboons who had been fed formula when compared with baboons that had been breast-fed as infants (24–26). Whether the long-chain fatty acids present in milk but absent from formula influence hepatic bile lipid metabolism is not known.

The objective of this study was to investigate the effect of supplementing infant formula with egg PL containing AA and DHA (i) on hepatic and bile lipids, (ii) on hepatic levels of mRNA for cholesterol 7-α-hydroxylase (C7H; EC 1.14.13.15) and HMG-CoA reductase (EC 1.1.1.34), the rate-limiting enzymes in bile acid and cholesterol synthesis, respectively, and (iii) on hepatic LDL receptor mRNA in formula-fed piglets. Egg PL has been used to provide AA and DHA to infants in studies considering the role of neonatal dietary AA and DHA on infant visual development (3,4).

MATERIALS AND METHODS

Animals and diets. Male Yorkshire piglets with birth weight >1 kg were obtained within 24 h of birth, from Peter Hill Holdings (Langley, British Columbia, Canada) and randomly assigned to be fed formula without (n = 9) or with egg PL to provide AA and DHA (n = 6), until 15 d of age. A group of

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Abbreviations: AA, arachidonic acid, 20:4n-6; ANOVA, analysis of variance; apo, apoprotein; C7H, cholesterol 7-α-hydroxylase; DHA, docosahexaenoic acid, 22:6n-3; HDL, high density lipoprotein; HMG-CoA, 3-hydroxy-3-methyl glutaryl CoA; LDL, low density lipoprotein; PCR, polymerase chain reaction; PL, phospholipid; RT, reverse transcription; VLDL, very low density lipoprotein.

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piglets ($n = 7$) left on the farm and fed by their natural mothers (milk) was also studied for reference. Piglets from the same litter were not assigned to the same diet group. The housing and procedures for feeding piglets have been described (27).

The formula fatty acids contained about 25% 16:0, 7% 14:0, 20% 18:2n-6, and 1.8–2.1% 18:3n-3 compared with 30% 16:0, 4% 14:0, 11% 18:2n-6, and 1.1% 18:3n-3 in the sow milk (Table 1). The levels of 18:2n-6 and 18:3n-3 in the formulas were similar to those in currently available term infant formula: human milk typically has about 12–16% 18:2n-6 and 1% 18:3n-3 (1,2). The formula with egg PL had 0.8% AA and 0.25% DHA, and the sow milk had 0.7% AA and 0.1% DHA.

At 15 d of age, between 0900 and 1030, the piglets were anesthetized and blood samples were drawn by cardiac puncture (27). Plasma was prepared by centrifugation, and with the exception of aliquot samples for high density lipoprotein (HDL) cholesterol, the samples were frozen at −80°C. After laparotomy, the liver was removed, bile was drawn from the gallbladder, the liver was weighed, and a portion was immediately frozen in liquid nitrogen and stored at −80°C until preparation of RNA. The remaining organ was homogenized and stored at −80°C for later lipid analysis. All 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.

Lipid analysis. The plasma and liver total cholesterol and triglyceride concentrations were determined using enzymatic reagents (Diagnostic Chemical Ltd., Charlottetown, Prince Edward Island, Canada; Boehringer Mannheim Diagnostics, Montréal, Québec, Canada) (27). Plasma HDL cholesterol was determined following precipitation of apoprotein (apo) B-containing lipoproteins with heparin-manganese chloride (28). The amount of cholesterol associated with apo B-containing lipoproteins (chylomicron + VLDL + LDL) was calculated as the difference between the total and HDL cholesterol. Plasma, liver, and bile lipid were extracted, the lipid fractions separated by thin-layer chromatography, and the PL, triglyceride, and cholesterol ester fatty acids analyzed by gas–liquid chromatography (29). The bile lipid extracts were

TABLE 1
Fatty Acid Composition (% total) of Formula Without and With Phospholipid (PL) and Sow Milk[a]

Fatty acid	Formula	Formula + PL	Milk[b]
14:0	7.6	6.5	3.8 ± 0.3
16:0	24.0	24.7	30.5 ± 2.9
18:0	5.1	6.6	9.4 ± 0.8
18:1n-9	38.6	37.0	37.5 ± 3.2
18:2n-6	20.7	20.0	11.1 ± 0.4
18:3n-3	2.1	1.8	1.1 ± 0.2
20:4n-6	0.0	0.8	0.7 ± 0.1
22:6n-3	0.0	0.2	0.1 ± 0.1

[a]The formula + PL contained 9.5 g/L egg PL. The formulas both contained 0.65 mmol/L cholesterol; the sow milk had 0.52 mmol cholesterol.
[b]Values for milk are means ± SD.

exposed to fluorescent light overnight to permit photodegradation of biliary pigments, prior to quantifying total cholesterol, total bile acids (30), and PL (31). Total protein was determined according to the method of Lowry *et al.* (32) using bovine serum albumin (BSA) as a standard.

mRNA assays. The reverse transcription polymerase chain reaction (RT-PCR) method, as well as the primer sequences used in the RT-PCR reactions, used to assess the levels of LDL receptor, HMG-CoA reductase, and C7H mRNA have recently been described (22). This procedure involved reverse-transcribing a known quantity of total RNA to generate a pool of cDNA representing the RNA in the original sample. PCR was used to amplify the cDNA corresponding to the mRNA of interest. The expression of β-actin was also determined for each sample and used as an internal control for the efficiency of each RT-PCR reaction.

Total RNA was isolated from liver tissue using TRIzol® (Gibco BRL, Burlington, Ontario, Canada) following the protocol described by the manufacturer. The RNA preparations were treated with 10U of RQ1 RNase-free DNase (Promega, Madison WI) to ensure no genomic DNA contamination, and 2.5 µg of total RNA was used for first strand cDNA synthesis as previously described (22). The resulting cDNA pool for each RNA sample was divided into aliquots (3 µL) with one aliquot used in each PCR reaction. Separate PCR reactions were run for LDL receptor, HMG-CoA reductase, C7H and β-actin (22). The number of cycles used for the PCR reactions for LDL receptor, HMG-CoA reductase, C7H and β-actin were gene specific and were determined to be in the exponential phase of the amplification process. The number of cycles for each gene was: LDL receptor 35 cycles, HMG-CoA reductase 35 cycles, C7H 35 cycles, β-actin 25 cycles. The PCR reactions (10 µL each) were resolved on a 1.5% agarose-1XTBE gel and stained with ethidium bromide. A photograph was taken with Kodak TRI-X pan film. The negatives were scanned with a video densitometer (model 620; Bio-Rad, Mississauga, Ontario, Canada) to determine the relative intensity of the bands, and this was expressed per microgram RNA used in the original RT reaction. The intra- and interassay variability between RT-PCR reactions was less than 10%.

Statistical analysis. Analysis to determine the effect of feeding formula in comparison with milk feeding, and of supplementing formula with PL AA and DHA was done with one-way analysis of variance (ANOVA). Fisher's least significant difference was used to determine specific group differences for ANOVA results with $P < 0.05$. All calculations were performed using the Statistical Package for the Social Sciences (SPSS) for windows, renewable version (release 6.1).

RESULTS

There were no differences in body weight or liver weight among the piglets fed the formula without ($n = 9$) or with PL ($n = 6$), or those fed sow milk ($n = 7$) (body weight, 5.15 ± 0.13, 4.83 ± 0.15, 4.78 ± 0.40 kg; liver weight, 28.3 ± 1.24, 26.6 ± 0.50, 24.7 ± 0.90 g, respectively). The piglets fed for-

TABLE 2
Plasma and Liver Lipids Concentrations in Piglets Fed Formula Without or With Egg PL or Milk[a]

	Formula	Formula + PL	Milk
Plasma (mmol/L)			
Cholesterol			
Total	2.36 ± 0.1[a]	2.49 ± 0.1[a]	3.96 ± 0.4[b]
HDL	1.05 ± 0.0[a]	1.36 ± 0.1[b]	1.56 ± 0.2[c]
Apo B containing	1.31 ± 0.1[a]	1.13 ± 0.1[a]	2.41 ± 0.3[b]
Triglyceride	0.41 ± 0.0[a,b]	0.50 ± 0.1[b]	0.32 ± 0.1[a]
Liver (μmol/g protein)			
Cholesterol	30.8 ± 0.7	30.4 ± 0.7	31.4 ± 1.3
Triglyceride	14.3 ± 1.0	16.9 ± .0.7	20.1 ± 2.4
Phospholipid	193.2 ± 7.0[a,b]	209.1 ± 5.9[b]	188.3 ± 5.4[a]

[a]Data are expressed as means ± SEM; formula, $n = 9$; formula + PL, $n = 6$; milk, $n = 7$. Values with a different roman superscript a–c are significantly different, $P < 0.05$. HDL, high-density lipoprotein; apo B, apoprotein B; for other abbreviation see Table 1.

mula all had significantly ($P < 0.001$) lower plasma total and apo B containing lipoprotein (VLDL + LDL) cholesterol concentrations than piglets fed milk (Table 2). Piglets fed the standard formula had a significantly lower plasma concentration of HDL cholesterol than the piglets fed milk. Although the plasma HDL cholesterol concentration was significantly higher in piglets fed the formula with PL than in piglets fed the standard formula, it was still significantly lower than in the milk-fed group. Piglets fed the formula with PL also had significantly higher plasma triglyceride concentrations than piglets fed milk.

There were no significant differences in the liver cholesterol, triglyceride, or PL concentrations among the piglets fed formula and those fed sow milk, with the exception that piglets fed the formula with PL had a significantly higher liver PL concentration than piglets fed milk (Table 2).

Piglets fed the formula with PL had significantly higher liver PL AA and DHA than piglets fed the standard formula or sow milk (Table 3). Supplementation with egg PL also resulted in significantly higher AA, but not DHA in the liver triglyceride when compared with piglets fed the standard formula or sow milk. Consistent with the lower 16:0 and higher 18:2n-6 in the formula than milk (Table 1), the liver PL, triglyceride, and cholesterol ester 16:0 was lower and the triglyceride and cholesterol ester 18:2n-6 was higher in the formula than milk-fed piglets.

Piglets fed the standard formula had significantly lower bile acid and PL in bile than piglets fed milk (Table 4). Including egg PL in the formula significantly increased concentrations of PL and bile acid in bile of the formula-fed piglets to concentrations not different from those of piglets fed milk. Piglets fed the formula with PL also had significantly higher bile PL AA and DHA than piglets fed milk or the standard formula.

The piglets fed formula had significantly higher levels of HMG-CoA reductase and C7H mRNA than piglets fed milk (Fig. 1). However, whereas the group comparisons found HMG-CoA mRNA was significantly lower in both the piglets fed the standard and those fed the PL supplemented formula, the differences in C7H mRNA from the sow milk group were not significant ($P = 0.07$). The levels of LDL receptor mRNA was not different between the milk-fed piglets and piglets fed the formulas.

DISCUSSION

Formula feeding results in lower plasma cholesterol, particularly in apo B-containing lipoproteins, and lower AA and DHA concentrations than breast feeding (3–10), and also produces short- and long-term changes in hepatic and plasma lipid metabolism in animals (20–25). Some evidence also suggests early nutrition may influence chronic diseases, including coronary heart disease, in adult humans (33). Previous studies have shown piglets fed formulas resembling infant formulas have lower plasma cholesterol, and higher hepatic C7H and HMG-CoA reductase mRNA and enzyme

TABLE 3
Liver PL, Triglyceride, and Cholesterol Ester Major Fatty Acids of Piglets Fed Formula, Without or With Egg PL Providing Arachidonic and Docosahexaenoic Acids, or Milk[a]

	Liver PL			Triglyceride			Cholesterol ester		
Fatty acid	Formula	Formula + PL	Milk	Formula	Formula + PL	Milk	Formula	Formula + PL	Milk
	g/100 g								
16:0	14.4 ± 0.3[a]	14.6 ± 0.2[a]	15.6 ± 0.2[b]	21.9 ± 0.6[a]	21.7 ± 0.4[a]	31.4 ± 0.9[b]	17.4 ± 0.8[a]	18.8 ± 0.8[a]	26.1 ± 0.7[b]
18:0	27.5 ± 0.3	26.9 ± 0.3	26.9 ± 0.2	7.5 ± 0.3	7.4 ± 0.2	6.5 ± 0.5	7.1 ± 0.4	6.8 ± 0.2	6.5 ± 0.9
18:1	11.3 ± 0.3[b]	9.8 ± 0.3[a]	9.5 ± 0.1[a]	32.1 ± 1.4[c]	28.5 ± 0.6[b]	26.5 ± 0.5[a]	34.4 ± 1.2[b]	27.4 ± 1.0[a,b]	25.7 ± 1.0[a]
18:2n-6	15.4 ± 0.3[b]	14.2 ± 0.3[a]	13.6 ± 0.3[a]	19.5 ± 0.5[b]	20.6 ± 0.3[b]	12.9 ± 0.4[a]	29.5 ± 1.0[b]	33.4 ± 0.8[c]	24.5 ± 0.9[a]
18:3n-3	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.3 ± 0.0[b]	1.1 ± 0.1	1.4 ± 0.1	1.4 ± 0.1	1.1 ± 0.0	1.2 ± 0.2	1.6 ± 0.3
20:4n-6	19.5 ± 0.3[b]	21.5 ± 0.3[c]	18.7 ± 0.3[a]	8.6 ± 0.9[b]	10.7 ± 0.4[c]	5.6 ± 0.1[a]	1.8 ± 0.2	2.5 ± 0.5	1.4 ± 0.2
20:5n-3	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.4 ± 0.0[b]	0.1 ± 0.0[a]	0.2 ± 0.0[b]	0.2 ± 0.0[b]	Trace	Trace	Trace
22:4n-6	0.6 ± 0.0	0.6 ± 0.0	0.6 ± 0.0	0.3 ± 0.0	0.4 ± 0.0	0.4 ± 0.0	Trace	0.1 ± 0.0	0.1 ± 0.0
22:5n-6	0.5 ± 0.1[a]	0.8 ± 0.1[b]	0.5 ± 0.1[a]	0.2 ± 0.0[a]	0.3 ± 0.0[b]	0.1 ± 0.0[a]	Trace	Trace	Trace
22:5n-3	1.3 ± 0.1[a]	1.3 ± 0.1[a]	2.7 ± 0.2[b]	0.5 ± 0.1[a]	0.6 ± 0.0[a]	0.8 ± 0.0[b]	Trace	Trace	Trace
22:6n-3	6.1 ± 0.1[a]	7.3 ± 0.2[c]	6.4 ± 0.1[b]	1.0 ± 0.1[a,b]	1.2 ± 0.1[b]	0.8 ± 0.0[a]	0.4 ± 0.1[b]	0.1 ± 0.0[a]	0.3 ± 0.1[b]

[a]Values given as mean ± SEM; formula, $n = 9$; formula + PL, $n = 6$; milk, $n = 7$. Values with different roman superscript letters (a–c) are significantly different, $P < 0.05$. See Table 1 for abbreviation.

TABLE 4
Bile Composition of Piglets Fed Formula Without or With PL or Milk[a]

	Diet		
	Formula	Formula + PL	Milk
Bile acids (mmol/L)	49.3 ± 4.4[a]	64.7 ± 6.9[b]	70.9 ± 4.6[b]
Cholesterol (mmol/L)	2.6 ± 0.2	2.9 ± 0.3	3.2 ± 0.3
Phospholipid (mmol/L)	12.7 ± 0.9[a]	16.0 ± 2.2[b]	16.4 ± 1.6[b]
Major fatty acids (% total)			
16:0	27.6 ± 0.3[a]	28.5 ± 0.3[a]	29.9 ± 0.4[b]
18:0	13.9 ± 0.5	13.6 ± 0.4	12.7 ± 0.4
18:1n-9	18.2 ± 0.2[b]	15.9 ± 0.4[a]	15.4 ± 0.2[a]
18:2n-6	23.9 ± 0.5	22.6 ± 0.5	21.5 ± 0.5
18:3n-3	0.3 ± 0.0[a]	0.3 ± 0.0[a]	0.5 ± 0.1[b]
20:4n-6	9.4 ± 0.4[a]	11.9 ± 0.7[b]	8.7 ± 0.4[a]
20:5n-3	0.2 ± 0.0[a]	0.2 ± 0.0[a]	0.4 ± 0.0[b]
22:4n-6	0.1 ± 0.0	0.1 ± 0.0	0.1 ± 0.0
22:5n-6	0.2 ± 0.0[a,b]	0.3 ± 0.0[b]	0.2 ± 0.0[a]
22:5n-3	0.5 ± 0.0[a]	0.5 ± 0.0[a]	0.8 ± 0.1[b]
22:6n-3	2.8 ± 0.1[a]	3.7 ± 0.2[b]	3.0 ± 0.1[a]

[a]Values shown are means ± SEM; formula, $n = 9$; formula + PL, $n = 6$; milk, $n = 7$. Values in a row with a different roman superscript a–c are significantly different, $P < 0.05$.

activity than piglets fed milk (22,23,34). Similarly, stable isotope measures of cholesterol synthesis have indicated that cholesterol synthesis is increased in infants fed formula (35). PL supplementation increased liver and bile PL AA and DHA, and increased the bile PL and bile acid concentrations of the formula-fed piglets such that the bile PL and bile acid concentrations were not different from those of piglets fed milk. Whether the increase in bile PL and bile acid was due to the AA and DHA or the PL is not clear from our studies.

Most studies have concluded that the secretion of PL in bile lipid is not increased by PL feeding (36,37), although short-term feeding with soybean-derived phosphatidylcholine high in 18:2n-6 increased bile cholesterol and PL output in rats (38). Dietary and biliary PL is hydrolyzed to lysophospholipid in the intestine prior to absorption, and subsequently reesterified for secretion to plasma in lipoproteins. This suggests the increased bile lipids secondary to PL feeding may be secondary to increased cholesterol or bile acid reabsorption, or hepatic lipoprotein PL or cholesterol uptake.

Inclusion of egg PL in the formula increased plasma HDL cholesterol concentrations in the formula-fed piglets. The concentration of plasma HDL cholesterol can be influenced by the rate of apo A-1 production (39). Further, recent studies reported that phosphatidylcholine increased apo A-1 secretion by newborn pig intestinal epithelial cells, possibly by a mechanism involving mobilization of a preformed pool of apo A-1 (40). However, it is not known if the plasma PL pool or turnover, or transfer of HDL cholesterol to the liver, which is thought to be important in the movement of cholesterol to liver for excretion (41), was increased by PL supplemention. The liver PL concentration of the formula-fed piglets, however, was not increased by PL supplementation, and no association was found between the liver and bile PL among piglets fed the milk and formula diets. Hepatic PL synthesis and secretion, on the other hand, have been shown to be in-

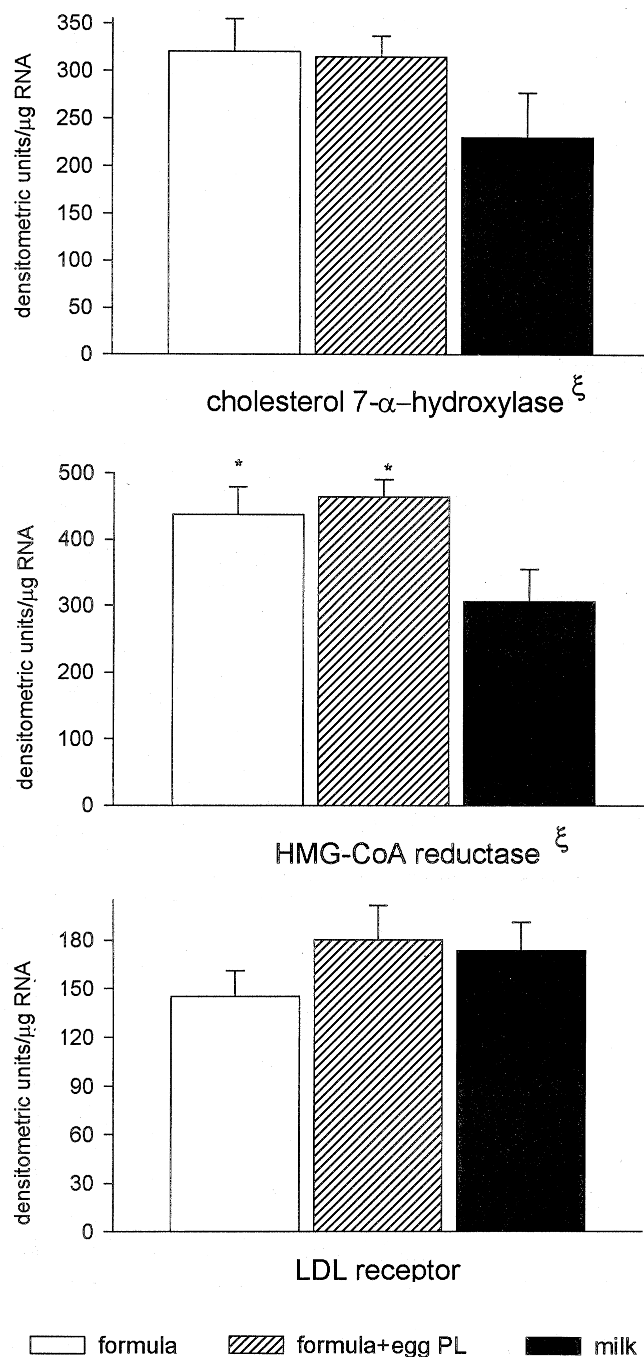

FIG. 1. Hepatic cholesterol 7-α-hydroxylase, low density lipoprotein (LDL) receptor, and 3-hydroxy-3-methylglutaryl (HMG)-CoA reductase mRNA levels in piglets fed formula without and with phospholipid (PL) or milk. Values shown as mean ± SEM; formula, $n = 9$; formula + PL, $n = 6$; milk, $n = 7$. ξ, Significant effect of feeding formula compared with milk; value for group fed formula significantly different from value for group fed milk ($P < 0.05$).

creased by bile salt administration (42), and the fatty acid composition of dietary lipid is known to influence intestinal membrane lipids and transport functions (43). Whether the quantity of PL or AA and DHA in the intestine influences bile acid reabsorption does not appear to be known.

Several studies have provided evidence that the fatty acid composition of dietary lipid influences the fatty acid composition of bile phospholipid, as well as biliary lipid and bile acid secretion (15–19,44). Thus, it is possible that the increase in bile PL and bile acid in piglets fed formula with egg PL was secondary to the increase in hepatic AA and DHA. However, the liver and bile AA and DHA in piglets fed formula with PL was higher than in piglets fed milk, whereas the bile acid and PL were not different. It seems unlikely therefore that the effects of the formula PL supplementation on the piglet bile components are explained by the n-6 and n-3 fatty acid components.

It is well appreciated that infants (as well as piglets) fed milk have higher plasma LDL cholesterol concentrations than infants fed formulas (3,22,23,34,35). The plasma LDL cholesterol concentration is determined by the rate of VLDL production and metabolism to LDL, and the rate of LDL receptor-dependent and -independent clearance (39). Dietary lipids are able to influence both LDL production and hepatic LDL receptor activity. However, substantial information is available to show that LDL receptor activity is regulated by the cholesterol content of the tissue (39). The liver cholesterol concentrations were not different among piglets fed formula and milk, consistent with the absence of a difference in LDL receptor mRNA. However, it is possible that the lower 18:2n-6 + 18:3n-3 and higher 16:0 content of milk, or some other component of milk not present in formula might have altered LDL receptor activity, without accompanying changes in LDL receptor mRNA. Future work might also consider whether VLDL synthesis and secretion are increased in neonates fed milk rather than formula.

Piglets fed formula have lower hepatic mRNA for the major rate-limiting enzymes in cholesterol and bile acid synthesis, HMG-CoA reductase and C7H, and lower plasma cholesterol than piglets fed milk (Table 2; Refs. 22,23,34). Supplementation with PL increased bile PL and bile concentrations in formula-fed piglets, but had no effect on HMG-CoA reductase or C7H mRNA or on plasma cholesterol. Studies in baboons have shown lower bile acid synthetic rates and higher hepatic LDL receptor mRNA, bile acid cholesterol saturation, cholesterol production rates, and neutral steroid excretion in juvenile and adult animals that had been breast-fed rather than fed formula (24–26.) The formula cholesterol and polyunsaturated/saturated fat ratio had no measurable effect on these long-term changes in cholesterol and bile acid metabolism in baboons (24–26). The possibility that early nutrition may affect chronic diseases, such as coronary heart disease in humans, has also been raised (33). Our results, which show that early milk feeding compared with formula feeding alters mRNA for regulatory enzymes of hepatic cholesterol metabolism, suggests diet-induced changes in expression of these proteins during early neonatal development may be an important site of lasting nutrient-gene interactions. Further studies are needed to determine the possible significance of early diet-induced changes in hepatic cholesterol and bile lipid metabolism for intestinal absorptive functions and the potential longer-term implications for cholesterol metabolism.

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Geometry of Conjugated Double Bonds of CLA Isomers in a Commercial Mixture and in Their Hepatic 20:4 Metabolites

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ABSTRACT: Rats were fed a fat-free diet for 2 wk. After this period, while maintaining the animals on the same diet, the rats were given intragastrically 180 mg per day of a mixture of conjugated linoleic acids (CLA) as triacylglycerols. Gas chromatography–mass spectrometry (GC–MS) analyses of this mixture, as well as hydrazine reduction and GC–MS and GC–Fourier transform infrared analyses of the resulting monoenes, revealed the presence of two major isomers, the 9*c*,11*t*- and the 10*t*,12*c*-18:2 accompanied by smaller amounts of the 8*t*,10*c* and the 11*c*,13*t*-18:2 isomers. Minor quantities of *cis,cis* and *trans,trans* conjugated isomers also were detected. The total fatty acid methyl esters from the liver lipids were fractionated by reversed-phase high-performance liquid chromatography, and the fraction containing the 20:4 isomers was further fractionated by silver nitrate thin-layer chromatography. A band containing two 20:4 conjugated isomers was submitted to hydrazine reduction and the resulting monoenes analyzed by GC–MS as dimethyl-oxazoline derivatives. The two conjugated isomers were tentatively identified as 5*c*,8*c*,11*c*,13*t*-20:4 and 5*c*,8*c*,12*t*,14*c*-20:4. These could be formed by desaturation and elongation of the 9*c*,11*t*- and 10*t*,12*c*-18:2 present in the commercial CLA mixture.

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A series of studies have shown that conjugated isomers of linoleic acid (CLA) have anticarcinogenic (1–6) and antiatherogenic (7,8) properties. CLA are present in foodstuffs such as milk and dairy products (9–14), meat from ruminants (15), and to a lesser extent, in partially hydrogenated oils (16) and frying oils as a result of heat treatment (17). In dairy products, the major isomer is 9*c*,11*t*-18:2 (13,14,18), whereas commercial samples of CLA mainly contain a mixture of *cis*/*trans* isomers of 8,10-, 9,11-, 10,12, and 11,13-18:2 (19).

The antioxidant activity of CLA has been suggested to explain in part the anticarcinogenic properties of these molecules (1,3). However, van den Berg *et al.* (20) recently showed that in model membranes, CLA did not exhibit any antioxidant properties. Furthermore, supplementation of CLA in MCF-7 breast cancer cells did not reduce lipid peroxide formation (21). Another possibility that has been suggested is that CLA may compete with linoleic acid for biosynthesis of arachidonic acid and eicosanoids (22). For example, CLA were shown to be absorbed and incorporated into the phospholipid fraction of different tissues (3,23–25) and to reduce the concentration of prostaglandin E_2 in serum and spleen. Like other *trans* 18:2 isomers of linoleic acid (26–28), CLA can be metabolized and converted to conjugated isomers of eicosatrienoic and eicosatetraenoic acids (24,29,30).

Recently, the feeding of rats with a commercial CLA mixture, as described elsewhere (19), permitted the identification by gas–liquid chromatography coupled with mass spectrometry (GC–MS) of three unusual conjugated C_{20} fatty acids—8,12,14-20:3, 5,8,12,14-20:4, and 5,8,11,13-20:4—in their liver lipids (30). These fatty acids are likely to arise from desaturation and elongation of the 9,11- and 10,12-18:2 isomers. Unfortunately, it is impossible to conclude at the moment which of the geometrical isomers (*cis*,*trans*, *trans*,*cis* or *trans*,*trans*) present in the commercial CLA samples are the precursors of the 20:3 and 20:4 conjugated acids. The purpose of this study was therefore to fully identify the starting CLA mixture as well as the conjugated isomers of arachidonic acid in liver lipids of rats fed the commercial CLA mixture.

EXPERIMENTAL PROCEDURES

Chemicals and isolation of the 20:4 metabolites in the biological samples. All solvents were supplied by SDS (Peypin, France), and distilled before use. Most chemical reagents were supplied by Sigma (L'Isle d'Abeau, France). Silica gel plates (20 × 20 cm, 2.5 mm thickness) were from SDS. The animal experiment, the liver lipid extraction, the fatty acid methyl ester (FAME) preparation, and the isolation of the 20:4 metabolites have been described recently (30).

Hydrazine reduction of 18:2 and 20:4 conjugated isomers. The CLA mixture (42 mg; Sigma) was dissolved in 30 mL of 96% ethanol in a round-bottom flask fitted with a condenser and

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Abbreviations: $AgNO_3$-TLC, silver nitrate thin-layer chromatography; CLA, conjugated linoleic acid; DMOX, dimethyl-oxazoline; FAME, fatty acid methyl ester; GC–FTIR, gas–liquid chromatography coupled with Fourier transform infrared spectroscopy; GC–MS, gas–liquid chromatography coupled with mass spectrometry; GLC, gas–liquid chromatography; HPLC, high-performance liquid chromatography.

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a magnetic stirrer (31) and immersed in a water bath at 40°C. A 95% hydrazine solution (1 mL) was added, and oxygen was passed over the stirred solution for 3 h. After addition of water, the fatty acids were extracted with diethyl ether and then converted to methyl esters with BF_3-MeOH at 95°C for 5 min (32).

Hydrazine reduction of the isolated 20:4 fraction was carried out on 12 mg of sample for 1 h at 40°C.

GLC. All analytical GLC analyses were carried out using a Hewlett-Packard (Palo Alto, CA) 5890 series II chromatograph fitted with a split/splitless injector and a flame-ionization detector. The column used was a CP-Sil 88 (100 m × 0.25 mm i.d., 0.2 μm film thickness; Chrompack, Middelburg, The Netherlands). It was programmed from 60 to 180°C at 20°C · min^{-1} and held at this temperature until completion of the analysis. The isolated 20:1 isomers and the resulting silver nitrate thin-layer chromatography ($AgNO_3$-TLC) fractions were analyzed on a DB-Wax column (J&W, Courtaboeuf, France; 30 m length, 0.25 mm i.d., and 0.2 μm film thickness), which was programmed from 60 to 180°C at 20°C · min^{-1}.

GC–MS. FAME were converted to dimethyl-oxazoline derivatives (DMOX) according to Fay and Richli (33) prior to GC–MS analysis. GC–MS analyses were carried out using a BPX column, as described previously, and a Hewlett-Packard 5970 mass spectrometer. The temperature was programmed from 60 to 170°C at a rate of 20°C · min^{-1} (30).

GLC–Fourier transform infrared spectroscopy (GC–FTIR). Samples (methyl esters) were analyzed by gas chromatography (HP 5890 gas chromatograph; a BPX70 column, 30 m × 0.25 mm i.d., film thickness 0.25 μm, SGE, Melbourne, Australia). The instrument was fitted with a splitless injector maintained at 250°C and coupled with a Fourier transform infrared spectrometer (FTS 60A, Bio-Rad, Cambridge, MA). The two instruments were connected by a Digilab Tracer® direct-deposition interface. The oven temperature was programmed from 60 to 200°C (20°C/min).

RESULTS AND DISCUSSION

Fatty acid composition of the CLA fraction. Based on equivalent chain length, the elution sequence of the geometric *cis/trans*, *cis/cis,* and *trans/trans* CLA isomers was determined by Scholfield and Dutton (34) and Scholfield (35) and verified by Ha *et al.* (11) and Sébédio *et al.* (17).

GC–MS of the DMOX derivatives, as described previously for identification of CLA isomers in cheese and in animal tissues (18,30,36,37) and in commercial mixture (38), permitted us to show that the two major mono-*trans* isomers of the CLA mixture were 9,11- and the 10,12-18:2 and two minor mono-*trans* isomers were 8,10- and 11,13-18:2. The first-eluting peak contained mainly the 9,11 isomer (37.4% of the total CLA) accompanied by smaller quantities of the 8,10 isomer (7.6% of the total) (Fig. 1A), the second peak was composed of the 11,13 isomer (13.6% of the total), and the third peak was the Δ10,12 isomers (33.5% of the total). All these compounds were the mono-*trans* isomers. The di-*trans* isomers, namely, the 8,10/9,11/10,12 (4.3% of the total) and 11,13 (0.8% of the total),

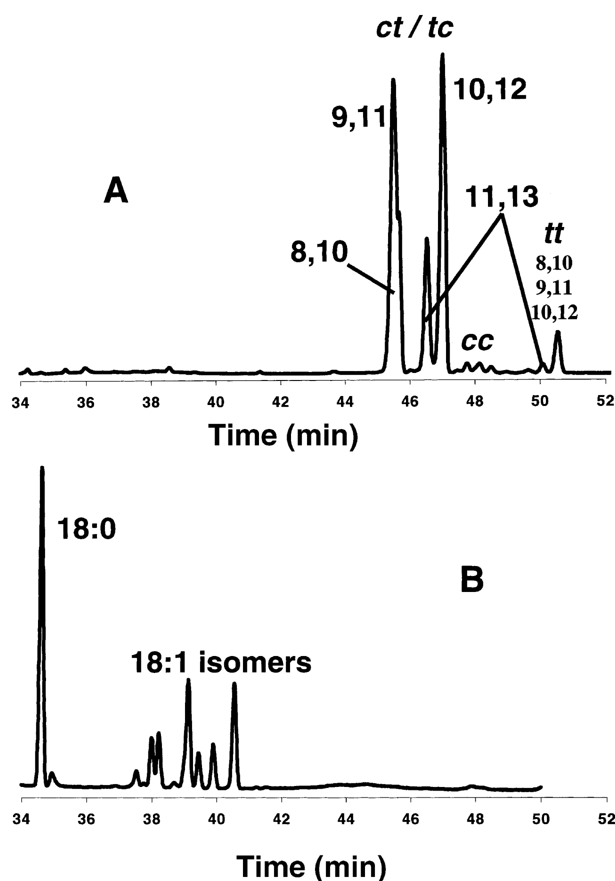


FIG. 1. Gas–liquid chromatographic analysis of (A) commercial conjugated linoleic acid (CLA) and (B) the commercial CLA mixture after hydrazine reduction. *c, cis; t, trans.*

were the two last eluting peaks. The minor peaks eluting between the di-*trans* and the mono-*trans* isomers contained the all-*cis* 8,10/9,11/10,12, and 11,13 isomers, representing 0.2, 0.9, 1.1, and 0.6% of the total, respectively, as previously described (38).

As GC–MS of the DMOX derivatives gave information only on the position of the double bonds and not on the geometry, the CLA mixture (Fig. 1A) was then submitted to hydrazine reduction. This method consists of partial reduction of the dienoic acid to give a mixture of saturates, monoenes, and unreacted dienes. Hydrazine reduction takes place without modification of either the position or the geometry of the ethylenic bonds, so the position(s) and geometry of the ethylenic bonds in the resulting monoenes represent those of the parent molecule (31). The resulting mixture (Fig. 1B) was submitted to high-performance liquid chromatography (HPLC) on a C18 reversed-phase column and the isolated monoene fraction submitted to $AgNO_3$–TLC. Three bands were obtained (Fig. 2). Each band was submitted to GC–FTIR, and aliquots of each fraction were also submitted to GC–MS after transformation into DMOX derivatives. Examination of GC–FTIR spectra revealed that all the 18:1 isomers present in the band of R_f 0.67 showed the characteristic absorption bands around 720 cm^{-1} and at 3,001 cm^{-1} of a

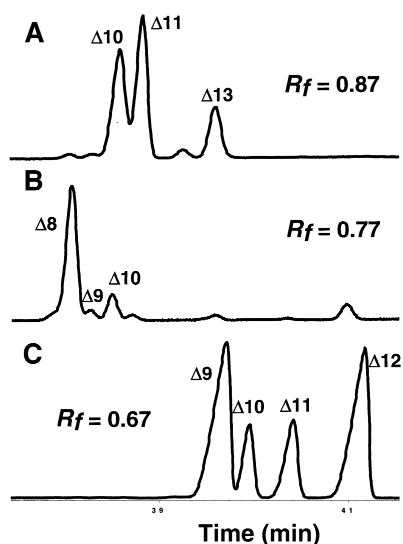


FIG. 2. Gas–liquid chromatographic analyses of the *cis* and *trans* 18:1 isomers isolated by a combination of high-performance liquid chromatography (HPLC) and silver nitrate thin-layer chromatography ($AgNO_3$-TLC) from commercial CLA which was submitted to a hydrazine reduction. For other abbreviation see Figure 1.

cis ethylenic bond, whereas the major isomers present in bands of R_f = 0.77 and 0.87 (Fig. 2) showed an absorption band at 968 cm^{-1} (Fig. 3), characteristic of a *trans* ethylenic bond (39).

The major MS fragments of the monoenes are reported in Table 1. As an example, for the Δ9 isomer in the R_f = 0.67 band, a mass interval of 12 units instead of the usual 14 units for a saturated chain occurred between *m/z* 196 (C8) and 208 (C9), indicating a double bond in the Δ9 position in the parent molecule. Examination of the other spectra of the R_f = 0.67 band permitted us to show that 18:1Δ9 and 18:1Δ12 were the major *cis* monoethylenic isomers. These were also accompanied by smaller amounts of the Δ10 and Δ11 *cis* isomers.

The two major *trans* isomers (band of R_f = 0.87) are the 18:1Δ10 and the 18:1Δ11. These are accompanied by a smaller amount of the Δ13 *trans* isomer. The major *trans* isomer contained in the band R_f = 0.77 is the 18:1Δ8. These data therefore indicate that the 9*c*,11*t*- and the 10*t*,12*c*-18:2 are the major CLA. These are also accompanied by smaller amounts of 8*t*,10*c*- and 11*c*,13*t*-18:2 isomers. The 8*t*,10*c*-18:2 is tailing the 9*c*,11*t* peak while the 11*c*,13*t* isomer is eluted ahead of the 10*t*,12*c*-18:2 isomer, as indicated by GC–MS of the DMOX derivatives (30,38). Examination of the other minor 18:1 isomers did not permit identification of the minor CLA peaks.

Identification of the 20:4 conjugated isomers. The combination of methods used to isolate and identify the 20:4 conjugated isomers is diagrammed in Scheme 1. Total liver lipids contained only 0.2% of conjugated 20:4 isomers (Table 2), identified previously (30) as 5,8,12,14- and 5,8,11,13-20:4. It was necessary to carry out one HPLC and two successive $AgNO_3$–TLC fractionations in order to isolate a fraction that was sufficiently enriched in these fatty acids. Along with the usual fatty acids, the total liver lipid FAME also contained the CLA given in the diet (0.6%) and one 20:3 conjugated isomer (0.1%), 8,12,14-20:3 (30). Arachidonic acid (20:4n-6) was also accompanied by small quantities of two mono-*trans* isomers, 5*c*,8*c*,11*c*,14*t*-20:4 and 5*c*,8*c*,11*t*,14*c*-20:4. These are known metabolites of 9*c*,12*t*-

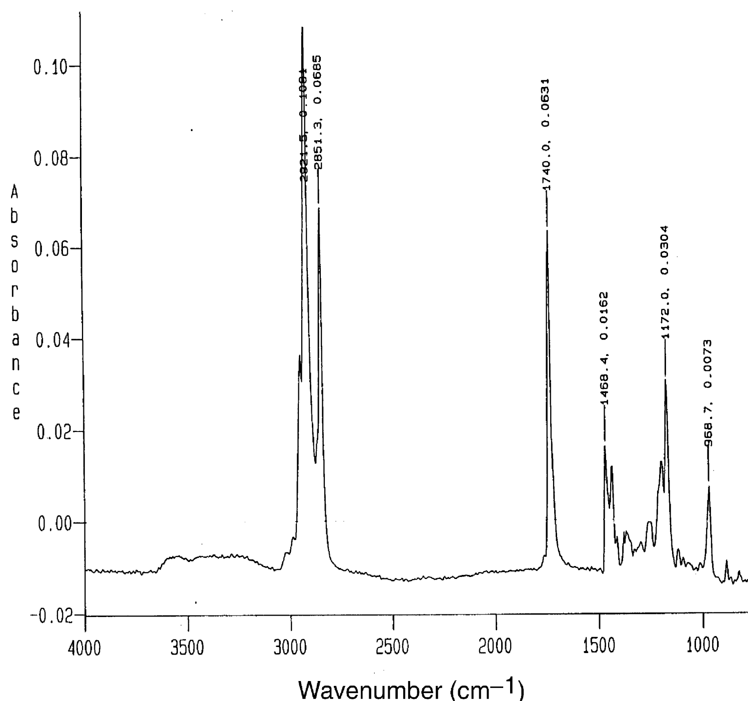


FIG. 3. Infrared spectrum of 18:1 Δ8 *trans* (R_f 0.77, Fig. 2) obtained after a combination of HPLC and $AgNO_3$-TLC from commercial CLA which was submitted to a hydrazine reduction. See Figures 1 and 2 for abbreviations.

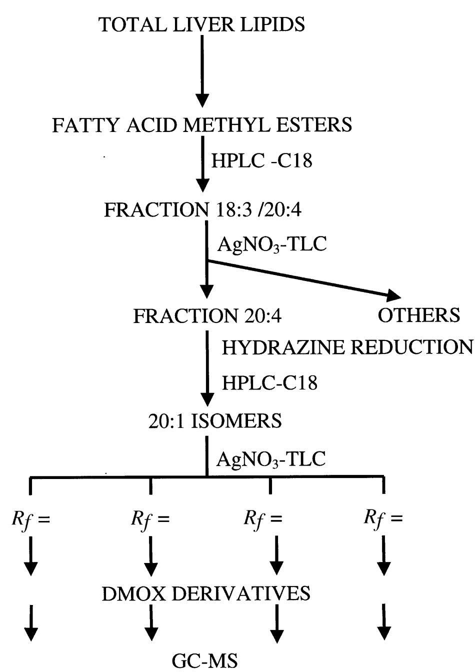
TABLE 1
Characteristic Ions in Mass Spectra of DMOX Derivatives of the Monoenes Obtained After Hydrazine Reduction of the CLA Mixture[a]

$AgNO_3$-TLC band (R_f)	Component	M^+ *m/z* (intensity %)	Fragments *m/z* (intensity %)
0.67	Δ9	335 (5)	113 (100); 126 (97); 182 (19); 196 (4); 208 (4); 222 (7); 236 (15); 250 (13)
	Δ10	335 (5)	113 (100); 126 (78); 196 (11); 210 (3); 222 (3); 236 (4); 250 (13); 264 (13)
	Δ11	335 (5)	113 (100); 126 (77); 210 (10); 224 (3); 236 (3); 250 (4); 264 (13); 278 (15)
	Δ12	335 (7)	113 (100); 126 (83); 224 (11); 238 (2); 250 (2); 264 (3); 278 (14); 292 (15)
0.77	Δ8	335 (4)	113 (54); 126 (100); 168 (24); 182 (7); 194 (5); 208 (7); 222 (20); 236 (9)
0.87	Δ10	335 (5)	113 (1`00); 236 (84); 196 (12); 210 (4); 222 (3); 236 (3); 250 917); 264 (16)
	Δ11	335 (7)	113 (100); 126 (84); 210 (9); 224 (3); 236 (3); 250 (5); 264 (19); 278 (19)
	Δ13	335 (9)	113 (100); 126 (91); 238 (8); 252 (2); 264 (2); 278 (3); 292 (16); 306 (16)

[a]DMOX, 4,4-dimethyloxazoline; CLA, conjugated linoleic acid; $AgNO_3$-TLC, silver nitrate thin-layer chromatography.

and 9*t*,12*c*-18:2 (27,28) which were present as minor components in the diet given to the rats (Juanéda, P., personal communication).

HPLC fractionation allowed isolation of a fraction enriched in 20:4 fatty acid isomers, the conjugated 20:4 isomers representing 3% of the total FAME. Two successive $AgNO_3$-TLC fractionations separated the conjugated 20:4 isomer from arachidonic acid. However, as the conjugated isomer migrated above 20:4n-6, this fraction also contained a large quantity of the 18:3 isomers (62%) (Fig. 4A).



SCHEME 1

Next, the isolated $AgNO_3$-TLC fraction was submitted to hydrazine reduction, yielding a complex mixture of 18:0, 18:1, 18:2, and unreacted 18:3 isomers accompanied by 20:0, 20:1, 20:2, 20:3, and unreacted conjugated 20:4 (Fig. 4B). The 20:1 monoenes, which represented 50% of the total C_{20} fatty acids, were isolated by HPLC and further submitted to $AgNO_3$-TLC. Four bands were obtained. The band of R_f 0.37 comprised two isomers, which were resolved as DMOX derivatives (Fig. 5); the bands at R_f 0.43 and R_f 0.50 constituted one major isomer each, while the band of R_f 0.60 revealed the presence of two 20:1 isomers.

The major MS fragments of the 20:1 monoenoic isomers are reported in Table 3. Sometimes the utilization of DMOX derivatives permits one to separate isomers that cannot always be resolved as methyl esters. This is the case for the two monoenes present in the band of $R_f = 0.37$. As previously reported, a gap of 12 mass units between ions containing n−1 and n carbon atoms indicates a double bond between carbons n and $n + 1$.

TABLE 2
Fatty Acid Composition (wt%) of the Total Liver Lipids or the HPLC and the $AgNO_3$-TLC Fractions[a]

	Total liver lipids	HPLC fraction	$AgNO_3$-TLC fraction
Σ Saturates	43.3		
Σ Monoenes	36.8		
Σ 18:2	4.6		
Σ CLA	0.6		
Σ 18:3	0.3	5.4	62.1
Σ 20.3	4.6		
Σ 20:3 conj.	0.1		
Σ 20:4	5.8	80.3	2.5
Σ 20:4 conj.	0.2	3.0	35.4
Σ 22:5	2.0	8.7	
Σ 22:6	1.7		

[a]HPLC, high-performance liquid chromatography; for other abbreviations see Table 1.

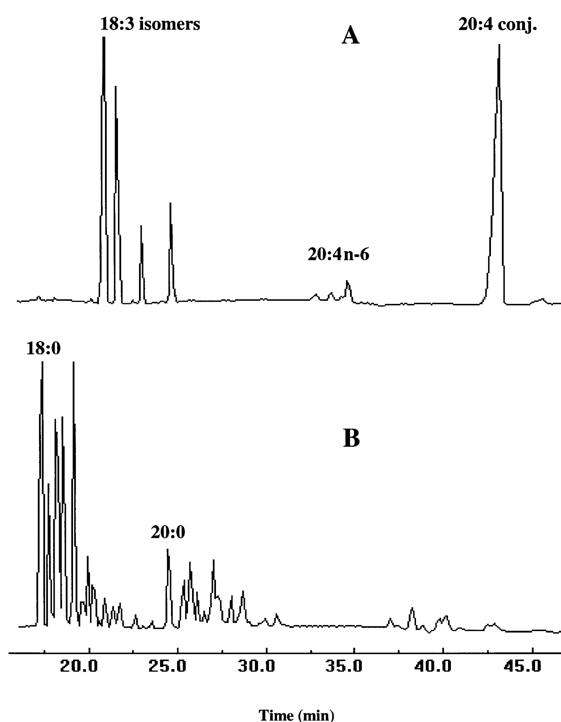


FIG. 4. Gas–liquid chromatographic analyses of the 18:3/20:4 fraction isolated by a combination of HPLC and $AgNO_3$-TLC from liver lipids before hydrazine reduction (A) and after hydrazine reduction (B). For abbreviations see Figure 2.

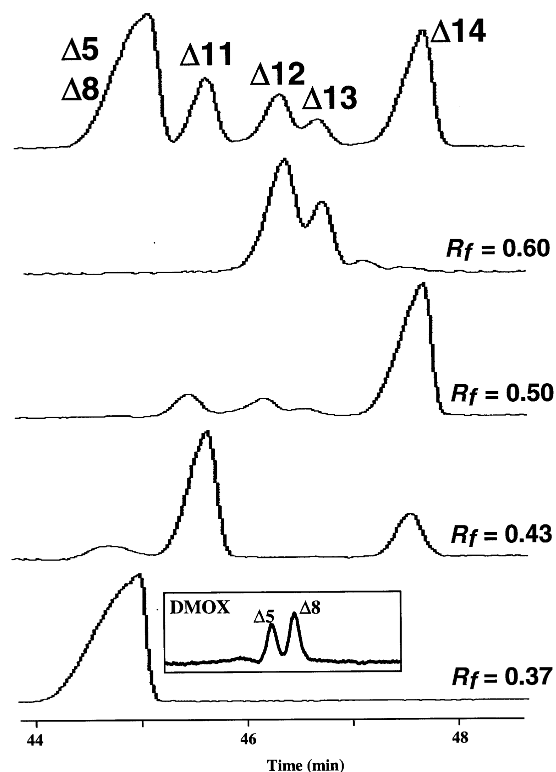


FIG. 5. Gas–liquid chromatographic analyses of the *cis* and *trans* 20:1 isomers isolated by a combination of HPLC and $AgNO_3$-TLC from the 18:3/20:4 fraction that was submitted to a hydrazine reduction (Fig. 4B). The band at $R_f = 0.37$ compressed two isomers, which were resolved as dimethyl-oxazoline (DMOX) derivatives.

Close examination of the spectra (Table 3) indicated that the band of $R_f = 0.37$ contained the Δ5 and Δ8 isomers; the band of $R_f = 0.43$, the Δ11 isomer and some of the Δ14 isomer present in band of $R_f = 0.50$; while the band with the highest R_f value contained the Δ12 and Δ13 isomers.

Unfortunately, considering that most of the monoenoic fraction available was used to prepare the DMOX derivatives for the GC–MS studies, FTIR spectra with bad signal-to-noise ratios were obtained. However, the position of the different monoenoic isomers on the $AgNO_3$-TLC plate is informative enough to enable the identification of both 20:4 conjugated isomers. First, the position of the band of $R_f = 0.60$ compared to a standard of elaidic acid indicates that both ethylenic bonds in Δ12 and in Δ13 are *trans*. Studies by Morris *et al.* (40) have shown that except for ethylenic bonds which are close to the carboxyl group (Δ2 position), *trans* isomers migrate ahead of all the *cis* isomers. Furthermore, for both the *cis* and the *trans* isomers (except for ethylenic bonds close to the carboxyl end),

TABLE 3
Characteristic Ions in Mass Spectra of DMOX Derivatives of the Monoenes Obtained After Hydrazine Reduction of the 20:4 Conjugated Isomers[a]

$AgNO_3$-TLC band (R_f)	Component	M^+ *m/z* (intensity %)	Fragments *m/z* (intensity %)
0.37	Δ5	363 (3)	113 (100); 126 (12); 153 (9); 166 (4); 180 (9)
	Δ8	363 (14)	113 (76); 126 (100); 168 (26); 182 (6); 194 (3); 208 (8); 222 (31); 236 (12)
0.43	Δ11	363 (15)	113 (100); 126 (59); 210 (10); 224 (4); 236 (3); 250 (4); 264 (42); 278 (48)
0.50	Δ14	363 (18)	113 (100); 126 (54); 252 (12); 266 (3); 278 (5); 292 (8); 306 (45); 320 (47)
0.60	Δ12	363 (21)	113 (100); 126 (60); 224 (30); 238 (3); 250 (4); 264 (8); 278 (32); 292 (51)
	Δ13	363 (12)	113 (100); 126 (61); 238 (6); 252 (4); 264 (3); 278 (10); 292 (33); 306 (30)

[a]For abbreviations see Table 1.

the R_f increases with increasing Δ number (40). This is observed for bands of $R_f = 0.37$ (Δ5, Δ8), $R_f = 0.43$ (Δ11), and $R_f = 0.50$ (Δ14). The band of $R_f = 0.60$, which includes the Δ12 and the Δ13 isomers, can therefore only be of *trans* geometry.

We have previously demonstrated that the 20:4 isomers are 5,8,11,13-20:4 and 5,8,12,14-20:4. Results of the hydrazine reduction show that these compounds are the 5*c*,8*c*,11*c*,13*t*- and 5*c*,8*c*,12*t*,14*c*-20:4 isomers. These fatty acids may be formed by desaturation and elongation of 9*c*,11*t*- and the 10*t*,12*c*-18:2, the major isomers present in the CLA mixture. The identification of the 8*t*,10*c* isomer using hydrazine reduction confirmed the GC elution order proposed by Kramer *et al.* (37). Similar conversions for the cis/trans isomers of linoleic and linolenic acids have already been proposed (26–28,41). Further *in vivo* experiments will now be carried out to study if CLA modifies the bioavailability of arachidonic acid and therefore may affect eicosanoid synthesis.

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Atorvastatin and Simvastatin Have Distinct Effects on Hydroxy Methylglutaryl-CoA Reductase Activity and mRNA Abundance in the Guinea Pig

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ABSTRACT: The effects of atorvastatin and simvastatin on hydroxy methylglutaryl (HMG)-CoA reductase activity and mRNA abundance were studied in guinea pigs randomized to three groups: untreated animals and those treated with 20 mg/kg of atorvastatin or simvastatin. Guinea pigs were fasted for 0, 6, 12, or 18 h in an attempt to remove the drug from their systems. Reductase activity and mRNA levels were analyzed after each time point. Reductase inhibitor treatment resulted in 50–62% lower cholesterol concentrations compared to untreated guinea pigs ($P < 0.0001$), while plasma triacylglycerol (TAG) concentrations did not differ among groups. Plasma cholesterol and TAG were 50–70% lower after 18 h fasting in the three groups ($P < 0.001$). In the nonfasting state, simvastatin and atorvastatin treatment did not affect HMG-CoA reductase activity compared with untreated animals. However, after 6 h of fasting, simvastatin-treated guinea pigs had higher HMG-CoA reductase activity than untreated animals ($P < 0.01$), suggesting that the drug had been removed from the enzyme. In contrast, atorvastatin-treated guinea pigs maintained low enzyme activity even after 18 h of fasting. Further, HMG-CoA reductase mRNA abundance was increased by sevenfold after atorvastatin treatment and by twofold after simvastatin treatment ($P<0.01$). These results suggest that simvastatin and atorvastatin have different half-lives, which may affect HMG-CoA reductase mRNA levels. The increase in reductase activity by simvastatin during fasting could be related to an effect of this statin in stabilizing the enzyme. In contrast, atorvastatin, possibly due to its longer half-life, prolonged inhibition of HMG-CoA reductase activity and resulted in a greater increase in mRNA synthesis.

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Hypercholesterolemia and hypertriglyceridemia are considered independent risk factors for the development of atherosclerosis and coronary heart disease (1). Because of the prevalence of hyperlipidemia in Western societies, different approaches are being used to reduce plasma low density lipoprotein (LDL) cholesterol and triacylglycerol (TAG) concentrations. Hydroxy methylglutaryl (HMG)-CoA reductase inhibitors are pharmacological agents often used in the treatment of hypercholesterolemia. Although they are quite effective in reducing plasma cholesterol levels, their effects on plasma TAG are less conclusive (2).

Atorvastatin, the newest of the statin class, is quite effective in decreasing both plasma LDL cholesterol and TAG, as well as increasing high density lipoprotein cholesterol in humans (3–5). Studies of the mechanism of action of atorvastatin in different animal models have demonstrated that atorvastatin reduces LDL cholesterol by two different means: decreased secretion of very low density lipoprotein particles from the liver (6–8) and increased LDL uptake (9,10). In addition, atorvastatin efficacy has been related to the prolonged inhibition of endogenous cholesterol synthesis as documented in cells, rats, and humans (11,12).

Studies in rats, mice, and cultured cells have demonstrated that statin treatment results in an up-regulation of HMG-CoA reductase activity and mRNA levels (11,13,14) as a compensatory mechanism to maintain cholesterol homeostasis. However, studies conducted on guinea pigs by our laboratory have indicated that atorvastatin and simvastatin treatment resulted in no effect or in lower HMG-CoA reductase activity compared to animals without treatment (8,9). These results suggest that in guinea pigs, atorvastatin and simvastatin may still be bound to the enzyme after isolation of hepatic microsomes, thus inhibiting activity. The present studies were conducted to further explore the effects of statins on HMG-CoA reductase activity and to determine whether HMG-CoA reductase gene expression was affected by statin treatment in guinea pigs. The guinea pig was used as an animal model because of its similarities in cholesterol metabolism to humans. In addition, guinea pigs have been proven to respond to drug treatment by decreasing the levels of LDL cholesterol as humans do. According to existing knowledge about statins and HMG-CoA reductase activity, we hypothesized that after removal of the drug, the activity of the enzyme would be increased and that the regulation of the enzyme by drug treatment would be in part at the level of gene expression.

MATERIALS AND METHODS

Materials. Glucose-6-phosphate, glucose-6 phosphate dehydrogenase, and NADP were obtained from Sigma Chemical

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Abbreviations: DEPC, diethylpyrocarbonate; HMG, hydorxy methylglutaryl; LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; TAG, triacylglycerol.

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Co. (St. Louis, MO). DL-Hydroxy-[3-^{14}C]methyl glutaryl CoA (1.81 GBq/mmol), DL-[5-^{3}H]mevalonic acid (370 Gbq/mmol), [^{32}P]-dCTP (18.5 GBq/mmol), and aquasol were obtained from Amersham (Clearbrook, IL). Simvastatin and atorvastatin were provided by Parke-Davis Research, Warner-Lambert Division (Ann Arbor, MI).

Diets. Isocaloric diets were prepared and pelleted by Research Diets Inc. (New Brunswick, NJ). Diets were designed to meet the nutritional requirements of the guinea pigs. The concentration of HMG-CoA reductase inhibitors in the diet was 0.1% or 20 mg/kg per day. The amount of cholesterol in the diet was adjusted to 0.04%, which is equivalent to 112 mg/1,000 kcal or less than 300 mg/day for a human diet (15). The fat mix was olive oil/palm kernel oil/safflower oil (1:2:1.8), which is rich in lauric and myristic acids, known to cause endogenous hypercholesterolemia in guinea pigs (8). Detailed composition of the diet has been published elsewhere (8).

Animals. Male Hartley guinea pigs weighing 350–400 g were purchased from Harlan Sprague Dawley Inc. (Indianapolis, IN). Sixty-three guinea pigs were randomly assigned to one of the three different diets—control, 20 mg/kg simvastatin or atorvastatin for 21 d. Guinea pigs were kept in a light-cycle room (light from 0700 to 1900) and had free access to water and foods/statin. At the end of the feeding period animals were subdivided into four different groups with nine guinea pigs in the nonfasting group and four for each fasting period, which was either 6, 12, or 18 h. All animal experiments were conducted in accordance with U.S. Public Health Service/U.S. Department of Agriculture guidelines. Experimental protocols were approved by the University of Connecticut Institutional Animal Care and Use Committee.

After the different fasting periods, guinea pigs were anesthetized under halothane vapors and blood was obtained by heart puncture. Plasma samples were collected and a preservation cocktail was added (aprotonin 0.5 mL/100 mL, phenylmethyl sulfony fluoride 0.1 mL/100 mL, and sodium azide 0.1 mL/100 mL). Plasma was analyzed for cholesterol (16) and TAG (17) using enzymatic methods. Livers were excised from guinea pigs after exsanguination and were used for microsome isolation and for RNA extraction.

Hepatic microsome isolation. Microsomes were isolated as previously described (8,9). Briefly, livers were pressed through a tissue grinder, placed in cold buffer (50 mmol/L KH_2PO_4, 0.1 mol/L sucrose, 50 mmol/L KCl, and 50 mmol/L NaCl, 30 mmol/L EDTA, and 2 mmol/L dithiothreitol, pH 7.2), and homogenized with a Potter-Elvejhem homogenizer for measurement of total activity. In addition, microsomes were isolated in the presence of 50 mmol/L NaF to inhibit endogenous phosphatases to determine HMG-CoA reductase expressed activity (18). The microsomal fraction was obtained after two centrifugations at $10,000 \times g$ for 15 min (JA-20 rotor in a J2-21 centrifuge; Beckman Instruments, Fullerton, CA), and 1-h centrifugation at $100,000 \times g$ at 4°C. Samples were further homogenized and centrifuged for an additional hour at $100,000 \times g$ at 4°C. Microsomal pellets were resuspended in cold buffer, homogenized, and stored at −70°C for enzyme analysis. The protein content in the microsomes was determined according to Markwell *et al.* (19).

3-Hydroxy-3-methylglutaryl-CoA reductase (HMG-CoA reductase) assay. The activity of HMG-CoA reductase (EC 1.1.1.34) was measured in hepatic microsomes according to Shapiro *et al.* (20). Microsomal protein (200 µg, approximately 10 µL) was incubated at 37°C with 50 µL of a solution containing 7.5 nmol (0.33 Gbq/nmol) [3-^{14}C]HMG-CoA, 4.5 mmol glucose-6-phosphate, 3.6 mmol EDTA, 0.45 mmol NADP, and 0.3 IU glucose-6-phosphate dehydrogenase. [^{3}H]Mevalonic acid (0.024 Gbq) was added as a recovery standard.

The reaction was stopped after 15 min with 10 M HCl (0.025 mL per tube). An excess of mevalonic acid was added, and samples were incubated for another 30 min at 37°C to allow for the conversion of mevalonic acid to mevalonalactone. After incubation, microsomes were pelleted by centrifugation for 1 min at $1,000 \times g$. An aliquot of the supernatant (0.1 mL) was applied to silica gel thin-layer chromatography plates and developed with acetone/benzene (1:1 vol/vol), and the area containing the mevalonate (R_f = 0.6–0.9) was scraped and mixed with 5 mL aquasol. Radioactivity was measured using a liquid scintillation counter. HMG-CoA activity is expressed as pmol of [^{14}C]mevalonate produced per min per mg microsomal protein. Recoveries of [^{3}H]mevalonate were 60–70%.

RNA extraction. Total RNA was extracted using a modification of the method described by Chomczynski and Sacchi (21). Briefly, 0.5 g of liver tissue was homogenized in 5 mL of guanidinium thiocyanate buffer [4 M guanidinium thiocyanate, 25 mM Na citrate (pH = 7.0), 0.5% *N*-lauroylsarcosine]. Homogenates were extracted with acid phenol and chloroform followed by a centrifugation to separate the phases. The aqueous phase containing the RNA was removed and precipitated with 5 mL of ethanol at −20°C overnight. Samples were then centrifuged at $3,500 \times g$ for 20 min and pellets were resuspended in 1 mL diethylpyrocarbonate (DEPC) water and then extracted with an equal volume of chloroform/isoamyl alcohol (49:1 vol/vol). After centrifugation, the aqueous phase was transferred to a microfuge tube and reprecipitated with 1 mL of ethanol for 2 h at −20°C. RNA was dissolved in DEPC water and concentration was determined by absorbance at 260 nm. The ratios of absorbance at 260 and 280 nm were in the range of 2.0–2.2.

Northern analysis. Total RNA was size-separated by electrophoresis in a 1% agarose gel with formaldehyde as reported by Yeh *et al.* (22). RNA was then transferred overnight to a nitrocellulose membrane (0.45 mm; MSI NitroPure, Westborough, MA) by the Northern procedure, using 20× standard saline citrate (SSC) buffer (1 × SSC = 150 mM NaCl, 15 mM Na citrate). After transfer was completed, RNA was fixed to the membrane by exposure to ultraviolet light for 30 s and baking for 2 h at 80°C.

The plasmid containing the cDNA insert for human HMG-CoA reductase was obtained from American Type Culture Collection (Rockville, MD). This plasmid contains regions homologous to the hamster HMG-CoA reductase gene (23) and thus appeared likely to hybridize with guinea pig mRNA.

Isolated insert was labeled with [^{32}P]-dCTP using the random primer method (Life Technologies, Rockville, MD). Unincorporated radioactivity was removed using Sephadex spin columns (TE MIDI select-D, G-50, 5 prime-3 prime, Boulder, CO).

Membranes were prehybridized for 2 h at 42°C in a solution containing 10% dextran sulfate, 50% formamide, 1 M NaCl, 1% sodium dodecyl sulfate (SDS), and 200 mg/mL of denatured salmon testis DNA. Labeled probe was added (2×10^6 PM/mL) and hybridization was continued at 42°C for 20 h. Membranes were washed twice in 50 mL of 0.25 M NaH_2PO_4, 2% SDS, and 1mM EDTA for 20 min at 42°C. An additional wash was performed with 50 mL of 0.05 M NaH_2PO_4, 1% SDS, and 1mM EDTA for 20 min at 55°C. Membranes were exposed to X-ray film for an average of 5 d. Autoradiographic signals were quantified with a GS-670 densitometer using Molecular Analyst Software (Bio-Rad, Hercules, CA). HMG-CoA reductase mRNA levels were corrected for 18S mRNA measured in the same blot.

Statistical analysis. Data were analyzed by two-way analysis of variance to test differences due to fasting, drug effects, and interactions. P values less than 0.05 were considered statistically significant. Fisher's least significant difference protected was used for *post-hoc* analysis. Data are presented as means ± standard deviation.

RESULTS

Atorvastatin vs. *simvastatin effects on plasma lipids.* Atorvastatin and simvastatin treatment resulted in significant reductions in plasma total cholesterol at the different fasting times (Table 1). In the nonfasting state, guinea pigs treated with statins had 51–53% lower plasma cholesterol concentrations compared to animals with no treatment ($P < 0.0001$). Fasting had different effects depending on the drug treatment. For untreated animals, plasma cholesterol was 31–35% lower after 18 h of fasting compared to the earlier times ($P < 0.001$). Guinea pigs treated with atorvastatin had significantly lower plasma cholesterol concentrations after 12 and 18 h of fasting compared to nonfasting guinea pigs and 6-h-fasted animals ($P < 0.001$) (Table 1). However, cholesterol levels for simvastatin-treated animals remained the same during the different fasting times (Table 1).

Similar to our previous report (9), no changes due to drug treatment were found for plasma TAG. However, fasting decreased plasma TAG such that after 18 h, levels were reduced by 68, 55, and 43% in control, atorvastatin-, and simvastatin-treated guinea pigs, respectively (data not shown).

Atorvastatin vs. simvastatin effects on HMG-CoA reductase activity. Total HMG-CoA reductase activity was altered after treatment with statins and by the different fasting times (Table 2). In addition, there was an interactive effect between drug treatment and fasting ($P < 0.05$). In the nonfasted state (0 h), atorvastatin caused a significant decrease in HMG-CoA reductase activity, while simvastatin treatment had no effect, similar to our reports for atorvastatin in the guinea pig. It is known that fasting decreases dramatically the activity of HMG-CoA reductase (24), which explains the 88% reduction in activity after 6 h of fasting in the control group. Fasting decreased HMG-CoA reductase activity in atorvastatin-treated animals to a similar extent as in nontreated guinea pigs. However, in simvastatin-treated guinea pigs, fasting did not decrease reductase activity to the same extent ($P < 0.05$) (Table 2).

In addition to total activity, expressed HMG-CoA reductase activity was determined in microsomes isolated in the presence of NaF. Expressed activity was not affected by statin treatment but was reduced after 6, 12, and 18 h of fasting (Table 3). HMG-CoA reductase expressed activity was reduced by 20, 36, and 36% after 6, 12, and 18 h of fasting, respectively (Table 3). The ratio of expressed to total activity was higher after statin treatment and this was due mostly to a net decrease in total enzyme activity. Fasting resulted in decreased expressed activity and increased ratios. This suggests that during fasting most of the enzyme present is in the expressed form.

TABLE 1
Plasma Cholesterol (mmol/L) of Guinea Pigs Not Treated (control) or Treated with 20 mg/kg Atorvastatin or Simvastatin After 0, 6, 12, or 18 h Fasting

	Fasting times (h)[a]			
Treatment	0	6	12	18
Control	3.98 ± 0.81	3.84 ± 0.62	3.80 ± 0.58	2.60 ± 0.74
Atorvastatin	1.94 ± 0.51	2.02 ± 0.51	1.20 ± 0.43	0.74 ± 0.23
Simvastatin	1.79 ± 0.51	1.71 ± 0.62	1.59 ± 0.23	1.55 ± 0.35
	Two-way ANOVA			
Statin effect[b]	$P < 0.0001$			
Fasting effect[c]	$P < 0.001$			
Interaction	NS			

[a]Data are presented as mean ± SD; n = 9 guinea pigs for nonfasting and 4 guinea pigs for 6, 12, and 18 h fasting. NS, not significant; ANOVA, analysis of variance.
[b]Simvastatin = atorvastatin < control.
[c]0 h = 6 h > 12 h > 18 h.

TABLE 2
Hydroxy Methylglutaryl (HMG)-CoA Reductase Activity (pmol/min-mg) of Guinea Pigs Not Treated (control) or Treated with 20 mg/kg Atorvastatin or Simvastatin After 0, 6, 12, or 18 h Fasting

	Fasting times (h)[a]			
Treatment	0	6	12	18
Control	7.2 ± 2.5	2.8 ± 0.8	1.3 ± 0.3	2.0 ± 0.9
Atorvastatin	3.6 ± 0.4	1.5 ± 0.3	1.2 ± 0.2	1.2 ± 0.4
Simvastatin	6.1 ± 2.8	5.4 ± 1.4	3.3 ± 0.7	3.6 ± 1.1
	Two-way ANOVA			
Statin effect[b]	$P < 0.001$			
Fasting effect[c]	$P < 0.0001$			
Interaction	$P < 0.05$			

[a]Data are presented as mean ± SD; n = 9 guinea pigs for nonfasting and 4 guinea pigs for 6, 12, and 18 h fasting. To test differences among means, Fisher's least significant difference (LSD) protected test was used as *post-hoc* analysis. See Table 1 for other abbreviation.
[b]Simvastatin, 0 h = atorvastatin 0 h < control 0 h.

TABLE 3
Expressed HMG-CoA Reductase Activity (pmol/min-mg) of Guinea Pigs Not Treated (control), or Treated with 20 mg/kg Atorvastatin or Simvastatin After 0, 6, 12, or 18 h Fasting

	Fasting times (h)[a]			
Treatment	0	6	12	18
Control	2.5 ± 0.2	2.0 ± 0.9	1.6 ± 0.3	1.6 ± 0.3
Atorvastatin	2.2 ± 0.3	1.8 ± 0.1	1.4 ± 0.3	1.1 ± 0.1
Simvastatin	2.3 ± 1.0	1.4 ± 0.3	1.6 ± 0.4	1.6 ± 0.4
	Two-way ANOVA			
Statin effect	NS			
Fasting effect[b]	$P < 0.001$			
Interaction	NS			

[a]Data are presented as mean ± SD; *n* = 9 guinea pigs for nonfasting and 4 guinea pigs for 6, 12, and 18 h fasting. To test differences among means, Fisher's LSD protected test was used as *post-hoc* analysis. See Tables 1 and 2 for abbreviations.
[b]0 h > 6 h > 12 h > 18 h.

TABLE 4
HMG-CoA Reductase mRNA Abundance (relative units) of Guinea Pigs Not Treated (control) or Treated with 20 mg/kg Atorvastatin or Simvastatin after 0, 6, 12, or 18 h Fasting

	Fasting times (h)[a]			
Treatment	0	6	12	18
Control	57 ± 28	135 ± 75	66 ± 6	76 ± 16
Atorvastatin	385 ± 146	160 ± 78	107 ± 20	120 ± 31
Simvastatin	119 ± 41	107 ± 47	89 ± 19	73 ± 28
	Two-way ANOVA			
Statin effect[b]	$P < 0.001$			
Fasting effect[c]	$P < 0.01$			
Interaction	$P < 0.01$			

[a]Data are presented as mean ± SD; *n* = 9 guinea pigs for nonfasting and 4 guinea pigs for 6, 12, and 18 h fasting. To test differences among means, Fisher's LSD protected test was used as *post-hoc* analysis. See Tables 1 and 2 for abbreviations.
[b]Control< simvastatin < astorvastatin.
[c]Fasting: 0 h > 6 h > 12 h = 18 h.

Atorvastatin vs. simvastatin effects on HMG-CoA reductase mRNA. The human cDNA probe used for measuring hepatic mRNA abundance for HMG-CoA reductase recognized two mRNA species 4.5 and 3.2 kb in size (Fig. 1). As indicated in Table 4, atorvastatin treatment resulted in a sevenfold increase in HMG-CoA reductase mRNA in the nonfasted animals, compared to the controls ($P < 0.001$). In contrast, simvastatin treatment produced only a twofold increase in the levels of mRNA compared to untreated guinea pigs. Fasting resulted in a significant decrease in mRNA levels in atorvastatin- and simvastatin-treated guinea pigs ($P < 0.01$), while fasting did not affect mRNA levels in untreated animals (Table 4).

DISCUSSION

HMG-CoA reductase activity is the main target for statin treatment. Since this enzyme is rate limiting and regulatory for cholesterol synthesis, the effect of atorvastatin and simvastatin on the activity of HMG-CoA reductase is of major importance. It has been reported in rat and cultured cell studies that HMG-CoA reductase activity is increased after treatment with statins (25,26). Cholesterol is a major regulator for HMG-CoA reductase synthesis (negative feedback regulation), and inhibition of reductase activity will reduce the amount of cholesterol being synthesized. Decreased intracellular cholesterol will lead to the production of more HMG-CoA reductase enzyme as a compensatory mechanism. Whether this increased enzyme amount will result in increased measurement of enzyme activity will depend on whether the statin is still bound to the enzyme during the assay procedure.

In the present study, both statins reduced or did not change reductase activity in nonfasted guinea pigs, suggesting that they are still bound to the enzyme at the time of assay. To further explore this hypothesis, studies were conducted with guinea pigs subjected to different fasting times in order to remove the drugs from the system.

Simvastatin treatment resulted in higher enzyme activity after 6, 12, and 18 h of fasting compared to nontreated guinea pigs. These data suggest that this statin was removed from the enzyme after 6 h of drug withdrawal. The results observed for simvastatin treatment agree with those reported by Del Puppo *et al.* (25), where, after removal of the drug, an up-regulation of reductase activity was observed. This may be the reason for the increased reductase activity observed after fasting in simvastatin-treated guinea pigs compared to untreated animals in the present study. The amount of HMG-CoA reductase mRNA was determined in liver of simvastatin-treated guinea pigs. In agreement with other studies (11,27), a twofold increase in mRNA levels was observed in HepG2 cells and rats with simvastatin treatment. The lower increase in reductase mRNA abundance with simvastatin compared to atorvastatin treatment in guinea pigs suggests that the observed increase in activity after fasting is probably due to activation of the already existing protein. Although the levels of immunoreactive protein were not determined in the pres-

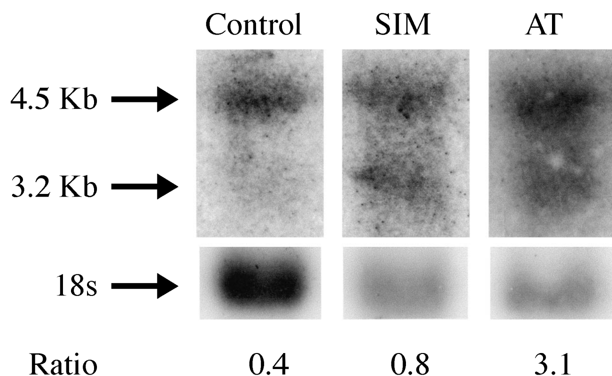

FIG. 1. Effect of atorvastatin and simvastatin on hydroxy methylglutaryl (HMG)-CoA reductase mRNA. AT, atorvastatin; SIM, simvastatin. Northern blot performed with human cDNA for HMG-CoA reductase. The sizes for HMG-CoA reductase and 18s mRNA are indicated. HMG-CoA reductase/18s mRNA ratio was used as a correction for amount of sample loaded. Figure shows a representative sample for each treatment.

ent study, it is possible that simvastatin conferred some protein stabilization as has been observed by Ness *et al.* (28).

In contrast to simvastatin, atorvastatin treatment continued to suppress HMG-CoA reductase activity even after 18 h of fasting, suggesting that either the drug is still tightly bound to the enzyme, or atorvastatin does not induce HMG-CoA reductase activity as do other reductase inhibitors. Ness *et al.* (26) have indicated in a recent study that atorvastatin binds very tightly to the enzyme active site and several washes of the microsomes were needed to remove some of the drug. Our data suggest that under our experimental conditions, 18 h of fasting were not sufficient to remove the drug from the enzyme.

It has been demonstrated in humans that atorvastatin has a half-life of 14 h compared to simvastatin's half-life of 1.9 h (2). In addition, atorvastatin metabolism results in the presence of two active metabolites, which are as potent as the parent drug (29). These properties may result in the lack of recovery of HMG-CoA reductase activity observed after fasting in atorvastatin treated guinea pigs. It has been observed that atorvastatin inhibits cholesterol synthesis longer than simvastatin in hypercholesterolemic patients (12) supporting the hypothesis that atorvastatin remains bound to the enzyme for longer periods of time.

HMG-CoA reductase mRNA abundance was sevenfold higher after atorvastatin treatment in nonfasted guinea pigs. An increase in reductase mRNA has also been observed in rats treated with atorvastatin (26). These results suggest that atorvastatin may be inducing the production of more protein at the transcriptional level through the compensatory mechanism known for statins. However, no increase in reductase activity was observed because atorvastatin was still bound to the enzyme.

Long-term fasting decreases the amount of HMG-CoA reductase mRNA as well as the amount of immunoreactive protein in rats (28). Ness *et al.* (26) also reported that rats fasted for 24 h had lower levels of HMG-CoA reductase mRNA. A fasting effect was observed for reductase mRNA in this experiment for guinea pigs treated with atorvastatin and simvastatin. However, fasting did not affect mRNA levels in untreated guinea pigs. At this point, the precise explanation for this discrepancy is not known, but it appears that in untreated guinea pigs, 18 h of fasting was not enough to cause a decrease in mRNA levels and the regulation of the enzyme activity may have taken place only at the level of phosphorylation. This may be similar to observations in the rat where with short fasting periods enzyme activity was dictated only by the degree of phosphorylation (30).

From these results, we may conclude that atorvastatin and simvastatin, despite similar cholesterol-lowering properties, have somewhat distinct effects on the regulation of HMG-CoA reductase activity and mRNA abundance. Either statins confer protein stabilization or they may interfere with translational efficiency of the reductase mRNA, as observed for dietary cholesterol in rats (31). Simvastatin effects on enzyme activity appear to be of shorter duration than atorvastatin and thus there is less up-regulation of mRNA levels compared to untreated guinea pigs. However, the observed increases in reductase activity after fasting in simvastatin-treated guinea pigs could be related to preservation of HMG-CoA reductase protein. In contrast, atorvastatin inhibits reductase activity for longer periods of time, which may be a plausible explanation for its higher efficacy observed in humans and for the greater up-regulation (sevenfold compared to twofold) of mRNA levels.

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Synthesis of Acetyl,docosahexaenoyl-Glycerophosphocholine and Its Characterization Using Nuclear Magnetic Resonance

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ABSTRACT: Docosahexaenoic acid (DHA) circulates in mammals in lipoproteins and bound to serum albumin as a nonesterified fatty acid as well as esterified in lysophosphatidylcholine (lysoPC). 1-Lyso,2-DHA-glycerophosphocholine (GPC) is an unstable isomer because of a primary alcohol at the *sn*-1 position. To keep DHA at the *sn*-2 position of lysoPC, its usual position for the corresponding lysoPC to be acylated into PC in tissues, we synthesized 1-acetyl,2-DHA-GPC and confirmed its structure by use of nuclear magnetic resonance (NMR) spectroscopy in comparison with its positional isomer, 1-DHA,2-acetyl-GPC. 1-Lyso,2-DHA-GPC was prepared from 1-stearoyl,2-DHA-GPC by enzymatic hydrolysis and purified by high-performance liquid chromatography. The isomerization of 1-lyso,2-DHA-GPC into 1-DHA,2-lyso-GPC was obtained by keeping the former overnight at room temperature under nitrogen. Both lysoPC isomers were acetylated by acetic anhydride into 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC, respectively, and the resulting phospholipids were fully characterized by NMR. In particular, the 1,2 substitution pattern of the acetyl and DHA chains could be easily detected by 2D heteronuclear multibond correlation. We conclude that 1-acetyl,2-DHA-GPC might be considered as a stable form of 1-lyso,2-DHA-GPC for its delivery to tissues, if the latter exhibits acetyl hydrolase activity.

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Since the pioneering epidemiological studies of Bang *et al.* (1), there has been a considerable interest in the potential health benefits of marine polyunsaturated fatty acids, namely eicosapentaenoic and docosahexaenoic (DHA) acids. In addition to the beneficial effects of those fatty acids in the circulatory cardiovascular system (2), DHA is of particular interest for the cerebrovascular system, especially the retina, in which it represents almost the only polyunsaturated fatty acid (3,4), and in brain grey matter, where it may be more prominent than arachidonic acid, depending on the area (5). Indeed, DHA is assumed to be required for proper visual functions and brain development (6,7). *In vivo*, DHA circulates in the bloodstream under various chemical forms attached to either albumin or lipoproteins (8). Previous studies in which ^{13}C-labeled DHA esterified in triglycerides or phosphatidylcholine (PC) was ingested as a single dose by human volunteers showed that nonesterified DHA and DHA esterified in lysophosphatidylcholine (lysoPC-DHA) are two chemical forms bound to albumin that could specifically bring DHA to platelets and erythrocytes, respectively (9,10). There is evidence that the incorporation of DHA into erythrocytes may serve as an indicator of DHA incorporation into the brain (11), and we consider this observation to be in agreement with the preferential brain uptake of DHA in the rat when DHA is injected as lysoPC-DHA compared to nonesterified DHA, both chemical forms being bound to albumin (12). Evidence for the preferential uptake of DHA has recently been provided using a reconstituted *in vitro* blood brain barrier with co-cultures of capillary endothelial cells and astrocytes (13). However, the metabolic fate of DHA in the brain is to accumulate at the *sn*-2 position of phospholipids. Although previous experiments have been done with 1-lyso,2-docosahexaenoyl-glycerophosphocholine (1-lyso,2-DHA-GPC), it is expected that part (if not the bulk) of this chemical form would have been isomerized into 1-DHA,2-lyso-GPC because of the primary alcohol reactivity at the *sn*-1 position.

The objective of the present study was to prevent such an isomerization by esterification of the *sn*-1 position with an acetyl group, keeping the molecule at a polarity closer to that of lysoPC-DHA. A stable molecule of this kind might be useful as a vehicle for delivering DHA through lysoPC-DHA, provided that the target tissues exhibit acetyl hydrolase activity.

EXPERIMENTAL PROCEDURES

Preparation of lysoPC-DHA. 1-Lyso,2-DHA-GPC was prepared by enzymatic hydrolysis from 1-stearoyl,2-DHA-GPC, which was provided by Avanti Polar Lipids (Alabaster, AL). The enzyme used was a lipase (EC 3.1.1.3) from *Rhizopus arrhizus* which exhibits phospholipase A_1 activity (14). The enzyme was furnished by Sigma (L'Isle d'Abeau, France). Two milligrams of substrate (PC) was dissolved in 1 mL of diethylether and sonicated for 4 min in a sonication bath with 1 mL of phosphoboric buffer (pH 6) containing 200,000 U of

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Abbreviations: COSY, correlation spectroscopy; DHA, docosahexaenoic acid; GPC, glycerophosphocholine; HPLC, high-performance liquid chromatography; HMBC, heteronuclear multibond correlation; HSQC, heteronuclear single quantum coherence; lysoPC, lysophosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; TLC, thin-layer chromatography.

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enzyme. This mixture was further incubated with vigorous shaking for 1 h at room temperature under nitrogen. The reaction was stopped in ice and total lipids were extracted according to Bligh and Dyer (15) at pH 3 and 4°C to minimize lysoPC isomerization.

The lysoPC formed was separated from the remaining PC substrate and the fatty acid was released by the lipase treatment using thin-layer chromatography (TLC) on silica gel 60G (Merck, Darmstadt, Germany) with the eluent mixture chloroform/methanol/water (65:25:4, by vol) at 4°C (16). The R_f of the compounds were 0.2, 0.47, and 0.91, respectively. The purity of 1-lyso,2-DHA-GPC obtained was checked by high-performance liquid chromatography (HPLC) as shown in Figure 1. The isomerization of 1-lyso,2-DHA-GPC into 1-DHA,2-lyso-GPC was induced by keeping the former overnight at room temperature under nitrogen. Under these conditions, further isomerization of 1-DHA,2-lyso-GPC into 1-DHA,3-lyso-GPC is very unlikely because of the lower reactivity of the *sn*-2 secondary alcohol. The positional isomers were analyzed by HPLC with a 5-µm Superspher C_{18} column (4.6 × 250 mm, i.d.; Hewlett-Packard, Les Ulis, France) isocratically eluted with meth-anol/water/acetonitrile (90:25:2.5, by vol) containing 0.35% choline chloride at a flow rate of 1.2 mL/min. Each lysoPC was detected at 210 nm. Figure 1 gives an example of chromatograms. If 1-DHA,3-lyso-GPC was formed subsequent to isomerization of 1-lyso,2-DHA-GPC, it would presumably have migrated in peak 1 of Figure 1B together with 1-lyso,2-DHA-GPC, the sum representing at most 12% of the two isomers. In addition, the nuclear magnetic resonance (NMR) spectrum of the acetylated derivative (2) appeared at least 95% pure, and no signal could be detected for a putative 1-acetyl,3-DHA-GPC.

Each lysoPC isomer was quantified on the basis of its DHA content by gas–liquid chromatography (9). LysoPC was treated with 5% H_2SO_4 in methanol for 90 min at 100°C in the presence of an appropriate amount of 1-heptadecanoyl,2-lyso-GPC as an internal standard. The mixture of DHA and heptadecanoic acid methylesters were separated and measured as previously described by gas chromatography, using an SP2380 capillary column (30 m × 0.32 mm; Supelco, Bellefonte, PA) (9).

Acetylation of lysoPC isomers. Both lysoPC isomers (1-lyso,2-DHA-GPC and 1-DHA,2-lyso-GPC) stored in chloroform were dried under reduced pressure at low temperature in the dark. Each lysoPC isomer was dissolved in 0.55 mL of a 0.135 M chloroformic solution of 4-pyrrolidinopyridine and 0.8 mL of a 0.138 M chloroformic solution of acetic anhydride and was continuously agitated for 16 h at 4°C to ensure complete reaction. Added to this were 1.7 mL of chloroform and 1.5 mL of water. After stirring, the mixture was centrifuged for 10 min at 800 × *g*. The chloroformic phase was separated and evaporated under reduced pressure. The 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC were separated from the remaining 1-lyso,2-DHA-GPC and 1-DHA,2-lyso-GPC, respectively, by TLC on silica gel 60G with the chloroform/methanol/water (65:25:4, by vol) mixture. The expected 1-acetyl,2-DHA-GPC and 1-DHA,2-acetyl-GPC at

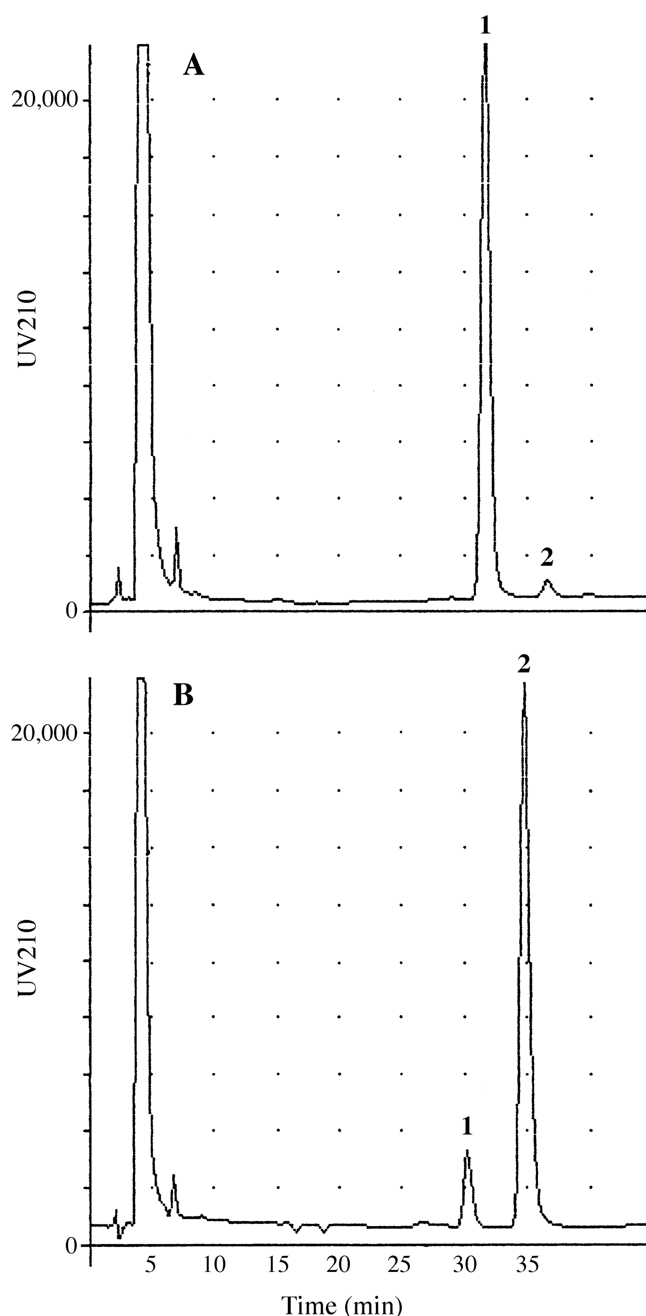

FIG. 1. Separation of 1-lyso,2-docosahexaenoyl-glycerophosphocholine (1-lyso,2-DHA-GPC) (peak 1) from 1-DHA,2-lyso-GPC (peak 2) by reversed-phase high-performance liquid chromatography. (A) Lysophosphatidylcholine (lysoPC) isomers obtained by enzymatic cleavage of 1-stearoyl,2-DHA-GPC were injected onto a 5-µm Superspher column (250 × 4.6 mm), and eluted at 1.2 mL/min by methanol/water/acetonitrile (90:25:2.5, by vol) containing 0.35% choline chloride. They were detected by ultraviolet light at 210 nm. (B) LysoPC isomers obtained by enzymatic cleavage were maintained overnight at room temperature. They were separated as indicated in A.

R_f 0.18 were scraped off the plate by extraction with chloroform/methanol/water (10:20:3, by vol) and the extract was dried under nitrogen before being dissolved in 0.4 mL of $CDCl_3$ for further NMR analysis.

NMR analysis. Nuclear magnetic spectra were recorded in $CDCl_3$ with a Bruker (Wissembourg, France) DRX-500 (500/125 MHz) spectrometer. Chemical shifts are given in ppm and are referenced to $CDCl_3$ resonances (7.26 and 77.0 ppm). Splitting pattern abbreviations are *s*, singlet; *d*, doublet; *t*, triplet; and *m*, multiplet. Resonance attributions were made according to the following experiments: Standard ^{1}H NMR, 2D H,H-correlation spectrocopy (COSY), 2D heteronuclear single quantum coherence (HSQC), and 2D gradient-selected heteronuclear multibond correlation (HMBC) with a mixing period of 60 ms.

SCHEME 1

RESULTS AND DISCUSSION

LysoPC isomers. The hydrolysis of 1-stearoyl,2-DHA-GPC by the *Rhizopus arrhizus* lipase led to 1-lyso,2-DHA-GPC with a yield recovery of about 93%, as based on ultraviolet quantitation. The lysoPC produced was extracted in acidic conditions to minimize its isomerization into 1-DHA,2-lyso-GPC. A typical HPLC profile of the lipase hydrolysis product is given in Figure 1. From this HPLC profile, it can be calculated that the isomerization was limited to around 4%. To prevent the 1-lyso,2-DHA-GPC isomer from its isomerization into 1-DHA,2-lyso-GPC, it was kept at pH 4 at 4°C. Leaving the 1-lyso,2-DHA-GPC isomer at room temperture overnight under neutral conditions was enough to provoke more than 85% isomerization into the 1-DHA,2-lyso-GPC isomer (Fig. 1). Both isomers have been taken into consideration in the present study as we found them bearing polyunsaturated fatty acids bound to human albumin, both forms being found in similar proportions (17). These lysoPC appear to be the main lysophospholipids released from the liver (18) and are considered to be the main form to provide polyunsaturated fatty acids in the brain (12,16). An important issue in this context is the biological relevance of 1-lyso,2-DHA-GPC, expected to result from liver phospholipase A_1 activity, compared to that of 1-DHA,2-lyso-GPC, which is unlikely to be acylated to yield PC, as that PC would contain DHA at the *sn*-1 position. In contrast, we may reasonably expect that 1-lyso,2-DHA-GPC could be easily acylated into physiological PC. In order to keep DHA stable at the *sn*-2 position, we aimed to chemically prepare 1-acetyl,2-DHA-GPC. This form could be then the precursor of 1-lyso,2-DHA-GPC, provided that it was first cleaved by an acetyl hydrolase.

NMR characterization of acetyl-DHA-GPC. 1-Acetyl,2-DHA-GPC **(1)** and its positional isomer, 1-DHA,2-acetyl-GPC **(2)** were obtained by acetylation of the corresponding DHA-lysoPC with acetic anhydride, and their structures (Scheme 1) were characterized by NMR spectroscopy, as described in the Experimental Procedures section.

As expected, the ^{1}H NMR spectra of compounds **1** and **2** were very similar and no distinction between them could be made on this basis. The 2D HMBC experiment, which is related to $^2J_{CH}$ and $^3J_{CH}$ connectivities (carbon–hydrogen connectivity through two or three bonds), appeared very useful to connect directly the carbon-1 of the glycerol backbone with the acetyl group in compound **1** or the DHA chain in compound **2**, thus revealing the 1,2 substitution pattern. In the 2D HMBC experiment with compound **1** (Fig. 2), cross peaks were observed between the carbon resonance at δ 171.13 (^{1}CH$_2$–O–<u>C</u>O–CH$_3$) and proton resonances at δ 2.06 (^{1}CH$_2$–O–CO–C<u>H</u>$_3$) and δ 4.17 (^{1}C<u>H</u>$_2$–O–CO–CH$_3$); a weak cross peak also was observed between the carbon resonance at

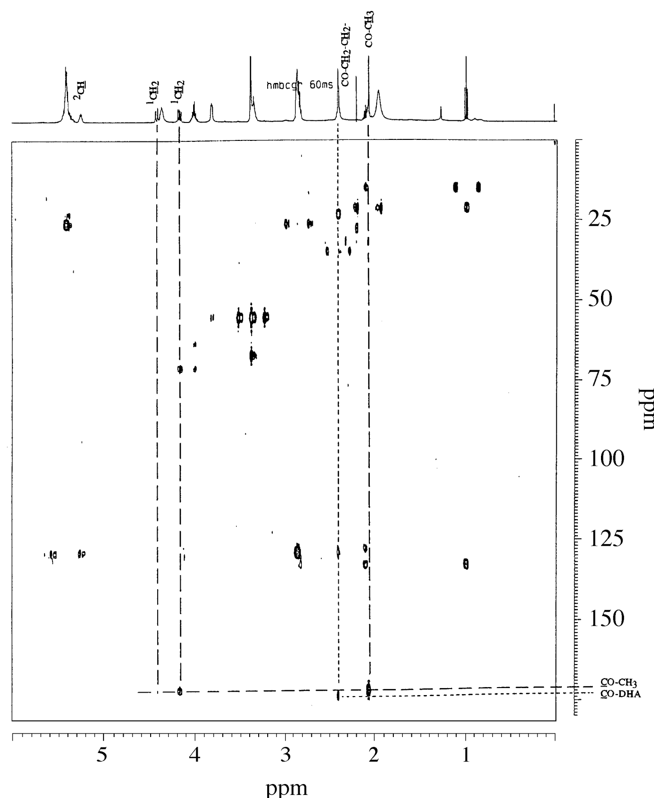

FIG. 2. 2D gradient-selected heteronuclear multibond correlation nuclear magnetic resonance spectrum of 1-acetyl,2-DHA-GPC (**1**). The presence of cross peaks on the $COCH_3$ line (horizontal) demonstrates the attachment of the acetyl group to C-1 of the glycerol backbone. The spectrum was obtained without filtering out the $^1J_{CH}$ coupling interaction since the expected information occurs in the carbonyl region where such a coupling is absent. Acquisition parameters: NS, 32; TD2, 2048; SW2, 6.008 ppm; relaxation delay, 1.5 s; mixing time, 60 ms; TDI, 128; SW1, 179.998 ppm. Processing parameters: F2 SI, 1024; F1 SI, 512. The time domain data were multiplied by a sine-bell function prior to Fourier transformation.

δ 171.13 and the other $^{1}CH_2$ proton resonance at δ 4.41 ($^{1}C\underline{H}_2$–O–CO–CH_3). Thus, the connection between the $^{1}CH_2$ and O–CO–CH_3 parts of the molecule was unambiguously established. A similar result was obtained with compound **2** since cross peaks were observed between the carbon resonance at δ 173.43 ($^{1}CH_2$–O–$\underline{C}$O–CH_2–CH_2) and proton resonances at δ 2.42–2.37 ($^{1}CH_2$–O–CO–$C\underline{H}_2$–$C\underline{H}_2$) and δ 4.18 ($^{1}C\underline{H}_2$–O–CO–CH_2–CH_2), establishing the connection between the $^{1}CH_2$ and O–DHA parts of the molecule (in this case, no cross peak was observed between the carbon resonance at δ 173.43 and the other $^{1}CH_2$ proton at 4.40). With chains bonded to carbon-2, DHA in **1** (Fig. 2), or acetyl in **2**, a cross peak was observed between the carbonyl carbon and the methylene or methyl protons of the chains, but we were unable to connect the chains with carbon-2 since the expected cross peaks between the carbonyl carbons and $^{2}C\underline{H}$ proton resonances at ~5.25 could not be observed. This missing observation is presumably related to the splitting of the $^{2}C\underline{H}$ proton resonances and to the small amount of product in the NMR sample. The following proton and carbon resonances were attributed to each compound from the different NMR experiments: 1-acetyl,2-DHA-GPC (**1**): ^{1}H NMR: δ 5.44–5.34 (*m*, 12H, $C\underline{H}$=$C\underline{H}$); 5.26–5.23 (*m*, 1H, $^{2}C\underline{H}$); 4.41 (*dd*, 1H, *J* = 12.0 and 3.2 Hz, $^{1}C\underline{H}_2$); 4.40–4.30 (*m*, 2H, O–$C\underline{H}_2$–CH_2–N); 4.17 (*dd*, 1H, *J* = 12.0 and 7.0 Hz, $^{1}C\underline{H}_2$); 4.04–3.96 (*m*, 2H, $^{3}C\underline{H}_2$); 3.84–3.79 (*m*, 2H, O–CH_2–$C\underline{H}_2$–N); 3.38 [*s*, 9H, $N(C\underline{H}_3)_3$]; 2.89–2.81 (*m*, 10H, =CH–$C\underline{H}_2$–CH=); 2.43–2.38 (*m*, 4H, O–CO–$C\underline{H}_2$–$C\underline{H}_2$–CH=); 2.12–2.03 (*m*, 2H, =CH–$C\underline{H}_2$–CH_3); 2.06 (*s*, 3H, CO–$C\underline{H}_3$); 1.00 (*t*, 3H, *J* = 7.5 Hz, =CH–CH_2–$C\underline{H}_3$). ^{13}C NMR: δ 172.81 (^{2}CH–O–$\underline{C}$O–CH_2–CH_2); 171.13 ($^{1}CH_2$–O–$\underline{C}$O–CH_3); 133.0–127.4 ($\underline{C}$H=$\underline{C}$H); 71.00 ($^{2}\underline{C}$H); 66.84 (O–CH_2–$\underline{C}H_2$–N); 63.55 ($^{3}\underline{C}H_2$); 63.37 ($^{1}\underline{C}H_2$); 59.40 (O–$\underline{C}H_2$–CH_2–N); 54.75 [$N(\underline{C}H_3)_3$]; 26.3–25.6 (=CH–$\underline{C}H_2$–CH=); 34.36 ($^{1}CH_2$–O–CO–$\underline{C}H_2$–CH_2); 22.86 (^{2}CH–O–CO–CH_2–$\underline{C}H_2$); 21.11 ($^{1}CH_2$–O–CO–$\underline{C}H_3$); 20.97 (=CH–$\underline{C}H_2$–CH_3); 14.47 (=CH–CH_2–$\underline{C}H_3$).

1-DHA,2-acetyl-GPC (**2**): ^{1}H NMR: δ 5.44–5.32 (*m*, 12H, $C\underline{H}$=$C\underline{H}$); 5.25–5.20 (*m*, 1H, $^{2}C\underline{H}$); 4.40 (*dd*, 1H, *J* = 12.0 and 3.2 Hz, $^{1}C\underline{H}_2$); 4.40–4.25 (*m*, 2H, O–$C\underline{H}_2$–CH_2–N); 4.18 (*dd*, 1H, *J* = 12.0 and 7.0 Hz, $^{1}C\underline{H}_2$); 4.04–3.98 (*m*, 2H, $^{3}C\underline{H}_2$); 3.80–3.74 (*m*, 2H, O–CH_2–$C\underline{H}_2$–N); 3.35 [*s*, 9H, $N(C\underline{H}_3)_3$]; 2.89–2.81 (*m*, 10H, =CH–$C\underline{H}_2$–CH=); 2.42–2.37 (*m*, 4H, O–CO–$C\underline{H}_2$–$C\underline{H}_2$–CH=); 2.13–2.02 (*m*, 2H, =CH–$C\underline{H}_2$–CH_3); 2.09 (*s*, 3H, CO–$C\underline{H}_3$); 1.00 (*t*, 3H, *J* = 7.5, =CH–CH_2–$C\underline{H}_3$). ^{13}C NMR: δ 173.43 ($^{1}CH_2$–O–$\underline{C}$O–CH_2–CH_2); 170.78 (^{2}CH–O–$\underline{C}$O–CH_3); 133.0–127.4 ($\underline{C}$H=$\underline{C}$H); 70.89 ($^{2}\underline{C}$H); 66.78 (O–CH_2–$\underline{C}H_2$–N); 63.79 ($^{3}\underline{C}H_2$); 63.25 ($^{1}\underline{C}H_2$); 59.45 (O–$\underline{C}H_2$–CH_2–N); 54.75 [$N(\underline{C}H_3)_3$]; 26.3–25.6 (=CH–$\underline{C}H_2$–CH=); 34.30 ($^{1}CH_2$–O–CO–$\underline{C}H_2$); 22.81 ($^{1}CH_2$–O–CO–CH_2–$\underline{C}H_2$); 21.34 (O–CO–$\underline{C}H_3$); 21.07 (=CH–$\underline{C}H_2$–CH_3); 14.48 (=CH–CH_2–$\underline{C}H_3$).

The carbonyl carbon chemical shifts deserve some comments. The carbonyl resonance of the C-1 DHA chain in **2** (173.43) appears at a higher chemical shift than its C-2 DHA chain counterpart in **1** (172.81), the same relationship being observed with the acetyl group (171.13 in **1** > 170.78 in **2**). This cross comparison may be related to the observed carbonyl chemical shifts in saturated or unsaturated α and β chains of triglycerides, the carbonyl of the α chain having the higher chemical shift (19–21).

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Nondestructive NMR Determination of Oil Composition in Transformed Canola Seeds

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ABSTRACT: Magic-angle spinning (MAS) ^{13}C nuclear magnetic resonance (NMR) spectroscopy is a convenient method for nondestructive, quantitative characterization of seed oil composition. We describe results for intact hybrid and transformed canola seeds. The MAS ^{13}C NMR technique complements and agrees with gas chromatography results. The spectral resolution approaches that of neat, liquid oils. MAS ^{13}C NMR data allow quantitative analysis of major oil components, including saturates and oleic, linoleic, and linolenic acyl chains. ^{13}C NMR directly and quantitatively elucidates, triglyceride regiochemistry and acyl chain *cis-trans* isomers that cannot be quickly detected by other methods. MAS ^{13}C NMR can serve as the primary method for development of near-infrared seed oil calibrations. These NMR methods are nondestructive and attractive for plant-breeding programs or other studies (e.g., functional genomics) where loss of seed viability is inconvenient.

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The analysis of plant seed oils is critical to assess the value of individual seeds as well as entire crops. Numerous methods are used to determine seed oil composition (1,2). Methods based on gas chromatography (GC), GC–mass spectrometry (GC–MS), and high-performance liquid chromatography–MS (HPLC–MS) are destructive and require chemical manipulation. GC analysis—the most common method—is inexpensive, precise, and efficient. By contrast, optical spectroscopy (near-infrared, NIR) and nuclear magnetic resonance (NMR) methods are nondestructive and attractive for plant-breeding programs or other studies where loss of seed viability is inconvenient. For example, an understanding of gene function relevant to oil composition could be gained from mutant plant screening. Single oilseeds would be screened to identify unique triglyceride (TAG) compositions. Linking the change back to the mutation could then lead to an understanding of gene function.

Whole seed NIR analysis is attractive from a cost per seed perspective. In addition, the information content of NIR is high. Total oil content and oil iodine number are measured with NIR. TAG compositions for multiple seed samples of *Brassica carinata* (mustard) are reported (3). However, NIR analysis is a secondary method and requires careful and comprehensive calibrations. In addition, seed variables, such as hull thickness, can complicate NIR data interpretation.

Low-resolution (wide-line) ^{1}H NMR spectroscopy is well known in seed oil analytical chemistry. Wide-line NMR measures total oil and moisture content for groups of seeds, but cannot determine oil composition. Low-resolution NMR is inexpensive; instrument calibration is straightforward and measurements are automated. High-resolution seed NMR studies to observe individual peaks from structurally distinct nuclei are less well known. The first high-resolution ^{13}C measurements on intact seeds appeared in 1974 (4). Reports on the application of ^{13}C NMR with a variety of intact seeds followed (5–12). Magic-angle spinning (MAS) methods give the highest spectral resolution (13–18). To date, published MAS seed spectra were acquired at moderate magnetic field strengths (^{1}H frequency ≤400 MHz) with sample probes designed to characterize amorphous solids. These probes are constructed with materials important for solid-state spectroscopy and do not emphasize high resolution. Solid-state probes are not readily adapted to provide high sample throughput. We report here results of initial seed oil studies using high-field NMR and a ^{13}C MAS probe designed specifically to obtain high-resolution spectra (19). Our experiments show high-resolution NMR quickly and accurately determines oil composition in whole seeds. This method can be automated to provide detailed seed oil compositions without the need for chemical extractions.

MATERIALS AND METHODS

Seeds. Canola seeds are from the Calgene hybrid and transgenic library. After NMR analysis, a sample of canola seeds germinated normally.

Sample preparation. Canola seeds were placed directly into wide-mouth MAS NMR sample tubes.

NMR spectroscopy. High-resolution spectra are measured at 11.7 tesla. (^{1}H = 500 MHz, ^{13}C = 125 MHz) using Varian NMR Instruments (Palo Alto, CA) Inova™ NMR spectrometers equipped with carbon-observe MAS Nanoprobes™. The ^{13}C spectra were acquired without a field-frequency lock at ambient

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Abbreviations: CPMAS, cross-polarization, magic-angle spinning; GC, gas chromatography; GC–FID, GC–flame-ionization detection; GC–MS, GC–mass spectrometry; HEAR, high erucic; HPLC–MS: high-performance liquid chromatography–MS; LEAR, low erucic; NIR, near infrared; NOE, nuclear Overhauser enhancement; NMR, nuclear magnetic resonance; MAS, magic-angle spinning; sat, saturated; TAG, triglyceride; TMS, tetramethylsilane.

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temperature (~21–22°C) in 10 min to 14 h using the following conditions: spectral width = 29.996 kHz, acquisition time = 2.185 s, $\pi/2$ pulse (3.8 µs) with no relaxation delay, $^{1}H\ \gamma B_2 = 2.5$ kHz with Waltz decoupling. The following conditions were typical of data processing: digital resolution = 0.11 Hz, 0.3 to 1.5 Hz line broadening, and time-reversed linear prediction of the first three data points. Chemical shifts are referenced by adding neat tetramethylsilane (TMS) to canola seeds and using the resulting referencing parameters for subsequent spectra. The ^{13}C resolution is 2–3 Hz for the most narrow seed resonances. Spectral resolution is independent of MAS spinning speeds (2.0–3.5 kHz) and data are typically obtained with 2.2 kHz spinning speeds. Spinning sidebands are <1% of the main resonance. TAG ^{13}C assignments are made from comparison with literature assignments or with shifts computed from a ^{13}C NMR database (20).

Preparation of fatty acid methyl esters and GC analysis. Twenty seeds from each Calgene canola line were crushed in a glass vial (12 × 32 mm) and extracted with toluene (500 µL) at room temperature for 3 h. An aliquot (100 µL) of the extracted seed oil TAG was subjected to methanolysis with 0.5 M sodium methoxide/methanol (500 µL) at 60°C for 20 min in another vial (capped). A saturated solution of NaCl (500 µL) and heptane (100 µL) was added to each sample and then centrifuged at 2,000 × *g* for 5 min. An aliquot (100 µL) of the upper phase containing methyl esters was recovered and subjected to compositional analysis using a GC–flame-ionization detector (FID) (model 6890; Hewlett-Packard, Wilminton, DE). The chromatographic conditions were 25 m × 0.25 mm fused-silica column (Omegawax 250; Supelco, Bellefonte, PA); 250°C injection and 270°C detection port temperatures; He was the carrier gas; H_2 at 40 mL/min; air at 450 mL/min; N_2 makeup at 35 mL/min; column temperature programmed from 172 to 242°C at 40°C/min; holding at 172°C for 1.7 min; column pressure programmed from 25 to 75 psi at 40 psi/min; holding at 25 psi for 1.7 min; 1.0 µL injected at a split ratio of 100. Peak retention time assignments were determined using authentic reference compounds (Sigma Chemical Co., St. Louis, MO) and the weight percent results were calculated from peak areas adjusted by theoretical FID response factors.

RESULTS AND DISCUSSION

High-resolution ^{13}C NMR of seeds. Figure 1 compares a ^{13}C spectrum acquired by placing about 80 canola seeds in a standard 5-mm NMR tube (Fig. 1A) with a spectrum of a single canola seed spinning at the magic angle (Fig. 1B). The resolution improvement is dramatic. The high-resolution MAS spectrum only contains carbon peaks from molecules with rapid molecular motions, i.e., molecules in liquid seed oil storage droplets. Long-chain saturated TAG are solids at room temperature, but they readily dissolve in seed oil droplets containing common amounts of unsaturated TAG. TAG and phospholipids immobilized in cell membranes do not appear in high-resolution MAS spectra. These components, and others with highly restricted global motion (proteins and carbohydrates), are seen in cross-polarization (CP) MAS ^{13}C NMR spectra of similar seeds (21). Figure 1C depicts a 10-min spectrum obtained from a single low-erucic (LEAR) canola seed. The 40-µL probe sample volume is larger than this seed, but the sensitivity is adequate to observe carbons from all oil components. This spectrum indicates that the MAS ^{13}C method can analyze hundreds of seed samples per week. The best compromise between sig-

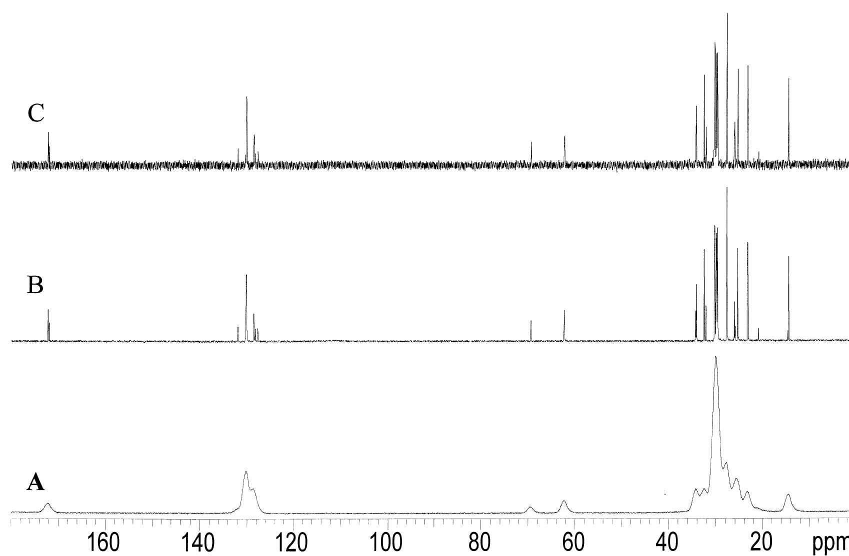

FIG. 1. The 125 MHz ^{13}C spectra of (A) about 80 canola seeds in a 5-mm nuclear magnetic resonance tube and (B) single (7.8 mg) canola seed collected with magic-angle spinning. Both spectra were signal-averaged for 4 h and processed with 1 Hz line broadening. (C) A 10-min (276 scans) single-seed spectrum represents the minimum signal-to-noise ratio suitable for seed screening.

nal-to-noise-ratio and time is achieved after 1 hr of signal averaging.

A set of canola seed and neat canola oil spectra obtained with the MAS probe (data not shown) demonstrates the importance of factors responsible for ^{13}C spectral resolution in seeds. The observed line widths in neat canola oil are about 0.5 Hz sharper than intact seed line widths. When the neat oil is diluted in $CDCl_3$ to ~20% (vol/vol), the line widths sharpen; at ~21–22°C, viscosity broadening in neat oil adds about 0.6 Hz to the sharpest lines. Heating seeds from 22 to 40°C decreases the line width by only 0.3 Hz. Taken together, these results verify earlier conclusions that the primary benefit of MAS is that it averages microscopic magnetic susceptibility gradients inherent in seed tissue (15,16).

Canola seed oil assignments. For initial analysis and carbon assignments, we discuss in detail the 4-h spectrum of a single LEAR canola seed. The terminal methyl, or ω_1, carbon region is well-separated from the other aliphatic resonances. This region (Table 1), along with the ω_2 methylene peaks (Fig. 2), is used to quickly determine the presence of n-3 and n-6 acyl chains. Methyls on saturated chains resonate just downfield of those on n-9 chains and are not resolved. Figure 2 shows how well MAS resolves the other aliphatic carbons at 125 MHz. The bulk methylene resonances (~29.9–30.8 ppm) are highly overlapped and will not be discussed further.

Methylenes near structural moieties relevant to oil composition (e.g., ω_1 methyls, olefin carbons, and ester groups) are readily assigned. Methylenes allylic to *cis* olefin carbons are found at ~26–26.5 ppm and at ~27.6–28 ppm (Figs. 2 and 3). This region is useful to identify mono-, di-, and trienes. The spectral region shown in Figure 3 contains all the peaks required for quantitative TAG analysis (*vide infra*). Identification of *cis* and *trans* isomers is problematic with GC methods. A 38-laboratory study concluded *trans* acyl chain analyses of unhydrogenated seed oils can give variable results (22). This study and another (23) found that canola oil contains 1 to 4% *trans* acyl chains. Allylic methylenes in *trans* olefins appear about 5 ppm downfield of their *cis* counterparts (24). Based on this *cis-trans* shift, we assign the small peak at ~32.5 ppm (Fig. 2) found in all spectra to *trans* acyl chain allylic carbons.

The olefin region (Fig. 4) is rich in important information about seed oil composition. The presence of 18:3n-3 acyl chains is determined by inspection. The 18:1n-9 C-10 and 18:2n-6 C-13 peaks overlap in the downfield (130.0–130.4 ppm) region. In the upfield region (128.4–128.8 ppm), the 18:3n-3 C-12 and C-13 peaks overlap. The other olefin methine carbons are well resolved. The 18:1n-9 C-9, 18:2n-6 C-9, C-10, and C-12 carbons, and the 18:3n-3 C-9 and C-10 carbons all separate into *sn*-1,3 and *sn*-2 regioisomer pairs (Fig. 5). The ability to identify all the olefin carbons for a given acyl chain reduces ambiguity due to resonance overlap.

The *sn*-1,3 and *sn*-2 glycerol backbone (~61, ~64 ppm, respectively) and ester carbonyl carbons (~172 ppm) have different chemical shifts, but do not contain information relevant to oil composition in these seeds.

Oil composition in transgenic canola seeds. Figure 6 compares the olefin region for LEAR, high erucic (HEAR) seeds, and seeds transformed to synthesize monoene acyl chains. The intense peak in the HEAR seed spectrum at ~130.2 ppm is from 22:1n-13 acyl chains. Seeds transformed to express 24:1n-15 acyl chains have a comparable resonance. The 24:1n-15 transformed seeds and the HEAR seeds have similar 18:1n-9, 18:2n-6, and 18:3n-3 compositions. Comparison with the LEAR spectrum shows the total amount of 18:3n-3 and 18:2n-6 chains is reduced in seeds with long acyl chains. A more subtle difference in the oil composition is apparent from comparison of allylic peak intensities (data not shown). Both hybrid and transformed seeds have a higher 18:3n-3 to 18:2n-6 molar ratio. Figure 6 indicates seeds transformed to express 18:1n-9 acyl chains do so at the expense of 18:3n-3 synthesis.

TABLE 1
^{13}C NMR Canola Seed Oil Peak Assignments

Carbon(s)	Acyl chain	Chemical shift (ppm)
CH_3, ω_1	Sat, 18:1n-9	14.60
	18:2n-6	14.56
	18:3n-3	14.75
CH_2, ω_2	18:3n-3	21.08
	Sat, 18:1n-6	23.22
	18.2n-6	23.34
CH_2 C-3	All	25.43
C=C–$\underline{C}H_2$–C=C–	C-14, 18:3n-3	26.05
	C-11, 18:3n-3	26.16
	C-11, 18:2n-6	26.19
CH_2–C=C–	Sat, 18:1n-9	27.78
	18:2n-6, 18:3n-3	27.81
n–CH_2	All	29.6–30.6
CH_2, ω_3	18:2n-6	32.18
$\underline{C}H_2$–C=C˜–, *trans* all		32.50
CH_2, ω_3	Sat, 18:1n-9	32.63
CH_2, C-2	All *sn*-1,3	34.24
	All *sn*-2	34.41
Glycerol CH_2	All *sn*-1,3	62.42
Glycerol CH	All *sn*-2	69.55
C=C–H	C-15, 18:3n-3	127.73
	C-10, 18:3n-3, *sn*-1,3	128.31
	C-10, 18:3n-3, *sn*-2	28.33
	C-12, 18:2n-6, *sn*-2	128.48
	C-12, 18:2n-6, *sn*-1,3	128.49
	C-10, 18:2n-6, *sn*-1,3	128.55
	C-10, 18:2n-6, *sn*-2	128.57
	C-13, 18:3n-3	128.61[a]
	C-12, 18:3n-3	128.62[a]
	C-9, 18:1n-9, *sn*-2	130.06
	C-9, 18:1n-9, *sn*-1,3	130.10
	C-9, 18:2n-6, *sn*-2	130.16
	All monoenes, 20:1	130.17
	C-9, 18:2n-6, *sn*-1,3	130.18[a]
	C-10, 18:1n-9, *sn*-1,3	130.23
	C-13, 18:2n-6, *sn*-2[a]	130.24
	C-10, 18:1n-9, *sn*-2[a]	130.26
	C-13, 18:2n-6, *sn*-2[a]	130.27
	C-9, 18:3n-3, *sn*-2	130.36
	C-9, 18:3n-3, *sn*-1,3	130.39
	C-16, 18:3n-3	132.03
C-1	All *sn*-2	171.97
	All *sn*-1,3	172.25

[a]Not resolved due to resonance overlap. NMR, nuclear magnetic resonance; sat, saturated.

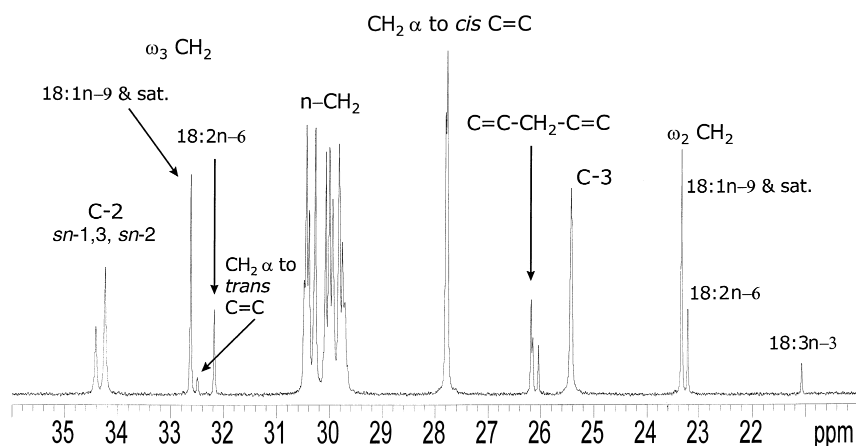

FIG. 2. The methylene-carbon assignments from a 4-h spectrum of a single 7.8-mg low-erucic (LEAR) canola seed.

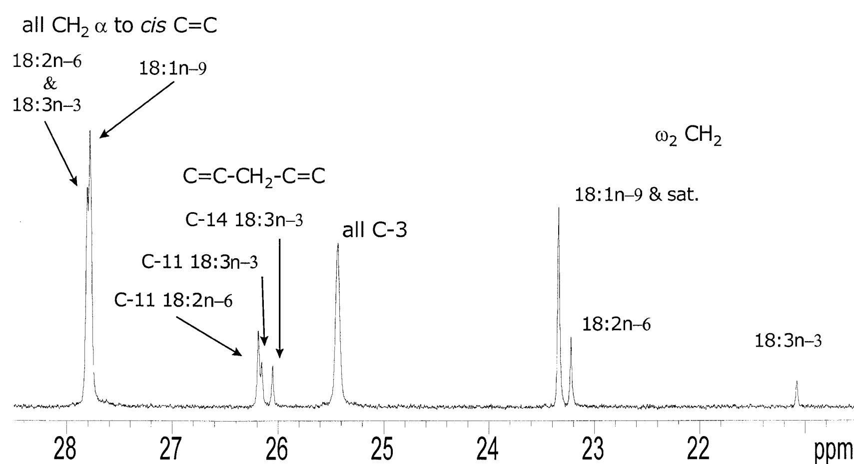

FIG. 3. Expansion of the spectrum in Figure 2 depicting the *cis*-allylic and ω_2 carbons.

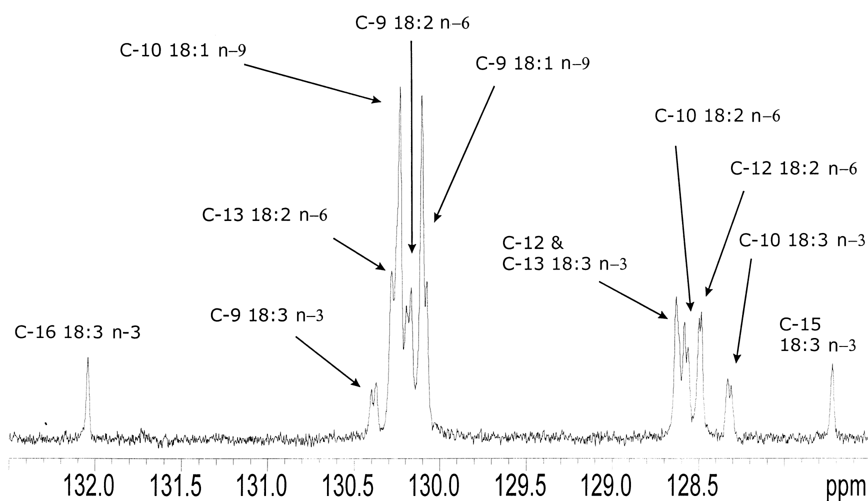

FIG. 4. The olefin-methine carbon assignments for four LEAR canola seeds. This is a 1-h spectrum with 0.3 Hz line broadening. See Figure 2 for abbreviation.

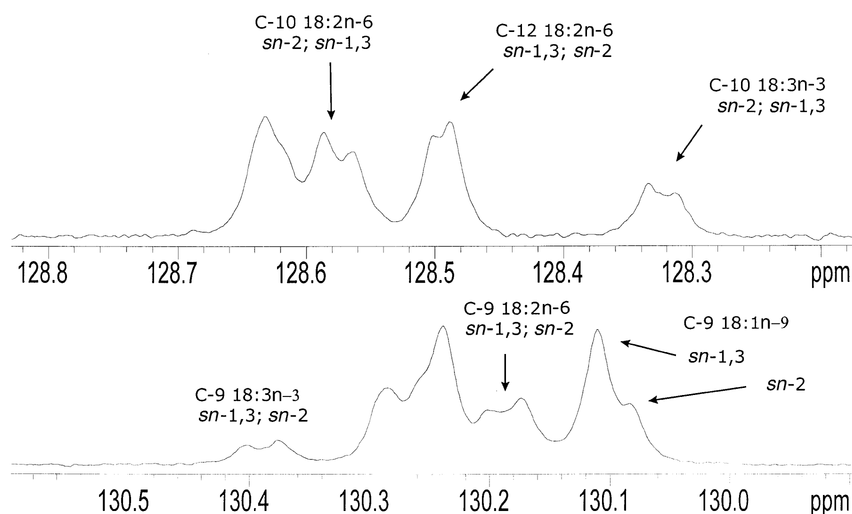

FIG. 5. Olefin-carbon regioisomer assignments for a LEAR canola seed. The *sn*-1,3 and *sn*-2 label order indicates downfield and upfield peak assignments. Unlabeled peaks do not have regioisomer shifts or resonance overlap obscures these shifts. See Figure 2 for abbreviation.

Figure 7 demonstrates how information in the olefin spectral region is used to assess oil composition in a set of closely related seed lines. Here, spectra for transgenic seeds high in 8:0/10:0, 12:0, 14:0, 16:0, and 18:0 acyl chains are shown. Based on the resonance assignments from Figure 5, it is possible to compare the 18:1n-9, 18:2n-6, and 18:3n-3 chain composition among these high-saturate seeds. Detail about saturated chain composition is difficult to extract from these spectra due to resonance overlap from similar methylene carbons in 8:0, 10:0, 12:0, 14:0, 16:0, and 18:0 chains. However, it is possible to compute the sum of all saturated chain concentrations (see below). These experiments demonstrate the potential of ^{13}C NMR in comparing TAG biosynthesis in transformed seed lines.

Canola seed oil quantitative analysis. In principle, the ^{13}C-peak area is directly proportional to the molar concentration of the corresponding carbon(s). In practice, ^{13}C peak areas also depend on relaxation parameters [e.g., nuclear Overhauser effect (NOE), and spin-lattice relaxation rates]. Use of suitable acquisition parameters provides quantitative results, but quantitative experiments require about a 10-fold increase in data collection time. As a result, ^{13}C NMR is often viewed as a qualitative method, well-suited for structure elucidation but inefficient for quantitative applications.

It is possible to correct ^{13}C peak areas to account for relaxation effects (24,25). Then, quantitative results are available with efficient data-collection times. For TAG mixtures in seed

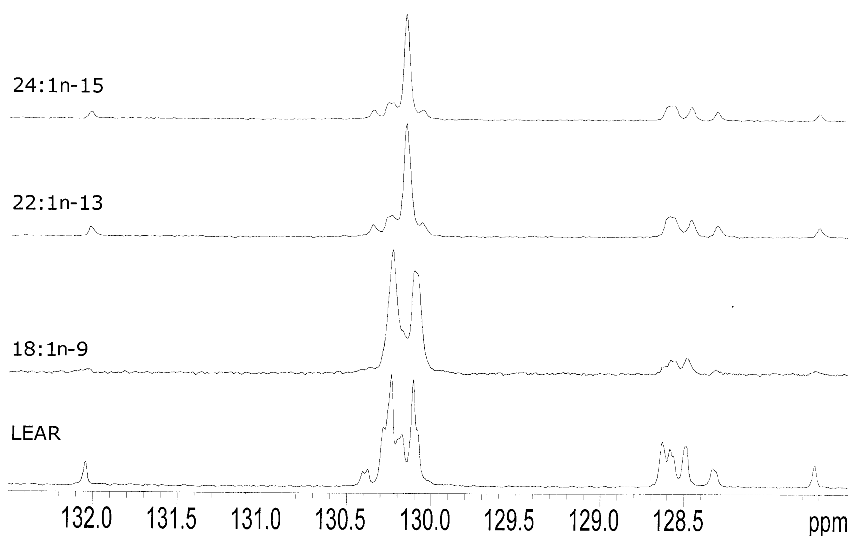

FIG. 6. The olefin region comparison of LEAR and high-erucic (HEAR, 22:1n-13) seeds and seeds transformed to synthesize monoene acyl chains. Assignments are given in Figure 4. For abbreviation see Figure 2.

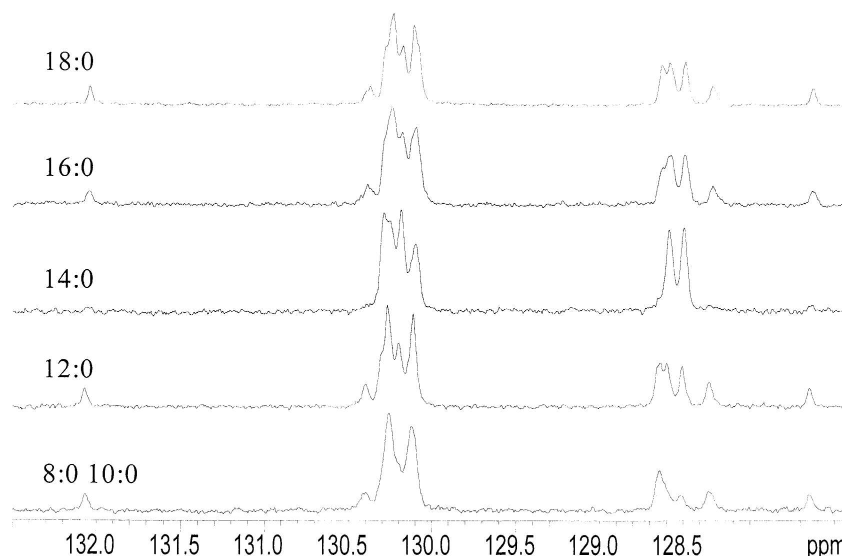

FIG. 7. The olefin region for seeds transformed to enhance saturated acyl chain composition. Assignments are shown in Figure 4.

oil storage droplets, NMR relaxation differences for similar carbons in similar molecules are comparable. If we collect a seed spectrum under both survey and quantitative conditions, we can establish a correction factor for each resonance to apply in survey spectra of similar seeds. Peak area correction factors for seeds for each carbon resonance in LEAR canola seeds are included in Table 2 (see below). The quantitative precision for ^{13}C NMR ranges from 1 to 15% and depends directly on the signal-to-noise ratio. Published studies comparing GC and ^{13}C NMR quantitative analysis indicate agreement between the two methods is 5 to 15% (9,11,16,24).

For intact seed oil composition quantitation, we focus on classes of CH_2 carbons found from 20–29 ppm (see Fig. 3 for assignments). With those assignments, we can describe how the acyl chain types contribute to each resolved peak (Table 3).

Algebraic combination of these relationships gives solutions for saturate (sat), 18:1n-9, 18:2n-6, and 18:3n-3 chain concentrations:

$$\text{sat} = P_{C\text{-}3} - \frac{P_{\omega 2}}{2} - \frac{P_{\alpha}}{2} \quad [1]$$

$$18{:}1\text{n-}9 = \frac{P_{\alpha}}{2} + \frac{3P_{\omega 2}}{2} \quad [2]$$

$$18{:}2\text{n-}6 = P_{v} - 2P_{\omega 2} \quad [3]$$

$$18{:}3\text{n-}3 = P_{18:3} \quad [4]$$

TABLE 2
Comparison of ^{13}C NMR and GC–FID Canola Seed Oil Compositions[a]

	Acyl chain ^{13}C NMR (mol%)[b] and GC–FID (wt%)			
Seed line[b]	18:3n-3	18:3n-6	18:1n-9[c]	Saturates
LEAR	9.3 (11.7)	24.0 (19.1)	60.6 (62.3)	6.1 (6.6)
8:0, 10:0	8.1 (9.3)	8.7 (8.4)	49.7 (54.2)	33.5 (27.6)
12:0	6.2 (7.1)	7.6 (9.8)	29.3 (30.8)	56.9 (52.3)
14:0	<1.0 (1.7)	21.8 (22.3)	20.7 (21.4)	57.5 (54.3)
16:0	3.8 (5.0)	21.3 (15.8)	35.0 (37.4)	39.8 (41.6)
18:0	6.8 (8.1)	19.6 (17.5)	46.0 (45.1)	27.6 (28.2)
18:1	<1.0 (1.9)	13.1 (7.8)	82.7 (84.0)	4.2 (6.0)
HEAR	10.8 (10.7)	17.6 (14.7)	69.3 (69.0)	2.3 (4.8)
24:1	9.3 (8.0)	18.2 (14.4)	69.0 (71.2)	3.4 (5.3)

[a]GC–FID, gas chromatography–flame-ionization detection; LEAR, low erucic; HEAR, high erucic; see Table 1 for other abbreviation.
[b]Intact seeds were used. Acyl chain mole percentages from peak area ratios were calibrated with no nuclear Overhauser enhancement. Delay = 7.1 or 12.1 s.
[c]Includes all *cis* monoenes (16:1, 20:1, 22:1, 24.1).

TABLE 3
Acyl Chain Type Contribution to Resolved Peaks

Peak area	ppm	Assignment (acyl chain molar amounts)[a]
$P_{18:3}$	21.1	ω_2 (18:3n-3)
P_{n-2}	23.3	ω_2 [(sat) + (18:1n-9) + (18:2n-6)]
P_{C-3}	25.6	C-3 [(sat) + (18:1n-9) + (18:2n-6) + (18:3n-3)]
P_v	26.2–0.5	vinyl allylic [(18:2n-6) + 2(18:3n-3)]
P_α	28.0	allylic [2(18:1n-9) + 2(18:2n-6) + (18:3n-3)]

[a]sat, saturated.

where each peak area (P_n) is multiplied by its empirical correction factor to correlate quantitative and survey peak areas.

A quantitative summary of canola seed TAG chain compositions from MAS ^{13}C NMR and GC–FID is found in Table 2. Several factors complicate direct comparison of the ^{13}C and GC data. The GC and NMR data are from the same seed lines, but were not done using the same individual seeds. The NMR results are in mole percentages and the GC data are in weight percentages. The most common seed oil TAG (C-18 acyl chains) have similar molecular weights. In this case, mole percentage–weight percentage differences may be neglected. For seed oils with shorter and longer acyl chains, the mole percentage–weight percentage differences become significant. Inspection of the olefin region (Figs. 4 and 6) establishes the importance of long-chain monoenes. The sample handling procedures used in the NMR and chromatography methods could account for some of the differences in Table 2. ^{13}C NMR directly observes liquid TAG in seed oil storage vacuoles. Chromatographic analysis is from whole seed extracts and involves chemical derivatization. Despite these complications, the results affirm that MAS ^{13}C oil characterization is both a qualitative and quantitative method.

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A Rapid and Quantitative Method for Total Fatty Acid Analysis of Fungi and Other Biological Samples

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ABSTRACT: A quantitative method for the one-step esterification and determination of absolute amounts of polyunsaturated fatty acids in biotechnologically produced fungal mycelia is proposed. A system of two internal standards was used to quantify the total fatty acid content of the samples by gas chromatography. The degree of methylation of the analyzed fatty acids was calculated using the internal standards, and subsequently this quotient was used to validate the derivatization and extraction reactions. By utilizing this degree of methylation, the corrected amounts of the various fatty acids in the sample can be calculated.

Paper no. L8282 in *Lipids 34,* 1347–1349 (December 1999).

For the biotechnological production of polyunsaturated fatty acids (PUFA) by fungi, a fast and reliable method for determining the fatty acids in the mycelia is necessary for the optimization of the production process. That the handling procedures of such a method are simple is just as important as the calculation of absolute amounts of fatty acids in the sample in stating specific productivities and optimizing the production process.

Fatty acids are usually determined by gas chromatography (GC) using the corresponding methyl esters, prepared either by base- or acid-catalyzed esterification, depending on the composition of the samples (1–12). All these methods imply a time- and solvent-consuming extraction step prior to methylation. For small samples, a two-step analysis with extraction of the fatty acids followed by esterification is inaccurate because of loss of material during both steps. Consequently, a one-step reaction that forms a product that can be injected directly into the gas chromatograph is required. Recently, some one-step fatty acid analysis methods have been published (4–7). The reliability of the data resulting from these methods bears comparison with the standard two-step extraction and methylation procedures. The one-step methods have in common either that they do not state the total amount of fatty acids contained in the sample (4,6) or that a significant difference occurs between the total amounts indicated by the one- and two-step methods (5). Furthermore, the absence of a means to validate the chemical reactions involved in these procedures characterizes all these methods.

The use of an internal standard, which is added to the sample, quantifies the amounts of different fatty acids in the sample relating to the total amount of fatty acids that are determined (4,5). However, the results of the fatty acid quantification vary with the method used (7). Moreover, the determination of the exact amount of fatty acids is difficult owing to the loss of material during the chemical reactions or incomplete derivatization.

The objective of this study was to develop a fast and easy analytical method for the determination of fatty acids in small amounts of fungal mycelia. This procedure should be applicable as a standard method for a high number of samples and should determine lipids in both the polar and neutral fractions of the mycelium. In the present work, the use of a second internal standard is proposed as a means to calculate the degree of methylation of fatty acids in a fungal sample and, subsequently, the total fatty acid content of the sample. Concurrently the absolute amount of each single fatty acid contained in the sample can be estimated. Furthermore, the methylation method used can be verified with the suggested degree of methylation.

MATERIALS AND METHODS

All used solvents were of analytical grade and purchased from Merck (Darmstadt, Germany). Methanolic HCl (3 M) was supplied by Supelco (Bellefonte, PA).

External standards. A PUFA methylesters (PUFAME) kit (Supelco) containing 14 PUFAME (C_{14}–C_{24}) supplemented with four saturated FAME (14:0–20:0) and methyl γ-linolenate (18:3n-6) (ICN Pharmaceuticals, Costa Mesa, CA) was used as external standard. For calibration, a mixture containing 100 µg of each fatty acid in 1 mL hexane was used. The flame-ionization detector (FID) responses were processed by HP 3365 series II ChemStation (Hewlett-Packard, Palo Alto, CA) chromatography software. The concentration of each fatty acid in the sample was calculated in relation to the response factor of the internal FAME standard.

Internal standards. Methyl margarate (17:0; 100 mg) dissolved in 1 mL hexane was used as internal FAME standard (IS 1), and 50 mg pentadecanoic acid (15:0) in 1 mL di-

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Abbreviations: FAME, fatty acid methyl ester; FID, flame-ionization detector; GC, gas chromatography; PUFA, polyunsaturated fatty acid; PUFAME, PUFA methyl ester.

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ethylether as internal fatty acid standard (IS 2), respectively, both purchased from Merck. One microliter of each standard solution was added to the sample.

Sample preparation. The filamentous fungus *Mucor circinelloides* CBS 108.16 (Centraal Bureau voor Schimmelcultures, Delft, The Netherlands) was cultivated in 500-mL Erlenmeyer flasks with 200 mL of glucose-containing growth medium for 3 to 7 d. The mycelium was filtered (Schleicher & Schüll filter paper, Dassel, Germany), washed twice with distilled water, and then immediately freeze-dried.

Direct esterification. At least three derivatization experiments per sample culture were made, each of which was analyzed in duplicate by means of GC. Of each sample culture approximately 5, 10, and 20 mg of lipid-containing, freeze-dried mycelium was precisely weighed and placed in a Pyrex® tube with a polytetrafluoroethylene screw-cap. Internal fatty acid standard IS 2 (50 µg) was added to the sample. Then 1 mL methanolic HCl (3 M) and 1 mL methanol were added to the sample in order to esterify the fatty acids. Finally, 0.5 mL hexane was added to aid the extraction of FAME from the mycelial tissue. After thorough mixing, the tubes were heated at 100°C for 1 h and shaken several times. The samples were cooled to ambient temperature and diluted with 2 mL hexane to achieve a suitable concentration for analyzing by GC. Internal FAME standard IS 1 (100 µg) was added to the sample. Afterward, 2 mL H_2O was added and carefully mixed to dissolve chloride ions (vigorous shaking of the sample causes gel formation and therefore loss of the sample). After phase separation, the upper organic layer was drawn off and an aliquot of 1 µL of this sample was analyzed by GC.

GC. A HP 5890 series II gas chromatograph equipped with FID (Hewlett-Packard) and WCOT fused-silica CP-WAX 52 CB capillary column, 25 m, 0.32 mm i.d., 0.2 µm film thickness (Chrompack, Middelburg, The Netherlands) was used. N_2 was the carrier gas, at a column head pressure of 35 kPa. Detector and injector temperatures were both 220°C. A temperature gradient was run from 160 to 190°C at 1°C/min. A sample amount of 1 µL was injected manually with split injection.

Degree of methylation. The degree of methylation (D_M) was calculated by analyzing the amount of methyl pentadecanoate ($C15_{FAME}$) of each sample and dividing it by the known amount of pentadecanoic acid ($C15_{FA}$), which was added to the sample.

$$D_M = \frac{[C15_{FAME}]}{[C15_{FA}]} \quad [1]$$

The D_M value was used as a validation factor to calculate the amounts of the various fatty acids contained in the sample cultures analyzed. The mean values of the amounts of each corrected fatty acid were summed to calculate the total amount of fatty acids in a sample culture.

RESULTS AND DISCUSSION

Because the determination of total amounts of a product is very important in fermentation technology, a system of two internal standards was developed to quantify the total content of saturated and unsaturated C_{14}–C_{18} fatty acids in fungal samples. Methyl margarate was used as internal standard (IS 1) to identify the detected peaks and simultaneously to calculate the individual response factors of the single fatty acids. As a second internal standard (IS 2), pentadecanoic acid was added to the sample before starting the derivatization. This fatty acid is esterified with the sample and is used for validation purposes assuming that the internal fatty acid standard (IS 2) is esterified in the same way as all other fatty acids in the sample. In contrast to enzymatic reactions, for example, there is only minimal dependency of the reaction kinetics on the chain length of the fatty acids examined. The reactivity of the derivatizing agents depends on the reactivity of the fatty acid carboxyl group, particularly on the p*K* value, which varies slightly within chain lengths from 14 to 18 carbon atoms (13).

A mixture of methanolic HCl/hexane (4:1, vol/vol) is used to combine the esterification step with the extraction step. After the GC analysis, the degree of methylation can be calculated by dividing the amount of analyzed methyl pentadecanoate by the amount of pentadecanoic acid initially added to the sample (Eq. 1). Thus this quotient can be used as a validation factor for both reaction steps of the combined method. In the case of incomplete methylation, the factor corrects the amount of analyzed fatty acids. As the more lipophilic FAME are mainly extracted by hexane, an insufficient extraction of the methyl esters can similarly be corrected by this factor to calculate the real amounts of the various fatty acids in the sample.

More than 60 different samples of freeze-dried mycelia of *M. circinelloides* were analyzed several times by GC with the proposed method. All FAME in the samples were completely separated by GC.

The proposed method was validated by analyzing (i) different sample quantities (5–25 mg), (ii) samples with different total amounts of fatty acids [3–11% (w/w)], and (iii) samples with different fatty acids compositions. As a selection of analyzed mycelia in Table 1 shows, the D_M correlated with none of these three factors.

TABLE 1
Total Amounts of Determined[a] and Corrected Fatty Acids and Degrees of Methylation (D_M) in Mycelia of Different Selected Cultures of *Mucor circinelloides* CBS 108.16

Sample culture	Total fatty acids (mg/$g_{dry\ mass}$)		
	Determined[b]	Corrected[b]	Mean D_M[b]
A	21.95 ± 1.30	31.03 ± 1.49	0.709 ± 0.050
B	41.65 ± 4.82	60.16 ± 4.18	0.691 ± 0.048
C	50.88 ± 1.79	59.41 ± 3.40	0.831 ± 0.047
D	52.02 ± 5.48	71.97 ± 1.73	0.722 ± 0.063
E	63.64 ± 5.80	84.77 ± 5.81	0.758 ± 0.114
F	77.91 ± 4.60	112.17 ± 8.85	0.701 ± 0.089

[a]Data achieved with proposed one-step esterification and extraction method.
[b]Values are means ± standard deviations of six experiments.

It can be deduced from the foregoing results that a combined esterification and extraction method without validation is not suitable for the fatty acid analysis of small amounts of fungal mycelia, as the degree of methylation of the fatty acids in the sample is not constant. Therefore, one cannot assume that all fatty acids will be esterified or extracted completely. However, no obvious explanation for the different degrees of methylation could be drawn as all experiments were carried out under identical conditions.

For the reasons discussed, a validation of a one-step esterification and extraction method, as proposed in this work, is imperative for the standard analysis of fungal fatty acids in order to achieve reliable results.

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Essentiality of Fatty Acids

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ABSTRACT: All fatty acids have important functions, but the term "essential" is applied only to those polyunsaturated fatty acids (PUFA) that are necessary for good health and cannot be completely synthesized in the body. The need for arachidonic acid, which is utilized for eicosanoid synthesis and is a constituent of membrane phospholipids involved in signal transduction, is the main reason why the n-6 class of PUFA are essential. Physiological data indicate that n-3 PUFA also are essential. Although eicosapentaenoic acid also is a substrate for eicosanoid synthesis, docosahexaenoic acid (DHA) is more likely to be the essential n-3 constituent because it is necessary for optimal visual acuity and neural development. DHA is present in large amounts in the ethanolamine and serine phospholipids, suggesting that its function involves membrane structure. Because the metabolism of n-6 PUFA is geared primarily to produce arachidonic acid, only small amounts of 22-carbon n-6 PUFA are ordinarily formed. Thus, the essentiality of n-3 PUFA may be due to their ability to supply enough 22-carbon PUFA for optimal membrane function rather than to a unique biochemical property of DHA.

Fatty acids carry out many functions that are necessary for normal physiological function and good health. Saturated fatty acids are involved in energy production, energy storage, lipid transport, the synthesis of phospholipids and sphingolipids needed for membrane synthesis, and the covalent modification of many regulatory proteins. Monounsaturated fatty acids also are involved in many of these processes and play a key role in maintaining optimal fluidity of the membrane lipid bilayer. Although these are clearly vital physiological processes, the term "essential" is not applied to the saturated or monounsaturated fatty acids. The designation is reserved for those polyunsaturated fatty acids (PUFA) that are required for good health and, in addition, cannot be completely synthesized in the body. As it is presently used, essentiality implies that the fatty acid not only performs a vital function, but it also is a required dietary nutrient.

Essential fatty acids. PUFA have important effects on the structure and physical properties of localized membrane domains and, in addition, are involved in eicosanoid production, signal transduction, and the activation of nuclear transcription factors. Two classes of PUFA, designated as n-6 and n-3, normally are present in the tissues and body fluids. Neither of these classes can be completely synthesized by mammalian cells and, therefore, they must be obtained from the diet. The usual Western diet contains 10- to 20-times more n-6 than n-3 PUFA, and the plasma and most other tissues also contain 10- to 20-times more n-6 fatty acid. The exceptions are the brain and retina which are rich in n-3 PUFA.

A very serious systemic illness called essential fatty acid deficiency occurs if the body becomes deficient in n-6 PUFA. A similar debilitating disease does not occur if there is a deficiency in n-3 PUFA. However, accumulating evidence indicates that n-3 PUFA are required for optimal health, especially for normal neural development and visual function (1,2), and there is an increasing consensus that they, too, are essential fatty acids.

Mechanism of action of the essential fatty acids. Table 1 lists the biochemical actions of the key members of the n-6 and n-3 classes of essential fatty acids. The need for arachidonic acid (20:4n-6) almost certainly is the primary reason why n-6 PUFA are essential. Arachidonic acid is the main substrate for synthesis of the eicosanoid mediators produced by the cyclooxygenase, lipoxygenase, and cytochrome P450 pathways (3), and it is highly enriched in the inositol phospholipids that are involved in signal transduction (4). Linoleic acid (18:2n-6) also probably is essential because it is utilized for the synthesis of the complex lipids that form the permeability barrier of the epidermis (5).

The biochemical reason that the n-3 PUFA are essential is not presently known. In fact, it is not certain whether eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), or both carry out the essential biochemical functions. Like arachidonic acid, EPA (20:5n-3) is a substrate for eicosanoid synthesis (6), and EPA competes with arachidonic acid for the same metabolic pathways (7,8). However, no requirement for the eicosanoids produced from EPA has so far been demonstrated, and very little EPA is present in the tissues of individuals who consume the usual Western diet. Therefore, competition with arachidonic acid seems improbable unless an individual is consuming supplemental amounts of EPA. Under ordinary conditions, DHA (22:6n-3) is the most abundant n-3 PUFA contained in the tissues, especially in the brain and retina. For this reason, it seems more likely that DHA rather than EPA is the essential n-3 component.

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Abbreviations: DHA, docosahexaenoic acid; n-6 DPA, n-6 docosapentaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid(s).

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TABLE 1
Biochemical Functions of the n-6 and n-3 Fatty Acids

Class	Fatty acid	Function
n-6	Arachidonic	Eicosanoid synthesis Component of the inositol phospholipids
n-6	Linoleic	Synthesis of lipids that form the epidermal permeability barrier
n-3	Eicosapentaenoic	Eicosanoid synthesis Structural analog and competitor of arachidonic acid
n-3	Docosahexaenoic	Structure of membrane lipid domains Modulation of integral membrane proteins Metabolism of phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidylserine Formation of free radicals Regulation of gene expression

DHA function. Considerable effort is being expended to determine the biochemical functions of DHA, and some recent progress has been made. DHA is contained primarily in three phospholipids, phosphatidylethanolamine, ethanolamine plasmalogens, and phosphatidylserine (9,10). This suggests that it may affect the trafficking or metabolism of these phospholipids, or it may produce structural changes in regions of the membrane lipid bilayer that are enriched in these phospholipids (11). Numerous effects produced by DHA are consistent with a membrane lipid effect. For example, DHA modulates the carrier-mediated transport of choline, glycine and taurine (12–14), the function of delayed rectifier potassium channels (15), and the response of rhodopsin contained in phospholipid vesicles (16). Specific interactions of phospholipid fatty acyl chains with membrane proteins could account for each of these effects. Other actions of DHA include the formation of free radicals in response to oxidative stress (17), transcriptional activation of genes (18), and the prevention of apoptosis (19). One or more of these effects probably constitutes an essential biochemical or developmental function and, hence, the molecular reason why DHA is essential.

Elongation and desaturation of n-3 and n-6 fatty acids. The same elongation and desaturation pathway involving 24-carbon intermediates and peroxisomal retroconversion is utilized by n-3 and n-6 PUFA (20). Studies of this process in cultured brain cells indicate that this pathway operates primarily in astrocytes and that the astrocyte pathway handles n-3 and n-6 PUFA differently (21). These differences are illustrated in Figure 1. DHA is the main product formed when the astrocytes are incubated with [1-^{14}C]linolenic acid, whereas arachidonic acid is the main product when they are incubated with an equivalent amount of [1-^{14}C]linoleic acid (21). DHA and arachidonic acid also are the main products released into the extracellular fluid during these incubations. About 20-times more DHA than n-6 docosapentaenoic acid (n-6 DPA), the corresponding 22-carbon fatty acid of the n-6 series, is formed by the astrocytes under these conditions. Furthermore, 10-times more radiolabeled DHA than n-6 DPA accumulates in the astrocytes, and 45-times more DHA is released into the medium.

Additional studies indicate that neurons cultured under similar conditions do not produce either DHA or DPA from n-3 or n-6 PUFA precursors, respectively (21). Therefore, the neurons apparently are dependent on an external source for these fatty acids, and it seems likely that they are supplied at least in part by release from the astrocytes (22). As described above (21), the astrocytes produce and release much more DHA than w-6 DPA. Taken together, these metabolic results probably explain why the brain accumulates DHA rather than n-6 DPA even though n-6 PUFA are usually much more abundant.

Basis for n-3 fatty acid essentiality. It is generally assumed that the n-3 class of PUFA is essential because the function of one of its components, probably DHA, is necessary and cannot be fully replaced by any other fatty acid. Because n-6 DPA has the same structure as DHA except for the Δ19 double bond, the unique action of DHA must somehow involve the methyl-end of its hydrocarbon chain.

While this remains the most likely biochemical explanation for the essentiality of the n-3 fatty acids, an alternative possibility should be considered in view of the astrocyte results

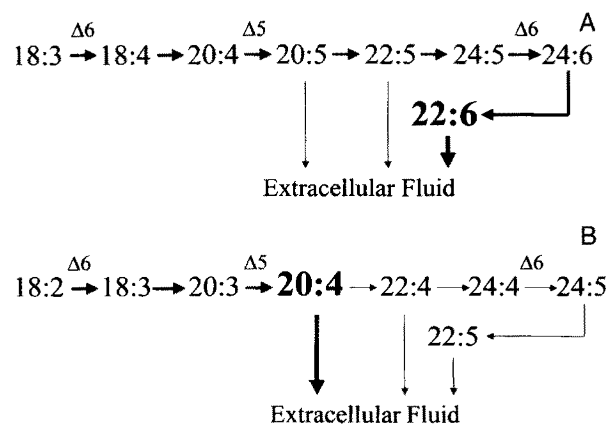

FIG. 1. Operation of the polyunsaturated fatty acid metabolic pathway in murine brain astrocyte cultures. A) illustrates the function of this pathway with n-3 fatty acids, B) with n-6 fatty acids. The fatty acids and arrows shown in bold type signify the main products that are formed and released to the extracellular fluid. Thus, docosahexaenoic acid (22:6) is the most abundant n-3 fatty acid product formed and released, whereas arachidonic acid (20:4) is the main n-6 product.

(21,22). It is possible that the fundamental need of the central nervous system is for a 22-carbon, highly unsaturated fatty acid containing a Δ4 double bond. Even though n-6 PUFA precursors are more abundant, enough n-6 DPA cannot be formed in the brain to fulfill this requirement because the astrocyte metabolic pathway utilizes n-6 PUFA primarily to produce arachidonic acid. By contrast, the pathway synthesizes mainly 22-carbon fatty acids from n-3 precursors, and DHA is the main product. Thus, n-3 PUFA may be essential because they are the only class that can generate a sufficient amount of a 22-carbon, unsaturated fatty acid containing a Δ4 double bond to satisfy the needs of the central nervous system.

The argument against this metabolic hypothesis is that although n-6 DPA replaces DHA when there is an n-3 PUFA deficiency, it does not completely overcome the functional deficit. However, recent findings in the rat pineal gland indicate that even though n-6 DPA increases during n-3 PUFA deficiency, it does not fully replace DHA (23). Although the decrease in total n-3 PUFA was almost entirely replaced by n-6 PUFA, arachidonic acid and docosatetraenoic acid (22:4n-6) accounted for about 50% of the replacement. There was a 12-fold increase in n-6 DPA, but the total pineal lipids contained only about half as much n-6 DPA as compared with the amount of DHA normally present. The reduction in n-6 DPA relative to the normal amount of DHA was especially evident in molecular species of phosphatidylethanolamine and ethanolamine plasmalogen that contain stearic acid.

Based on these observations, it appears that the functional abnormalities in n-3 PUFA deficiency may be due to an inability to fully replace the loss of DHA with n-6 DPA. If so, the quantitative differences in the products formed by the elongation and desaturation pathway with n-3 and n-6 fatty acids may be the underlying biochemical reason why n-3 PUFA are essential.

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Sphingolipid Metabolism in the Regulation of Bioactive Molecules

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A living organism is an extraordinary complex system in which harmony and thus communication between parts is one of the most important goals to pursue. Communication within an organism occurs at different levels: intercellularly and intracellularly. In both cases one of the main targets involved in this process is the cellular membrane. From or through this compartment of one given cell, a wide variety of signals can be sent either to interact with other cellular units or to specifically direct the behavior of the same given cell. Important players in this communication system are not only the proteins present in the membrane but interestingly also the lipid counterpart. In this way the concept of lipids as bioactive molecules and not only as structural components of the membranes has evolved. At first, this concept was mainly applied to the glycerolipid class, such that the signal transduction pathways regulated by enzymes of glycerolipid metabolism are now well defined (1). More recently another class of lipids has been proposed to play a key role in the cellular communication system: the sphingolipids.

SPHINGOLIPIDS: STRUCTURE AND METABOLISM

Sphingosine and ceramide are the two structurally simplest molecules belonging to the sphingolipid family, from which all other complex sphingolipids derive (2). Sphingosine represents the sphingoid backbone, and ceramide carries the fatty acyl group in amide linkage to the sphingoid moiety. The wide variety of the naturally occurring sphingolipid species is due to the different chain length of either the sphingoid unit or the fatty acyl moiety, and to the nature of the functional group at the 1-position. For instance, sphingomyelin (SM) carries a phosphorylcholine moiety, the cerebroside subclass carries sugar residues (such as glucose and galactose), and the gangliosides contain acidic sialic acid residues in addition to the sugar units (3).

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Abbreviations: A-SMase, a lysosomal acid SMase; DAG, diacylglycerol; GSH, glutathione; ICE, interleukin-converting enzyme; N-SMase, neutral magnesium-dependent; PC, phosphatidylcholine; PKC, protein kinase C; PLC, phospholipase C; Rb, retinoblastoma gene product; SM, sphingomyelin; SMase, sphingomyelinase; TNFα, tumor necrosis factor α.

Sphingolipid metabolism includes a series of biosynthetic and catabolic reactions in which ceramide plays a very central role (Scheme 1). The *de novo* synthesis starts with the condensation of palmitoyl-CoA with serine (forming 3-ketosphinganine) through the rate limiting action of serine palmitoyl transferase (3). Reducing and acylating reactions, through a NADPH-dependent reductase and ceramide synthase, then follow yielding ceramide formation probably by the final action of a dehydrogenase (4). At this point there are multiple fates of the newly synthesized ceramide. Ceramide can be converted into SM through the transfer of the choline phosphate group from phosphatidylcholine (PC) through the action of PC:ceramide phosphocholine transferase (SM synthase) (5,6). On the other hand, ceramide can be utilized as a precursor for cerebrosides and gangliosides through the sequential addition of carbohydrate units (from sugar nucleotide donors) and/or sialic acid residues (from CMP-*N*-acetylneuraminic acid donors) (7). Moreover, ceramide can be phosphorylated at the 1-position through the action of a distinct ceramide kinase yielding ceramide-1-P (8).

In sphingolipid catabolism clearly ceramide plays once again a crucial role. In fact, ceramide is the breakdown product of complex sphingolipids which are sequentially hydrolyzed in their head groups. Likewise, ceramide is the product of SM catabolism through the action of different sphingomyelinases (SMase), which hydrolyze the phosphocholine head group releasing choline phosphate (9). Ceramide, in turn, is the target of ceramidases which cleave off the fatty acyl chain, yielding sphingosine and its phosphorylated metabolite sphingosine-1-P (10).

SPHINGOLIPIDS AS SECOND MESSENGERS

As evidenced from the previous description, the web of sphingolipid metabolism is complex and leads to a large variety of molecules. In spite of this, it is only recently recognized that many of these metabolites have a role as bioactive molecules. The demonstration of inhibition of protein kinase C (PKC) activity by sphingosine was the first step in this direction (11). The discovery of SM hydrolysis and ceramide formation in response to certain stimuli represented a further move into the understanding of sphingolipid biology (12). Nowadays, significant effort is directed at the elucidation of the role of ceramide.

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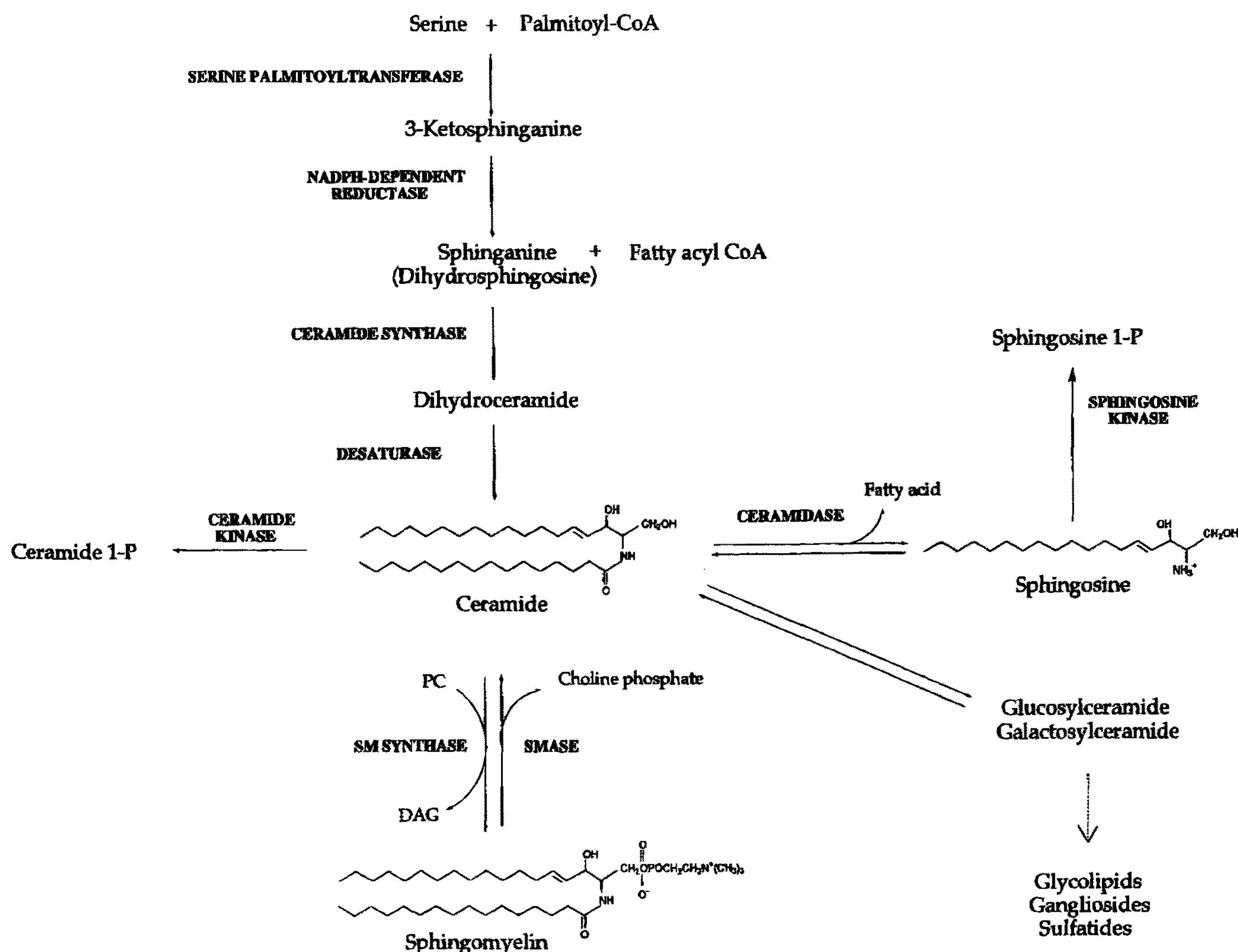

SCHEME 1. Shown is a schematic of the blueprint of sphingolipid metabolism focusing on the earlier steps leading to and from ceramide and on the steps that relate ceramide to sphingosine, sphingosine 1-phosphate, and DAG. Abbreviations: PC, phosphatidylcholine; DAG, diacylglycerol; SM, sphingomyelin.

sphingosine, and sphingosine 1-P as key regulators of these emerging signal transduction pathways.

Growth regulation by ceramide. Ceramide accumulates in response to several different inducers such as cytokines [tumor necrosis factor α (TNFα), interleukin β1 (ILβ1), interferon-γ (Infγ) and Fas], cytotoxic agents (chemotherapeutic agents, irradiation), and finally stressful conditions (such as serum deprivation and heat shock) (13). The general response to these stresses is a growth-suppressed condition which can be due either to cell cycle arrest or to apoptosis. The choice between the two fates is determined mostly by the cell type. Cellular treatments with exogenously added short-chain ceramide analogs (C2-, C6-ceramide) mimic the same biological effects observed when natural stress inducers are used. The effects of ceramide on cell cycle arrest were studied mostly in response to serum deprivation (14). This condition induced elevation of intracellular ceramide level with time, a progressive arrest of cell cycle in the G0-G1 phase and dephosphorylation of the retinoblastoma gene product (Rb). Treatment with exogenously added ceramides also induced dephosphorylation of Rb (15). On the other hand, ceramide-mediated apoptosis was studied upon treatment with several different agonists, in particular with TNFα and after addition of short-chain ceramide analogs. The latter treatment was able to induce DNA fragmentation similar to TNFα treatment (16). Also recent studies established the involvement of pro-interleukin-1 converting enzyme (ICE) family members upstream (such as FLICE) and downstream (such as caspase 3) to ceramide signaling (17). Interestingly, activation of the PKC pathway by treatment with phorbol esters seems to enhance viability and thus to counteract ceramide mediated apoptosis (16). Importantly, treatment with exogenous ceramide increased proliferation of quiescent Swiss 3T3 fibroblasts, indicating that eventually ceramide may positively regulate cell proliferation (18).

Regulation by sphingosine and sphingosine-1-P. Sphingosine was first discovered to be a PKC inhibitor (11), and in line with this observation it has been shown to inhibit cell growth (19). Moreover, sphingosine was implicated as endogenous me-

diator of apoptosis during phorbol ester-induced differentiation of HL-60 cells (20). On the other hand, sphingosine stimulated the proliferation of growth-arrested Swiss 3T3 (21) and Rat-1 fibroblasts (22). The mitogenic effects exerted by sphingosine may be due to its conversion to sphingosine-1-P (23). One of the mechanisms by which sphingosine and sphingosine-1-P may elicit mitogenic effects could be the induction of phosphatidic acid accumulation (19). This accumulation could be the result of diacylglycerol (DAG) kinase activation (24), inhibition of phosphatidic acid phosphohydrolase (25), and most importantly activation of phospholipase D (26).

Other metabolites. Other sphingolipid metabolites, such as ceramide 1-P (8) and GD3 gangliosides (27), seem to be also bioactive molecules in the regulation of cellular proliferation and/or apoptosis, but further investigations are in process.

The web of sphingolipid signaling. In addition to the general paradigm that ceramide negatively regulates cell growth, it is clear that there are several interrelated events, such as competing functions of ceramide, sphingosine, sphingosine-1-P, ceramide-1-P, gangliosides and DAG, which create a more complex picture. The emerging scenario is best understood as an intricate web of interconnected metabolic reactions, which modulate the relative intracellular concentrations of the several different species. These metabolic pathways may play a critical role in the fate of a signaling pathway by differential regulation of the key enzymes. So, in order to better understand the biological behavior of a given pathway as part of a more general picture, it becomes extremely important to study and seriously characterize each enzymatic activity and try to relate it to the cellular environment, in terms of inhibitors or activators.

ENZYMES OF SPHINGOLIPID SIGNALING

Until now, great efforts have been spent in order to better understand the biological functionality of the sphingolipid pathway. On the other hand, this approach cannot replace investigations directed to study the biochemical characteristics of the reactions themselves, and thus of their regulatory enzymes. Researchers normally find several obstacles when approaching the problem. First, sphingolipid metabolism is based on a number of interconnected reactions; different enzymes may share the same precursor or lead to the same product, possibly influencing each other. In this case it is difficult to discriminate between enzymatic activities. Second, very few enzymes of the pathway have been purified and cloned; thus most of the studies conducted *in vitro* use mixed preparations (homogenates or membrane pools) which may yield misleading results. The involvement of different enzymes regulating the levels of specific sphingolipid species in response to external stimuli is becoming more and more evident. In this report, we will be focusing on a few of them, such as ceramide synthase, sphingomyelinases (SMase), ceramidases, and SM synthase.

CERAMIDE SYNTHASE

Ceramide synthase catalyzes the acylation of long-chain sphingoid bases by transfer of a fatty acid from the CoA thioester (28). This activity is localized in the endoplasmic reticulum, with the catalytic site thought to face the cytosol and an *in vitro* pH optimum of 7.4 (29). The most popular approach followed to study the role and biological importance of ceramide synthase is the use of specific inhibitors. Since ceramide synthase is one of the early enzymes involved in sphingolipid *de novo* synthesis, eventual *in vivo* experimental manipulations of this activity may result in a general alteration of sphingolipid metabolism. One may predict that inhibition of ceramide synthase activity would lead either to sphinganine accumulation and/or loss of complex sphingolipids. In many cell types both free sphinganine and specific inhibition of sphingolipid metabolism are growth inhibitory and cytotoxic. It follows that results obtained by altering/inhibiting ceramide synthase activity may not be easy to interpret. Among the inhibitors, fumonisin B1 is the best studied (29). Treatment with fumonisin B1 mainly induces accumulation of sphinganine, which is then partially phosphorylated to sphinganine-1-P. The most rapid intracellular effect of fumonisin B1 treatment is sphinganine elevation rather than depletion of complex sphingolipids, but the relative magnitudes of these two effects vary between cell types (30). Fumonisin B1 treatment alters the behavior of cell-surface proteins and changes cell morphology, inhibits PKC, and induces Rb dephosphorylation and cell cycle arrest (29). On the other hand, fumonisin B1 was shown to induce cell proliferation in growth-arrested Swiss 3T3 cells (28). Moreover, fumonisins were recognized to play an important role in the induction of different diseases such as esophageal cancer, equine leukoencephalomalacia, and porcine pulmonary edema (31).

SMASE

SMase is a SM-specific phospholipase C (PLC)-type activity, hydrolyzing SM phosphodiester bond and yielding ceramide and phosphorylcholine. Evidence for the importance of SMase activity arose with the discovery of the so-called "sphingomyelin cycle." Several different inducers (TNFα, INFγ, IL-1β, nerve growth factor, Fas ligand, dexamethasone, and others) were found to cause SM hydrolysis in a variety of cell types (monocytes, lymphocytes, fibroblasts, glioma cells) (13). SM hydrolysis leads to ceramide production which mediates at least part of the biological effects exerted by these inducers. SM is then regenerated by the action of the SM synthase. So far, five types of SMases have been described: a lysosomal acid SMase (A-SMase), a plasma membrane-bound neutral magnesium-dependent (N-SMase), a cytosolic neutral magnesium-independent, a zinc-stimulated acid SMases and finally an alkaline SMase. At least two of these SMases, the A-SMase, and the N-SMase, may regulate ceramide intracellular levels (32).

N-SMase. The N-SMase activity can be detected in a variety of organs even if its distribution is not uniform. For example, in rat, the highest activity can be found in brain; less activity is found in testis, adrenal gland, liver, spleen, and kidney (9). Subcellular fractionation studies showed that N-SMase is a plasma-membrane bound enzyme with a possible intracellular

orientation (33). Its activity requires the presence of magnesium and its optimal pH values range between 6.0 and 8.5. Phospholipids (in particular phosphatidylserine (34)) and free arachidonic acid can stimulate N-SMase activity (35). *In vitro* (direct enzymatic assay) and *in vivo* (changes in SM and ceramide levels) measurements of N-SMase activity showed enzyme activation after cellular treatments with inducers of the SM cycle (9). The N-SMase time-course activation varies between cell lines, such that after TNFα treatment of monocytic U937 cells, human skin fibroblasts and mesangial cells a peak of activity is reached after few minutes (2–60 min), while in murine L929 fibro-blasts a significant increase is evident only after several hours (12 h) (13). Studies conducted using a potent ICE inhibitor (crm A) seem to suggest a possible role of this family of proteases in the events that lead from TNFα/receptor interaction to N-SMase activation (17). Recently another forwarding step toward a better understanding of the biochemical/biological N-SMase regulation has been made. Remarkably, glutathione (GSH), at concentrations similar to intracellular levels, was shown to actively modulate N-SMase activity (36). *In vitro* studies on a partially purified N-SMase showed that physiologic intracellular GSH concentrations (1–20 mM) were inhibitory of the enzymatic activity, whereas at lower GSH concentrations, N-SMase activity was recovered. The GSH inhibitory effect was not due to the sulfhydryl group as other general reducing agents used (dithiothreitol, β-mercaptoethanol) were ineffective. Instead, the γ-glutamyl-cysteine moiety of GSH seems to be required. Moreover, cellular treatment with L-buthionine-(*SR*)-sulfoximine, a GSH synthesis inhibitor, caused, as expected, intracellular GSH depletion and induced the SM cycle. Since GSH depletion is a general cellular phenomenon observed in response to stressful conditions, it can be an important mechanism of N-SMase activation, while under physiological conditions GSH would control N-SMase activity. GSH/N-SMase model is also a useful example of a regulatory system involving cross-talk between different cellular areas implicated in stress signaling. Finally, the recent reported cloning of the enzyme will be extremely helpful in the near future to get a better understanding of the processes just discussed (37).

A-SMase. A-SMase is distributed in all mammalian tissues analyzed, and compared to the N-SMase, A-SMase activity is much higher (except for the brain) (32). Its pH optimum sets around 5.0, and its activity localizes with the lysosomal fraction (9). A-SMase was the first of the SMases to be described, purified, and cloned (38). A-SMase defect was found to be the biochemical determinant of Type A and Type B Niemann-Pick disease, resulting in SM accumulation and causing neurological disorders (32). Divalent ions and chelating agents do not affect the activity of this enzyme, while dithiothreitol and AMP are inhibitors (9). On the other hand, ApoC-III, an apolipoprotein from human very low density lipoprotein and a group of proteins named saposins (sphingolipid activator proteins), in particular saposin D, stimulate A-SMase activity (9). The biological role of A-SMase, and in particular its importance in the regulation of the apoptotic signal, is still controversial. Schutze *et al.* (39) reported A-SMase activation after TNFα treatment: through the activation of a putative PC-specific PLC, TNFα induced DAG release, turning on A-SMase. A-SMase has been proposed also to mediate Fas-induced apoptosis (40). On the other hand, evidences against the involvement of A-SMase in the TNFα and IL-1β induced SM cycle were reported by other authors (41). Finally, A-SMase knock-out mice have reproduced a phenotype similar to the type A Niemann-Pick disease and they showed resistance to radiation-induced apoptosis (62).

CERAMIDASES

Ceramidase activity hydrolyzes ceramide into free sphingoid base (sphingosine) and fatty acid. Therefore, it may represent a very important tool in order to modulate the switch between ceramide- and sphingosine-regulated biology. In spite of this potential major role, not much is known about their biochemical and biological regulation even if some of the forms have been purified and cloned. In the literature, different forms of ceramidases have been reported, mainly based on different pH profiles.

Acid ceramidase. Acid ceramidase activity is assumed to be ubiquitous (rat brain, liver, kidney, and human skin fibroblasts, spleen, kidney, brain, leukocytes) and to play a housekeeping role being localized in the lysosomes (42). Its optimal pH ranges between 4.0 and 5.0. This type of activity was the first ceramidase to be purified and cloned (43). Acid ceramidase activity does not seem to be affected by phospholipid addition, while saposin D was shown to activate *in vivo* and less clearly *in vitro* (44). On the other hand, *N*-oleoylethanolamine is recognized as an acid ceramidase inhibitor even if its inhibitory effect is rather weak and at very high concentrations (44). Substrate specificity studies showed preference for ceramides with unsaturated fatty acids (45); moreover, the preferred fatty acyl chain would carry 12 carbons, while increasing or decreasing chain length would result in loss of activity (46). The main biological significance for acid ceramidase is its association with lipogranulomatosis or Farber's disease, evolving when this ceramidase activity is impaired (42). Interestingly, this kind of disease is also observed when normal saposin D is absent (47).

Alkaline ceramidase. Less is known about alkaline ceramidase. Its activity was reported in rat cerebellum and liver, in human fibroblasts and brain, in guinea pig skin, and porcine epidermis (44). It is a membrane-bound enzyme, possibly localized in the plasma membrane, with an *in vitro* pH optimum ranging between 8.0 and 9.0 (44). Nowadays, two alkaline ceramidases were purified from guinea pig skin (48): the first one (CDase I) purified to homogeneity with pH optimum around 7–10, the second (CDase II) partially purified with pH optimum around 8–9. Contrary to acid ceramidase, saposin D, as well as the A, B, and C forms, do not have any effect on alkaline activity (49). On the other hand, a ceramide analog (1*S*,2*R*)-D-erythro-2-(*N*-myristoylamino)-1-phenyl-1-propanol was shown to be a potent inhibitor of alkaline ceramidase in HL-60 cells (at lower concentrations than *N*-oleoylethanolamine), with no effect on acid ceramidase activity (50). The implication of alkaline ceramidase in mediating sphingolipid signaling became evident when the cellular response to growth factors ([Platelet Derived Growth

Factor (PDGF), Fibroblast Growth Factor (FDF), Epidermal Growth Factor (EDF)] was investigated in Swiss 3T3 and vascular smooth muscle cells (44). In this model, ceramide degradation and sphingosine accumulation accompanied the growth factor- induced proliferative effect. Induction of alkaline ceramidase by growth factors seems to be subsequent to tyrosine kinase phosphorylation (44). Cytokines (TNFα, IL-1β), on the other hand, are more directly involved in SMase induction and do not stimulate ceramidases activation.

Neutral ceramidase. Neutral ceramidase activity is poorly characterized from every point of view. Ceramidase activity, ranging in neutral pH, is reported in rat liver plasma membrane and microsomes and rat intestinal brush border membrane (44). One problem in studying neutral and alkaline ceramidases is the rather broad range of pH in which these enzymes work, so that it is difficult to discriminate between the two forms considering only this parameter. On the other hand, the high affinity shown by the neutral ceramidase toward very short- (C2) or long-chain ceramides (C16) represents a tool which can be efficiently used for this purpose (51). Until now, there are no reports on neutral ceramidase involvement in signal transduction events.

SM SYNTHASE

Mechanisms of *in vivo* SM biosynthesis have been somewhat controversial over the past years, and different biochemical routes have been proposed. At first, CDP-choline was suggested to be the donor of the phosphocholine moiety to ceramide (52); then, the acylation by stearoyl-CoA of sphingosylphosphorylcholine was considered (53). Finally, *in vitro* (5,6) and most importantly *in vivo* cellular labeling studies (6,54) established the existence of a preferred biochemical pathway which transfers the phosphorylcholine unit directly from PC to ceramide. This reaction is catalyzed by the PC:ceramide phosphocholine transferase (SM synthase) and leads to SM and DAG formation. SM synthase activity has been reported in mouse and rat kidney, lung, liver, and spleen and in monkey liver and heart (5,32,55); on the other hand, in the brain, SM synthesis may pass through phosphatidylethanolamine instead of PC (5). Subcellular fractionation studies characterized SM synthase as a membrane-bound enzyme with an intracellular orientation of the active site (56). In liver most of the activity seems to be localized in the Golgi apparatus (>80%), with the remainder mainly distributed between plasma membrane and endoplasmic reticulum (55). On the other hand, SM synthase localization in cellular models shows more variability. For instance, it was reported that in SV40-transformed fibroblasts (57), oligodendrocytes (58), and BHK cells (59), plasma membrane SM synthase shows a higher relative activity compared to the one that resides in the Golgi. *In vitro* studies indicated no strong dependency of the activity on the presence of divalent cations in the incubation mixture (6). The enzyme shows a pH optimum around 7.4 when Tris buffer is used and around 6.5 in the presence of imidazole buffer (5). SM synthase is one of the less-studied enzymes along the sphingolipid pathway, and little is known about its involvement in ceramide-mediated signal transduction events. On the other hand, because of the direct regulation of ceramide and DAG levels, its biological importance in directing a cell toward cell cycle progression or growth arrest is more than reasonable. Elevation of SM synthase activity (seven- to ninefold) was recently observed in malignant conditions, such as Morris hepatomas (60). Moreover, recent studies (60) showed that SV40-transformation induced a significant increase of SM synthase activity in human lung fibroblasts. *In vivo* metabolic labeling with a short-chain ceramide analog and *in vitro* measurements showed a twofold increase of SM synthase activity in SV40-transformed fibroblasts compared to the normal counterpart. Interestingly, when elevation of a specific pool of cellular ceramide, localized in the plasma membrane, was induced by bacterial SMase treatment, normal fibroblasts were completely unable to produce SM from it, while the SV40-transformed cells actively metabolized this ceramide, converting it back to SM. These results may suggest the possibility that SV40 transformation would induce a form of SM synthase that resides in the plasma membrane or in functional proximity to it. This would indicate that different forms of SM synthase may have different targets and eventually different biological effects (*de novo* synthesis for the enzymatic activity in the Golgi and signal transduction for the one in the plasma membrane). As a consequence of the elevated SM synthase activity in SV40-transformed cells, a decrease of the intracellular ceramide levels and an increase of DAG levels occurred. The regulation of intracellular ceramide/DAG ratio makes SM synthase an important "biostat" in the regulation of cell viability/cell death. Another important feature of this enzyme is its reported inhibition by the putative PC-PLC specific inhibitor, D609 (60). PC-PLC is thought to play an active role in signal transduction events regulating DAG formation (39). On the other hand, no genuine evidence of the real existence of the PC-PLC has been produced. Interestingly, SM synthase and PC-PLC share several biochemical and biological characteristics (DAG formation from PC, increased activity in transformed phenotype (61), inhibition by D609); thus, these considerations raise the question whether the biological events that until now have been attributed to the PC-PLC may be partially due to the SM synthase.

CONCLUSIONS

In conclusion, the study of signaling and cell regulation through ceramide has now evolved to the biochemical level by focusing on specific enzymes of ceramide metabolism. A general hypothesis can be presented whereby individual enzymes of ceramide metabolism serve as input points in the regulation of ceramide levels (Scheme 1). For example, activation of SMases or ceramide synthase would elevate ceramide levels and activate ceramide-induced responses. On the other hand, activation of enzymes of ceramide degradation or incorporation such as ceramidases or SM synthase decreases and attenuates ceramide levels. Also, as an important

corollary, some of these enzymes may play an additional fundamental role in interconverting lipid signals. For example, SM synthase has the capacity of interconverting a ceramide signal into a DAG signal, whereas ceramidases can transform a ceramide signal into a sphingosine or sphingosine-phosphate signal. This area of research promises great future insight into important areas of cell studies.

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Lipids in Vascular Function

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ABSTRACT: Physiological and pathological vascular responses depend on the action of numerous intercellular mediators, ranging from hormones to gases like nitric oxide, proteins, and lipids. The last group consists not only of the different types of lipoproteins, but also includes a broad array of other lipophilic signaling molecules such as fatty acids, eicosanoids, phospholipids and their derivatives, sphingolipids and isoprenoids. Due to space limitations, it is impossible to discuss all the vascular effects of lipophilic mediators or compounds. Therefore, we will focus on one of the most important lipid-mediated diseases, atherosclerosis. Lipoproteins and especially their native or oxidized lipid compounds affect vascular function in many different ways, and these effects do not only modulate atherogenesis but are of paramount physiological and pathophysiological importance in other diseases, such as inflammation, tumor metastasis, or normal wound healing.

Hypercholesterolemia, as defined by increased levels of low density lipoproteins (LDL), was found to alter vascular function in an atherogenic way. This includes disregulated endothelium-dependent vasodilation or contraction, hypercoagulability, enhanced platelet reactivity, as evidenced by increased *in vivo* formation of thromboxane A_2 and increased recruitment of inflammatory cells into the vessel wall. Lowering cholesterol not only reverses these adverse effects but also reduces the incidence of major cardiovascular events, demonstrating a key role for LDL and/or its oxidized derivatives mildly modified LDL (mmLDL) and thoroughly oxidized LDL (oxLDL) in atherosclerotic vascular dysfunction. On the cellular level, a complex network of molecular processes associated with plaque formation and progression is activated by LDL and oxidatively modified LDL, and their bioactivity can at least in part be mimicked by native or oxidized lipid components present in these lipoproteins.

LDL and intracellular arachidonic acid. Native LDL, which is taken up by almost all cell types *via* receptor-mediated endocytosis, induces the activation of key intracellular signaling pathways, such as protein kinase C (PKC), mitogen-activated protein (MAP) kinases, and the transcription factors activating protein-1 (AP-1) and nuclear factor κB (NFκB). These in turn trigger the expression of proatherosclerotic factors and cellular responses (for reviews, see Refs. 1–4). The most relevant are the increased synthesis of platelet-derived growth factor (PDGF) and of the adhesion molecule intracellular adhesion molecule 1 (ICAM-1) by endothelial cells, which stimulate growth of endothelial and smooth muscle cells and increase monocyte adhesion, respectively. In parallel, production of the vasodilator nitric oxide (NO) is reduced, whereas formation of superoxide anion (O_2^-) is increased, shifting the vascular response to a more contractile state.

In addition to these cellular processes, native LDL also delivers polyunsaturated fatty acids (PUFA), especially linoleic acid and arachidonic acid, to cells. These fatty acids can be incorporated and stored in membrane phospholipids, serve as precursors for eicosanoids, or may act as intra- or intercellular messengers, modulating inflammatory, atherogenic and mitogenic responses. Accordingly, arachidonic acid derived from intracellular stores or from extracellular sources was found to enhance O_2^- generation in monocytic cells by increasing the assembly of the NADPH oxidase (5). In fibroblasts, the 5-lipoxygenase products of arachidonic acid were implicated in the transduction of growth factor-induced cytoskeletal rearrangements and possibly in cell migration by the activation of rho (6). Arachidonic acid and its enzymatically oxygenated derivatives, the eicosanoids, were also found to modulate the activity of other important intracellular signaling pathways, such as adenylate and guanylate cyclases, G-proteins, or various protein kinase pathways including PKC, MAP kinases, tyrosine kinases, and protein kinase A (PKA; for reviews, see Refs. 7,8). Downstream of these signaling pathways, transcription factors such as AP-1, NFκB or the peroxisome proliferator-activated receptors (PPAR) are activated (9). These transcription factors may then stimulate expression of the immediate early response genes c-fos, c-jun or Egr-1 or late response genes, coding for tumor necrosis factor-α, the receptor for the macrophage colony-stimulating

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Abbreviations: AP-1: activator protein 1; EET: epoxy-eicosatrienoic acid; HDL, high density lipoprotein; HETE: hydroxy-eciosatetraenoic acids; HMG-CoA: 3-hydroxy-3-methyl-glutaryl CoA; ICAM-1: intercellular adhesion molecule 1; LDL: low density lipoproteins; Lp(a), lipoprotein (a); LT, leukotriene; LX, lipoxin; MAP kinase: mitogen activated protein kinase; mmLDL, mildly modified low density lipoprotein; NFκB: nuclear factor kappa B; NO: nitric oxide; oxLDL, oxidized low density lipoprotein; PAF: platelet-activating factor; PDGF: platelet-derived growth factor; PG, prostaglandin; PKA: protein kinase A; PKC: protein kinase C; PUFA: polyunsaturated fatty acids.

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factor (M-CSF) or interleukin-8 (7). Increased expression of c-fos and Egr-1 by cellular as well as extracellular arachidonic acid was linked to the induction of cell growth in fibroblasts (10), whose increased proliferative response may be associated with the early restenosis of atherosclerotic vessels after deep injury of the vascular wall by angioplasty.

Eicosanoids. LDL-derived or cellular arachidonic acid can be enzymatically oxygenated by cyclooxygenases, lipoxygenases, or cytochrome P450 monooxygenases, resulting in the formation of eicosanoids. These are highly active local mediators which affect vascular responses in often complex and opposing ways (for reviews, see Refs. 11–14). Depending on the cell types involved and on the transcellular exchange of precursors, eicosanoids may promote and increase inflammatory and atherogenic responses or may inhibit them. The classical vasoactive eicosanoids are the endothelium-derived antiaggregatory and vasodilatory prostacyclin and its direct platelet- or monocyte-derived counterpart thromboxane A_2 which induces platelet aggregation and vasoconstriction. In addition, prostaglandin E_1 (PGE_1) and PGE_2 have vasodilatory effects, whereas $PGF_{2\alpha}$ and the leukotrienes C_4 and D_4 are vasoconstrictors. Formation of leukotriene C_4 (LTC_4) was not only observed in monocytes or macrophages, but also in endothelial cells after transcellular uptake of the epoxide precursor LTA_4 produced in neutrophils. The other main leukotriene, LTB_4, is synthesized in activated neutrophils and monocytes and is a primary mediator of inflammatory processes, stimulating neutrophil degranulation, chemotaxis, and O_2^- generation. LTB4 also facilitates neutrophil adhesion to the endothelium by increasing the expression of the granulocyte adhesion molecule CD11b/CD18 (MAC-1; for review, see Ref. 13) and by enhancing the binding affinity of the endothelial counterreceptor ICAM-1 (15). Increased adhesion of neutrophils to endothelial cells is the first step in neutrophil extravasation into the subendothelial space such as in inflammatory responses and enables transcellular arachidonic acid metabolism.

A novel group of lipoxygenase-derived eicosanoids whose vascular activity was only recently identified is the lipoxins, LxA_4 and LxB_4 (for review, see Ref. 16) These trihydroxylated derivatives of arachidonic acid are generated through the dual oxygenation by 15- and 5-lipoxygenases or 5- and 12-lipoxygenases, respectively. Since these lipoxygenases are usually expressed in different cell types, Lx synthesis depends mainly on the transcellular exchange of intermediates between monocytes and neutrophils or neutrophils and platelets. Such cellular interactions will arise in inflammatory or atherogenic activation and after endothelial wounding. Lx are vasodilatory and anti-inflammatory, since they reduce neutrophil activation, expression of CD11b/CD18, and neutrophil transmigration across the endothelium. In contrast, they were found to be chemotactic for monocytes and to increase monocyte adherence, a response which may be important in early atherogenesis.

Another not yet well-characterized group of eicosanoids, the cytochrome P450 monooxygenase-derived metabolites epoxyeicosatrienoic acids (EET) and 19- or 20-hydroxyeicosatetraenoic acids (19- or 20-HETE), also deserve brief discussion because of their putative vascular effects. Apart from their enzymatic generation by the cytochrome P450 monooxygenase system, EET may also be formed by a free radical attack of arachidonic acid. Furthermore, LDL in atherogenic concentrations stimulated EET synthesis in endothelial cells (17) and EET were detected in LDL (18). EET were found to induce vasodilation (19) and to reduce platelet aggregation (20), but also to enhance monocyte adhesion to the endothelium (21) and to increase cell growth in nonvascular cells (22). 19- and 20-HETE, which can also be formed by nonenzymatic oxidative modification of arachidonic acid, function as vasoconstrictors in different animal models (for review, see Ref. 23). Further investigation is required to definitely delineate the vascular effects of these compounds *in vivo*, but it is indicated that they may exert an important modulatory role in inflammation and atherogenesis, especially with regard to their formation by free radical attack of lipoproteins or cellular phospholipids.

n-3 PUFA. Epidemiologic studies indicate that long-chain n-3 PUFA reduce cardiovascular events and possibly atherosclerosis. Given the important roles of prostacyclin and thromboxane A_2 in vascular function and the observations that n-3 PUFA reduce the formation of active thromboxanes while not altering the production of prostacyclins (as PGI_2 and PGI_3 are equally active), this shift in prostanoid synthesis may be one decisive mechanism through which n-3 PUFA protect from cardiovascular disease. Additional studies revealed that n-3 PUFA do not only affect the vascular tone and platelet aggregation (for reviews, see Refs. 24–26). They reduce the formation of cytokines and growth factors in monocytes, decrease the expression of adhesion molecules and of PDGF in endothelial cells, and subsequently also reduce adhesion of monocytes to endothelial cells (27). They increase NO production in endothelial cells and inhibit mitogenesis in smooth muscle cells and fibroblasts. Similar to arachidonic acid, n-3 PUFA were also found to act as intracellular second messengers (for review, see Refs. 8,28). They are at least in part antagonistic to arachidonic acid and reduce the activation of cellular signaling pathways involved in inflammatory and mitogenic responses, such as the formation of inositol phosphates, release of intracellular calcium, activation of PKC and growth-related early gene expression (29). On the other hand, activation of the NADPH oxidase and generation of superoxide anion are stimulated by both n-3 and n-6 PUFA in monocytic cells. In addition, when incorporated into LDL at high levels, n-3 PUFA may render LDL more susceptible to oxidation and thus may trigger proatherogenic responses. Whether this effect is relevant *in vivo* depends on cellular and plasma antioxidant status (30). Thus, n-3 PUFA may have the potential to reduce atherogenic vascular dysfunction as long as they are in balance with other protective factors, especially with lipid-soluble antioxidants.

Oxidized LDL. LDL deposited in the subendothelial space are oxidatively modified by cell-derived oxygen free radicals or cellular enzymes such as monocytic 15-lipoxygenase, re-

sulting in the oxidation of cholesterol and the esterified PUFA. The oxidative modification of LDL can be mimicked *in vitro*, leading to the formation of either mmLDL or thoroughly oxLDL. It was suggested that in the early stages of atherogenesis, primarily mmLDL may be generated, whereas in later stages with a pronounced inflammatory reaction, oxLDL will be formed. Although mmLDL binds to the LDL receptor and oxLDL to a member of the scavenger receptors, their effects on vascular cells are very similar and result in the activation of prothrombotic, proinflammatory, and mitogenic responses (Table 1; for review, see Refs. 1–4). Through the activation of intracellular protein kinase systems and transcription factors such as NFκB or AP-1, they induce the expression of cellular adhesion molecules on endothelial cells and monocytes, and stimulate the synthesis of monocyte chemoattractant protein-1, as well as of cytokines and growth factors, in particular PDGF, and procoagulant factors like tissue factor or plasminogen activator inhibitor-1 in vascular cells. Oxidized LDL induces a vasoconstrictive state, reducing the formation of the endothelium-derived vasodilators NO and PGI_2, while enhancing the production of the vasoconstrictor endothelin. In the subendothelial space, the unregulated uptake of oxLDL by monocyte-derived macrophages reduces macrophage migration and leads to their transformation to one of the hallmarks of atherosclerotic lesions, the foam cells. In low concentrations, oxLDL induces smooth muscle cell growth, whereas in high concentrations it is cytotoxic. Furthermore, oxLDL is chemotactic for monocytes and T-cells. Monocytes or macrophages may then present antigenic epitopes of oxLDL to B-cells, inducing the formation of anti-oxLDL antibodies and an immune reaction toward deposited oxLDL.

Vasoactive components of oxidatively modified LDL. The vascular effects of oxidized LDL, well-characterized *in vitro* and to a great part *in vivo*, clearly delineate key pathophysiological events leading to atherosclerotic lesions. However, it is not yet well-understood which components present in mmLDL or oxLDL activate these processes. During oxidation of LDL, the apolipoproteins, cholesterol, and the unsaturated fatty acids esterified in phospholipids or present as cholesterol-esters can be modified. Whereas modification of the apolipoproteins seems to play only a minor role in the vascular responses, oxidized lipid components of LDL were found to activate cellular responses similar to those of mmLDL or oxLDL. Oxidatively modified LDL contains various oxidized derivatives of cholesterol, the oxysterols 7-hydroxycholesterol, 7-ketocholesterol or 5α,6α-epoxycholesterol, as well as a number of highly active oxidation products of PUFA, such as aldehydes, monohydroxylated fatty acids, epoxides or isoprostanoids (31–33). During oxidation of LDL, endogenous phospholipase A_2 activity is increased, leading to the cleavage and release of these oxidized vasoactive metabolites and to the formation of lyso-phosphatidylcholine. In addition, vasoactive platelet-activating factor (PAF)-like phospholipids (34) and phosphatidylcholine derivatives in which arachidonic acid in the *sn*-2 position was oxidized to oxovaleric or glutaric acid were detected in oxidatively modified LDL (35). Table 2 summarizes important vascular effects of oxidized lipid components in mmLDL or oxLDL. Taken together, most of these derivatives activate endothelial cells, smooth muscle cells and monocytes, resulting in increased monocyte and endothelial cell adhesiveness, the generation of proinflammatory mediators and growth factors, and in the induction of cell growth. They also facilitate vasoconstriction, thrombosis, and platelet aggregation and thus stimulate *in vitro* many of the processes associated with atherogenesis.

Statins and isoprenoids. Lowering cholesterol by any kind of intervention will reduce the incidence of cardiovascular events not only in hypercholesterolemic subjects but also in normocholesterolemic persons with additional risk factors. The most effective drugs available today are the statins, which inhibit HMG-CoA reductase and thus cholesterol and also isoprenoid synthesis. It is assumed the beneficial effects of statins derive from the reduction in cholesterol levels alone. However, additional clinical and laboratory investigations demonstrated that statins modulate vascular responses by other mechanisms as well (for review, see Refs. 36,37). Statins reduce the expression of the monocyte adhesion molecule CD11b and monocyte adhesion to endothelial cells; they inhibit cell growth in smooth muscle cells and induce differentiation of monocytic cells. In hepatic and monocytic cells, statins increase desaturation and elongation of n-6 and

TABLE 1
Vascular Effects of mmLDL and oxLDL[a]

Cell type	mmLDL	oxLDL
Endothelium	Increased expression of CAM, MCP-1, M-CSF, G-CSF, tissue factor	Increased expression of CAM, MCP-1, tissue factor, PAI-1, endothelin cytotoxicity
		Reduced formation of NO, PGI_2, t-PA
Smooth muscle	Increased expression of MCP-1 cell retraction	Increased expression of MCP-1, PDGF, tissue factor
		Cell growth or cytotoxicity, chemotaxis
Monocytes	Increased expression of CD11b, IL-1 cytokines	Increased expression of CD11b, MCP-1, tissue factor, TNF-α chemotaxis
Plateles	Aggregation	

[a]CAM, cellular adhesion molecules; MCP-1, monocyte-chemoattractant protein-1; M-CSF, monocyte colony-stimulating factor; G-CSF, granulocyte colony-stimulating factor; PAI-1, plasminogen activator inhibitor 1; t-PA, tissue-type plasminogen activator; PDGF, platelet-derived growth factor; IL-1, interleukin 1; TNF-α, tumor necrosis factor-α; NO, nitric oxide; PG, prostaglandin; LDL, low density lipoprotein; oxLDL, oxidized LDL; mmLDL, mildly modified LDL.

TABLE 2
Vascular Effects of Oxidized Lipid Components in Oxidatively Modified LDL[a]

Oxidized lipid	Vascular effects
Oxysterols	Apoptosis in endothelial cells
Aldehydes (e.g., 4-hydroxynonenal)	Mitogenic for vascular smooth muscle cells; cytotoxic for endothelial cells; chemotactic for neutrophils and monocytes; stimulation of platelet aggregation and foam cell formation
19- or 20-HETE	Vasoconstriction
9- or 13-HODE	Increased eNOS expression, vasorelaxation
Epoxides	Vasodilation; induction of cell growth; increased monocyte adhesion
Isoprostanoids	Vasoconstriction; induction of smooth muscle cell and fibroblast growth; stimulation of platelet activation; priming platelet aggregation
Lyso-phosphatidylcholine	Increased expression of adhesion molecules, PDGF, MCP-1 and COX-2 in endothelial cells; increased expression of CD11b in monocytes; monocyte chemotaxis; induction of smooth muscle cell growth
PAF-like phospholipids	Increased neutrophil adhesion to endothelial cells
Oxidized phosphatidylcholine	Increased monocyte adhesion to endothelial cells

[a]HETE, hydroxy-eicosatetraenoic acid; HODE, hydroxy-octadecadienoic acid; PAF, platelet-activating factor. See Table 1 for other abbreviations.

n-3 PUFA, increasing the formation and cellular availability of arachidonic acid (38). Statins also decrease the expression of the scavenger receptors type I and II and of CD36 in monocytic cells, reduce the expression of tissue factor in macrophages, and inhibit superoxide anion generation in macrophages. *In vivo*, treatment with statins reduces platelet aggregation and plasma fibrinogen levels, and normalizes endothelial dysfunction, the latter probably by increasing NO formation. Many of these effects are most likely secondary to the lowering of serum cholesterol levels. However, some may be a consequence of a reduced generation of isoprenoids. Isoprenoids, such as all-*trans*-geranylgeranyl or all-*trans*-farnesyl moieties, bind to proteins, making the latter more lipophilic and enabling their interaction with lipophilic molecules (for review, see Ref. 39). Isoprenylation of signaling molecules such as heterotrimeric G-proteins or small G-proteins of the *ras* family allows for their translocation to the plasma membrane and transduction of receptor-dependent signals to the cytosol and nucleus. Accordingly, it was found that the growth inhibitory effects of statins in mesangial cells are based on reduced isoprenoid formation, since only all-*trans*-farnesol and all-*trans*-geranylgeranyl which are not precursors of cholesterol were able to overcome the inhibition (40). Since a reduction in cardiovascular events by statins is often observed already after a few months of therapy and not after some years as would be expected by plaque regression, altered cell activation by reduced isoprenylation of signaling molecules may be an additional mechanism underlying the protective effects of statins in vascular function.

HDL. HDL play a protective role in cardiovascular disease. This has been mainly attributed to their involvement in reverse cholesterol transport, enabling the removal of cholesterol from peripheral cells and the reduction of LDL entry into the vessel wall. However, HDL was found to have additional beneficial effects on vascular function. HDL reduces LDL oxidation and cellular responses to oxidized LDL, such as generation of PAF (41) or adhesion molecule expression in endothelial cells (42), while restoring prostacyclin synthesis (43). Further research will probably uncover additional molecular mechanisms which may explain the protective effects of HDL.

Other vasoactive lipids. In addition to the lipids discussed above, numerous other lipid molecules modulate vascular function either on an intercellular or intracellular level. Two other atherogenic lipoproteins, lipoprotein (a) [Lp(a)] and triglycerides in very low density lipoproteins or chylomicrons were found to directly affect vascular responses. Lp(a) was suggested to inhibit fibrinolysis due to its kringel-like domains which resemble plasminogen (for review, see Ref. 44). Lp(a) can compete with plasminogen for its activation by tissue-type plasminogen activator and increases endothelial cell expression of plasminogen activator inhibitor. Lp(a) also induces the expression of ICAM-1 on endothelial cells (45), and oxidized Lp(a) stimulates adhesion of monocytes to the endothelium (46), effects which may explain in part its role in atherogenesis. Similar effects were observed for triglycerides in endothelial cells. They induce expression of the adhesion molecules vascular cell adhesion molecule 1 and E-selectin and increase monocyte adhesion (47). Interestingly, triglyceride levels are lowered by n-3 PUFA *in vivo*. A further important mediator in vascular processes, in particular in inflammation, is the PAF (for review, see Ref. 48). PAF does not only stimulate platelet activation and aggregation but also induces vasoconstriction, expression of endothelial cell adhesion molecules, and the activation of neutrophils and monocytes as evidenced by their formation of LTB_4, O_2^- and cytokines and their adhesion to endothelial cells. Furthermore, saturated fatty acids or *trans* fatty acids also modulate vascular responses by as yet not well-characterized mechanisms. At the intracellular level, signaling in vascular cells is not only affected by PUFA or isoprenoids but also by the classical lipid second messengers inositolphosphates or by the activation of the sphingomyelin/ceramide pathway, leaving room for additional mechanisms for lipids to modulate vascular function.

Concluding remarks. Lipids in their broadest sense can affect vascular responses and vascular function in numerous ways, stimulating or inhibiting cellular processes which are associated with atherogenesis, inflammation, wound healing, or tumor metastasis. These effects of lipids help to maintain the physiologic vascular responses or restore normal vascular function after injury, but may also promote vascular damage if additional factors, such as hypercholesterolemia and oxidation of lipoproteins, are active. Further delineation of mechanisms underlying the vasoactivity of lipids will help to better understand pathophysiological mechanisms and to design effective therapeutic or preventive strategies in vascular diseases.

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Dietary Fatty Acids and Coronary Heart Disease

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ABSTRACT: The effects of dietary fats have been established in epidemiological and intervention studies and through relationship to risk factors for development of coronary heart disease (CHD). During a period where the impressive effects of hydroxymethylglutaryl-CoA reductase inhibitors on the mortality of CHD dominate the medical journals, it is important to realize the major effects of dietary fatty acids on a series of events included in the multifactorial disorder of CHD.

During the last few years, four secondary and primary prevention studies on the morbidity and mortality of coronary heart disease (CHD) documented the effects of statins, the hydroxymethylglutaryl-CoA reductase inhibitors (1–4). A meta-analysis on the effects of cholesterol reduction after the introduction of statins predicts that for every 10 percentage points of cholesterol lowering, CHD mortality risk is reduced by 15% ($P <$ 0.001) and total mortality risk by 11% (5).

During the last 50 yr, many dietary intervention studies (for review see Ref. 5) were designed to establish potential effects on CHD by modulating the dietary intake of fatty acids and cholesterol. In summary, these studies showed that by reducing the intake of saturated fatty acids and cholesterol in the diet and partly substituting them with mono- or polyunsaturated fatty acids, both serum cholesterol and the risk for developing CHD are reduced. In the present situation, the question arises. Do the new potent hypocholesterolemic drugs make dietary intervention less important? This question is certainly relevant in a period where both patients and physicians are overwhelmed by the impressive results of the drug intervention trials.

It is important to stress that CHD still is the most common cause of death globally in both males and females. Less than 40% of the fatalities, however, can be associated with traditional coronary risk factors, including high levels of serum cholesterol. Also the statin intervention studies suggest that even with these drugs effects other than cholesterol lowering may be of significance. The reduction in mortality could not be explained on the basis of only cholesterol. Furthermore, looking upon the geographical distribution of CHD and relating that to dietary traditions, the Seven Countries Study clearly indicates that the risk associated with a certain cholesterol level is much higher in the northern European countries than in the countries in southern Europe and even more so in Japan (6). This study clearly indicates that factors other than cholesterol may be heavily involved in the pathogenesis of CHD.

Dietary fats and CHD. Only anecdotal reports associated dietary fats with development of atherosclerosis and cardiovascular disease until after World War II (7). The fall and subsequent rise in CHD mortality during and after the war in Norway were correlated with lower consumption of animal fats and higher consumption of cereals, vegetables, and fish during the war years (8). This change was also associated with the fall and rise in the frequency of postsurgical venous thrombosis, probably indicating a relationship between the occurrence of venous thrombosis and arterial vascular disease. During the following years, a series of studies associating dietary fats with serum cholesterol levels initiated by the studies of Keys and the Framingham Study later confirmed a close relationship between intake of dietary fats and the occurrence of CHD (9,10). Also the dietary changes among the Japanese due to westernization of the traditional diet or to emigration to areas with different dietary habits support these associations (11). More than 20 yr ago the studies by Dyerberg *et al.* (12) in the Greenland Eskimos suggested that the intake of n-3 polyunsaturated fatty acids was significant in preventing CHD. In more recent studies, the development in eastern European countries was of special interest. The morbidity and mortality of CHD were increased, and at the same time diets rich in fats from animals sources were dominant in regard to nutrition (13).

Recently, the Nurses Health Study including more than 80,000 nurses confirmed the association between intake of saturated fatty acids and also of *trans* unsaturated fatty acids and CHD, whereas monounsaturated fatty acids (MUFA) and polyunsaturated fatty acids (PUFA) had the opposite effect (14). Also other studies showed a similar pattern; however, the protective relationship with MUFA was not confirmed in all studies; the beneficial effects of very high intake of n-6 PUFA were questioned (15). Of special interest are the epidemiological studies suggesting that intake of small amounts of n-3 fatty acids in certain populations seems to have a protective effect against development of CHD, whereas in other populations with a traditionally high intake of n-3 fatty acids no such relationship was established. These last epidemiological observations certainly indicate that the interplay between the various dietary fatty acids is of prime importance for understanding the relationship between dietary fatty acids and CHD (for review see Ref. 16).

It has been estimated that at least half of the deaths caused by CHD result from electrical instability of the heart, culminat-

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Abbreviations: CHD, coronary heart disease; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

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ing in ventricular fibrillation. Studies in cardiac myocytes and in animals following partial occlusion of coronary arteries indicate that saturated fatty acids could promote ventricular arrhythmia, whereas n-3 and n-6 PUFA have antiarrhythmogenic effects (17,18); n-3 PUFA seem to be more potent. A population-based case–control study including 334 patients with primary cardiac arrest and 493 control subjects seems to confirm the experimental studies (19). Compared with no intake of n-3 PUFA, an intake of 5.5 g/mon or one fatty fish meal per week was associated with a 50% reduction in the risk of primary cardiac arrest. Compared with the red blood cell membrane n-3 PUFA levels of 3.3% of total fatty acids, a level of 5.0% of total fatty acids was associated with a 70% reduction in the risk of primary cardiac arrest. Two dietary intervention studies seem to confirm this observation (20,21).

Intervention studies. The intention of all the intervention studies performed to date in humans was to determine whether reduction in CHD can be produced by modulation of the diet. No distinction was made as to whether thrombosis, athersclerosis, or both or other factors were altered. As thrombosis was shown to be present in more than 90% of patients with acute coronary events, the results of the intervention studies may reflect effects on atherosclerosis, thrombosis, and possibly also on other factors like inflammation and myocardial vulnerability.

The dietary intervention studies included reduction in blood cholesterol as secondary endpoints. An analysis of randomized trials evaluating the effect of the dietary-induced cholesterol reduction on total mortality and incidence of CHD concluded that for 1% reduction in cholesterol an estimated 2.5% reduction in CHD incidence was observed (5). The subjects were included from both secondary and primary prevention trials. Many of these trials were designed on the use of combined drug and dietary interventions.

A large multiple risk factor intervention trial was designed to reduce over a period of 6 yr three major risk factors in middle-aged men who were at increased risk for CHD: elevated serum cholesterol, increased blood pressure, and excessive cigarette smoking (22). A significant reduction in risk factors was accomplished in the intervention group, but there was also a less-pronounced reduction in the control group. In both groups the survivors had a greater reduction in serum cholesterol than in those participants who ultimately died from CHD, suggesting that the reduction of serum cholesterol induced by changes in the diets was associated with a lower mortality from CHD. However, the MRFIT study was inconclusive in respect to overall mortality from CHD (22). The primary intervention trial carried out by the Oslo Study Group lasted 8.5 yr and included 1,200 high-risk men (23,24). This trial showed that the reduction of total fat intake from 41 to 28% of calories and an assumed change in the polyunsaturated/saturated ratio from 0.4 to approximately 0.7 reduced CHD events, including CHD death and sudden death by approximately 50%. The reduction in polyunsaturated/saturated ratio was caused mainly by a reduction of saturated fatty acids. Approximately 75% of the beneficial effects observed in the Oslo Study appeared to be due to the changes in long-term dietary habits of the participants, whereas 25% were related to changes in smoking habits.

In most dietary intervention studies, saturated fat was replaced by an increased intake of PUFA of the n-6 family. Subsequent analysis, however, indicated that most of the beneficial effects of these dietary manipulations were related to a reduced intake of saturated fats. The Diet and Reinfarction Trial (DART) including 2,033 men who had recovered from myocardial infarction showed that subjects advised to eat fatty fish had a 29% reduction in 2 yr all causes mortality compared with those not so advised or advised to reduce fat intake only (20). The incidence of sudden death plus death rate from CHD was not significantly affected. The Lyon Study including 605 survivors of a recent first myocardial infarction (303 controls and 302 study patients) who were followed over a period of 27 mon (21). Those in the study group had been on a Mediterranean-type diet which included less than 10% of energy as saturated fat, less than 5% of energy as linoleic acid, and more than 0.6% of energy as α-linolenic acid. When primary and secondary end points were combined, the study showed a risk reduction of 76%. Interestingly, no significant reduction in cholesterol levels was observed compared with the control group.

Finally, dietary intervention with n-3 fatty acids on graft potency in patients undergoing coronary artery bypass and followed by angiographic evaluation showed more open grafts 1 yr after the procedure in a study, including 600 patients given 4 g of 85% eicosapentaenoic acid and docosahexaenoic acid (ethylester) in addition to aspirin or warfarin. No conclusive evidence indicates that supplement with n-3 fatty acids prevents the occurrence of restenosis after percutaneous transluminal coronary angioplasty (for review see Ref. 16).

Dietary fatty acids and risk factors for CHD. It has been well documented not only that dietary fatty acids may modify well-established coronary risk factors like blood lipid levels, blood pressure, and hemostatic variables associated with increased tendency to thrombosis but also that a high intake of saturated fatty acids in the diet has been documented as an independent risk factor for CHD.

In the present context it is significant that, whereas lipid-lowering drugs like the statins lower low density lipoprotein cholesterol levels, increase high density lipoprotein-cholesterol, and reduce triglycerides, modulation of the dietary fatty acids with reduction in saturated fatty acids and eventually an increase in MUFA and/or PUFA particularly of the n-3 family may induce similar or occasionally even more pronounced changes in blood lipid levels. In addition these fatty acids have metabolic effects which may reduce blood pressure in patients with hypertension, reduce the consequences of postprandial hyperlipemia which in itself represents an independent risk factor, and modulate cell structure and function leading to metabolic consequences in every cell in the body. It may turn out that these cellular adaptations to nutrition may be of greater importance for the development of coronary events than the reduction of traditional risk factors.

The combined effect of n-3 fatty acids and statins. In recent studies the combined effects of long chain n-3 fatty acids from

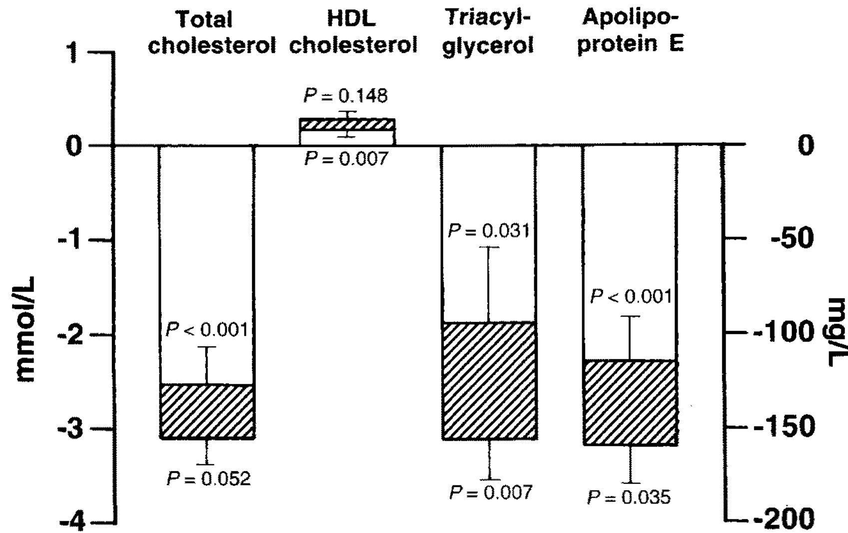

FIG. 1. Effects of Simvastatin (20 mg/d) and additional effects of n-3 fatty acids (Omacor[R]) (4 g/d) in 21 patients with combined hyperlipemia treated for 5-wk periods compared to 20 patients treated with Simvastatin plus placebo (corn oil) on serum total cholesterol, high density lipoprotein (HDL)-cholesterol, triglycerides, and apolipoprotein E (Ref. 26); open box, effect Simvastatin; cross-hatched box, additional effect of n-3 fatty acids.

fish oil and simvastatin in patients with combined hyperlipemia were evaluated (25,26). As can be observed from Figure 1, 4 g/d of n-3 fatty acids given as ethylesters of eicosapentaenoic acid and docosahexaenoic acid (Omacor[R]; Pharmacia & Upjohn K/S; Oslo, Norway) in addition to 20 mg/d of Simvastatin reduced serum total cholesterol and triglycerides more than Simvastatin only. In addition, apolipoprotein E levels were further reduced. The postprandial hyperlipemia after a standardized fatty meal was similarly reduced. Also risk factors related to the hemostatic system were modulated by the supplement of n-3 fatty acids. This study indicates that even minor changes in the intake of n-3 fatty acids may efficiently reduce the risk profile in addition to what can be induced by the statins in this patient group with a very high risk for development of CHD.

Concluding remarks. In a period where secondary but also primary prevention for development of CHD is dominated by the new potent cholesterol-lowering drugs, it is imperative not to forget the traditional interventions related to lifestyle and dietary habits. It is definitely easier to swallow one tablet a day than to interfere with dietary preferences and tradition. Thus, in the 4S Scandinavian Simvastatin Study, dietary advice was followed by a $^1/_2$% decrease in total cholesterol (Pedersen, T., personal communication). It should be stressed that dietary changes may have profound structural and metabolic effects, interfering with a series of risk factors not modulated by statins. CHD definitely is a multifactorial disorder involving atherosclerosis and thrombosis in addition to inflammation, cell proliferation, and a disharmony in the orchestra of cytokines and eicosanoids. A reappraisal of good dietary habits and further research evaluating the effects of the individual fatty acids separately and in combination with other fatty acids are mandatory.

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Antioxidant and Other Activities of Phenolics in Olives/Olive Oil, Typical Components of the Mediterranean Diet

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The impact of the environment on human physiology and pathology has been recognized for decades. In this respect, the diet certainly plays a major role, as indicated by the well-known observation that the incidence of certain diseases is quite different in geographical areas where populations have traditionally been exposed to somewhat unique diets. "Niches" characterized by distinct dietary conditions maintained throughout the centuries can be recognized throughout the world, such as those of populations in the Far East, or living on sea coasts, where fish is a major component of the diet, and those in the Mediterranean area. In the Mediterranean basin, several components of the diet are derived from plants and fruits and, among them, olives and grapes have been, at least originally, rather unique to this area.

Due to the particular climate, plants in the Mediterranean basin are subjected to prolonged exposure to sunlight. Thus, many fruits, including olives and grapes, have developed an array of protective compounds that limit the effects of ultraviolet light and the subsequent oxidative damage. Accordingly, the levels, dependent upon the rates of synthesis, of anthocyanins, flavonoids, and phenols in dark-colored fruits are enhanced by sunlight. Wine and olive oil, which originate from the Mediterranean area, are obtained by pressure techniques from ripe fruits; the compounds, responsible for the organoleptic and other properties of the fruits are thus transferred to the fluids.

The observation of a lower incidence of coronary heart disease (CHD) (1,2) and of certain types of cancers (3,4) in the Mediterranean area lead to the hypothesis that a diet rich in grain, legumes, fresh fruits and vegetables, wine in moderate amounts, and olive oil had beneficial effects on human health. To date, this effect has been mainly attributed to the low saturated-fat intake of the Mediterranean diet and to its high monounsaturates proportion, which indeed may favorably affect the plasma lipid/lipoprotein profiles. Nevertheless, other components of the diet, such as fiber, vitamins, flavonoids and phenols, may play an important role in disease prevention, acting on different cardiovascular variables.

Olive oil, in particular, is the principal source of fat of the Mediterranean diet. The production of olive oil for 1997–1998 in the European Community is estimated to be 1,941,000 tons, which represents 85% of the world production. Due to the increasing popularity of the Mediterranean diet, its consumption is expanding to nonproducer countries such as the United States, Canada, and Japan. Abundance of oleic acid, a monounsaturated fatty acid, is the feature that sets olive oil apart from other vegetable oils. In particular, oleic acid (18:1n-9) ranges from 56 to 84% of total fatty acids, while linoleic acid (18:2n-6), the major essential fatty acid and the most abundant polyunsaturate in our diet, is present in concentrations between 3 and 21%. Depending on its chemical and organoleptic properties, olive oil is classified into different grades (5) that also serve as guidelines for the consumer's preference. In many producing countries, extra virgin olive oil, i.e., the one with the highest quality, accounts for just 10% of the whole production. In addition to triglycerols and free fatty acids, olive oil contains a variety of nonsaponifiable compounds that add up to 1–2% of the oil and are important for its stability and unique flavor and taste. In contrast, other edible seed oils lose most of their minor compounds during the refining stages.

The absolute concentration of phenols in olive oils is the result of a complex interaction between several factors, including cultivar, degree of maturation, and climate. It usually decreases with overmaturation of olives, although there are some exceptions to this rule. For instance, olives grown in warmer climates, in spite of a more rapid maturation, yield oils that are richer in phenols. Analysis of the "minor constituents" is very important for the identification of the area of production of each batch and allows the disclosure of possible adulterations. Most phenols confer a very bitter and pungent zest to the oil. The effect of bitterness and pungency is the result of complex interactions between the "minor constituents" and the taste buds. In particular, phenolic acids, such as phenol and cinnamic acid, are responsible for the bitter sensation felt on the lateral and posterior areas of the tongue, while secoiridoids confer a peculiar pungency. As a result, organoleptic feelings that remind one of pepper or chili peppers can be found in phenols-rich

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Abbreviations: HT, hydroxytyrosol; iNOS, inducible form of the enzyme nitric oxide synthase; LDL, low density lipoprotein; NO, nitric oxide; OE, oleuropein.

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TABLE 1
Inhibition of Superoxide Anion Formation Rates by Olive Oil Phenolics

	Superoxide-producing system	
	Xanthine-xanthine oxidase	PMN + PMA[a]
Hydroxytyrosol (EC_{50})	9.1 μM	3.2 μM
Oleuropein (EC_{50})	14.3 μM	29.3 μM

[a]PMN, phorbol 12-myristate 13-acetate; PMA, polymophonucleate.

olive oil, which is most favored by gourmets. This is confirmed by the higher scores usually obtained in panel tests by oils produced from greener olives (6) because of the "fruity" and complex aroma provided by their high phenol content. Conversely, "sweet" oils are almost devoid of phenols. Notably a very high load of phenols may result in an excessive and unpleasant bitterness and is not synonymous with quality. The continuous systems employed to extract the oil sampled in the panel test reported by Cimato *et al.* (6), in spite of removing a portion of phenols, prevented the development of off-flavors that can derive from dirty fiber mats or molds and hence lower the percentage of defective oils. In turn, phenol-rich virgin olive oils very likely exhibit greater stability and a strong, fruity flavor, indicating a high, but not necessarily the most preferred, organoleptic quality of the oil.

The presence of phenolic compounds confers on olive oil a particular resistance to the development of rancidity, and the major contributor to this effect has been attributed to hydroxytyrosol (3,4-dihydroxyphenylethanol), an orthodiphenol that derives from the secoiridoid oleuropein (OE), the bitter principle of olives. In contrast, tyrosol appears to contribute very little, if any, to the stability of olive oil.

Thanks to the recent availability of pure compounds, we investigated the biological activities of some olive oil phenols including, but not limited to, their antioxidant capacity. Both hydroxytyrosol (HT) and OE potently inhibit copper sulfate-induced oxidation of low density lipoprotein (LDL) in a dose-dependent manner, when incubated from 10^{-6} to 10^{-4} M (7,8). The protective effects of HT and OE are demonstrated through the assessment of various markers, such as a reduced formation of short-chain aldehydes (evaluated as thiobarbituric acid-reacting substances) and of lipid peroxides, by the persistence of a higher vitamin E content in the residual LDL (indicating sparing of endogenous antioxidants), and by a reduced formation of malondialdehyde-lysine and 4-hydroxynonenal-lysine adducts, indicating protection of the apoprotein moiety (7,8). Although the antioxidant activity of olive oil phenols on LDL oxidation was observed mainly when copper sulfate was used as the oxidative agent, suggesting that these compounds at least acted as metal chelators, they also prolonged the lag phase of conjugated diene formation when LDL oxidation was induced by a metal independent oxidative process, such as the use of free radical generators (7). This suggests that additional activities of HT and OE, among the antioxidant properties, may include chain-breaking in radical propagation and/or radical scavenging (9).

More recent studies focused on the scavenging activities of olive oil phenols with respect to various free radicals (10). In particular, OE and HT were shown to potently inhibit superoxide production by either a cell-free system or by activated human neutrophils (Table 1 and Fig. 1). Superoxide is an oxygen-centered radical that is produced by enzyme sys-

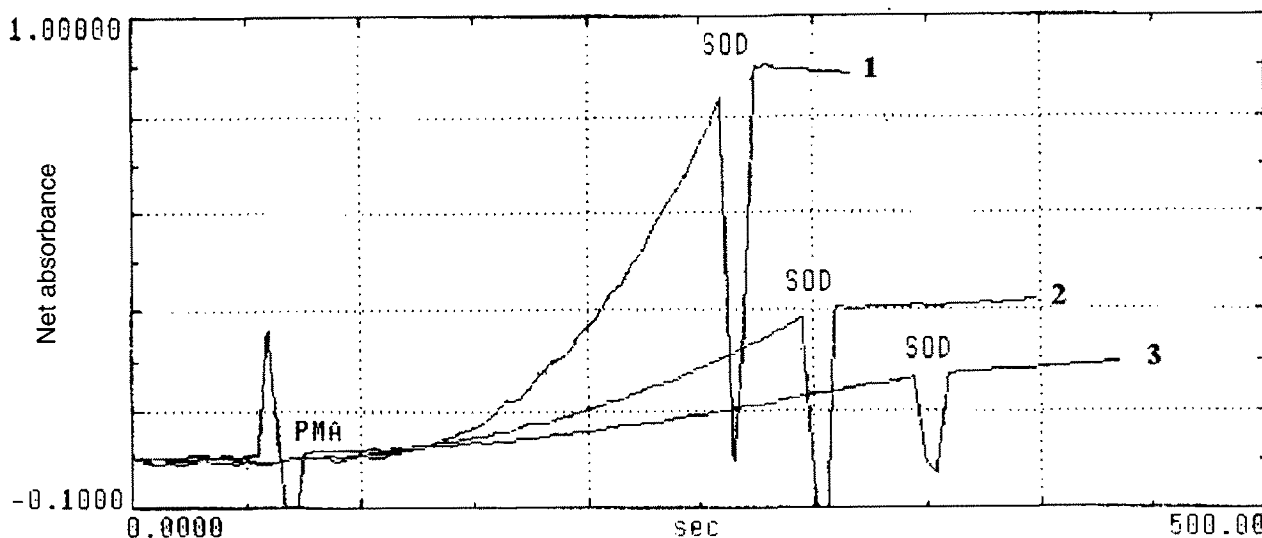

FIG. 1. Phorbol 12-myristate 13-acetate-induced superoxide production by human neutrophils. Samples were as follows: (1) control; (2) hydroxytyrosol 10^{-5} M; (3) hydroxytyrosol 10^{-4} M. SOD, superoxide dismutase 50 ng/mL.

tems, by autoxidation reactions, and by univalent oxygen reduction caused by electron transfers. It is highly reactive (half-life 10^{-5} s) and, in biological systems, can oxidize ascorbic acid and reduce cytochrome C. It is also a key factor in the Haber-Weiss reaction from which it generates the highly reactive hydroxyl radical. Superoxide dismutase dismutases superoxide to hydrogen peroxide and molecular oxygen. It is noteworthy that vitamin E and the established synthetic antioxidant butylated hydroxytoluene do not scavenge superoxide, suggesting that olive oil phenols are better antioxidants than the above-mentioned molecules in processes involving Haber-Weiss reactions.

Also, olive oil phenols were shown to scavenge hypochlorous acid (Fig. 2). HOCl is produced at the site of inflammation by activated neutrophils, through activation of the enzyme myeloperoxidase. Among other actions, hypochlorous acid inactivates α-1-antiproteinase, the major inhibitor of proteolytic enzymes in human plasma, such as elastase. Therefore, since elastase is also released by activated neutrophils, chlorination and oxidation of α-1-antiproteinase may greatly potentiate tissue damage (11). HOCl is also a component of household bleaches. This particular aspect may bear some relevance to industrial application of phenolic antioxidant, since bleaches are often employed to disinfect items with which food comes into contact.

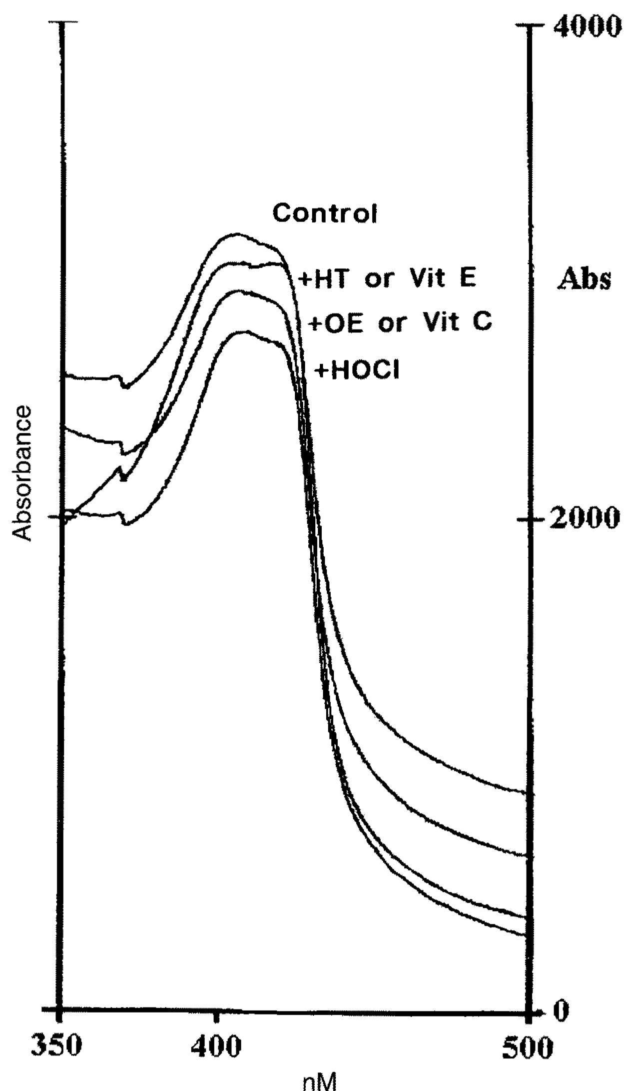

FIG. 2. Absorption spectra of catalase incubated with HOCl and the compounds under investigation. HOCl was 80 μM, whereas the compounds under investigation were added at the final concentration of 10^{-5} M. Abbreviations: OE, oleuropein; HT, hydroxytyrosol; Vit C, vitamin C; Vit E, vitamin E.

Finally, as demonstrated by chemiluminescence, both OE and HT were able to inhibit phorbol ester-induced neutrophil respiratory burst, a process involved in bronchoconstriction and airways inflammation.

HT and OE were also tested for other biological activities, namely, the effects on cells which appear to be involved in various functions in the cardiovascular systems, such as platelets and polymorphonuclear leukocytes. As to the activity on platelets, they exerted marked inhibition of the aggregation induced by various agonists, with an IC_{50} of the same order (10^{-5} M range) of magnitude as that of aspirin, and they also inhibited the accumulation of the proaggregatory compound thromboxane in serum which takes place during blood clotting obtained under controlled conditions. In human leukocytes, HT and OE markedly inhibited the production of the proinflammatory molecule leukotriene B_4, an eicosanoid generated from arachidonate through a 5-lipoxygenase, following activation with a calcium ionophore (12). The potent inhibitory effects of HT and OE toward these parameters suggest that they are able to interfere with cellular oxygenases, thus disclosing unpredicted biological activities in addition to the antioxidant activities.

Finally, when added to murine macrophages together with a bacterial lipopolysaccharide, OE increases the functional activity of these immune-competent cells, as evaluated by a significant increase (+58.7 ± 4.6%) in the production of the bactericidal and cytostatic factor nitric oxide (NO) (Table 1). A tonic effect of OE on the inducible form of the enzyme nitric oxide synthase (iNOS) was demonstrated by Western blot analysis of cell homogenates and by the use of the iNOS inhibitor L-nitromethylarginine methyl ester (Table 1) (13). On the other hand, OE alone was unable to affect the production of NO and the expression of iNOS, indicating that it behaves as a modulator of the NO system, and not as a direct activating agent.

Macrophage-derived NO during acute sepsis and inflammation represents an adaptive response of the organism that reacts to the endotoxin challenge by increasing the production of this mediator. In fact, NO inhibits platelet aggregation and adherence to vessel walls, and it maintains a proper perfusion rate through increased vasorelaxation. The protective effects of NO on cardiovascular function are further documented by the observation that inhibition of NO synthesis during sepsis increases cellular damage and animal mortality (14).

In conclusion, the interest in minor components, providing a vast array of different chemical structures, in plants and veg-

etables is rapidly growing in the areas of both nutrition and pharmacology and pharmaceutics. From the point of view of nutrition, it should be pointed out that minor compounds in cells and tissues, which are components of our diet, may play important functions, and their activities may thus be transferred to the recipient organisms.

This applies to the complex mixture of minor components, including those of phenolic nature, that are present in olives and consequently in the oil.

Although they all play a role in the drupe, it should be recognized that not all of them appear to be active, at least in the biological assays devoted to test the antioxidant and cellular activities. Accurate evaluations of the composition of minor components, affected by cultivars, climate, ripeness and processing, indifferent oil batches, and of the relationships between structure and biological activities (nature and potency) are essential for the characterization of different oils in terms of organoleptic and nutritional properties.

The biological activities disclosed in *in vitro* studies need obviously to be confirmed after administration, and information on the absorption, distribution, and metabolism of these compounds in animals and humans is an essential prerequisite. Notably, however, possible interactions at different steps in the kinetics (intestinal lumen, hepatic metabolism, cardiovascular and peripheral tissues and organs), with endogenous lipid and water-soluble antioxidants, considering the amphiphilic nature of phenolics, represent an area of additional important research.

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Fatty Acids, Antioxidants, and Coronary Heart Disease from an Epidemiological Perspective

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ABSTRACT: Oxidized low density lipoproteins (LDL) play a major role in the development of atherosclerosis. Saturated fatty acids, especially fatty acids with 12–16 carbon atoms, are the most important determinants of the LDL cholesterol level. The LDL lipoprotein fraction can be oxidized by, e.g., smoking. Oxidative damage of LDL lipoproteins can be prevented by nutritive, e.g., vitamin E, and nonnutritive antioxidants, e.g., flavonoids. It can therefore be hypothesized that fatty acids and antioxidants are important determinants of coronary heart disease (CHD). There is a large body of evidence from prospective studies that LDL cholesterol-lowering is associated with a lower CHD risk. The evidence for a protective effect of antioxidants on CHD risk is much weaker and is most promising for vitamin E and flavonoids. The Seven Countries Study showed that at the population level saturated fat, cigarette smoking, and flavonoids are important determinants of long-term CHD mortality. These results suggest that a diet low in saturated fat and rich in antioxidants in combination with no smoking is associated with low CHD risk.

In research carried out since the 1950s it became clear that dietary fatty acids are important determinants of serum total and low density lipoprotein (LDL) cholesterol. More recently evidence is accumulating that not native LDL but oxidized LDL is of major importance in the development of atherosclerosis. This raised the interest in a possible protective role of antioxidants in the occurrence of atherosclerotic complications like coronary heart disease (CHD). This presentation will give an overview of the associations between dietary fatty acids, antioxidants, and the occurrence of CHD from an epidemiological perspective.

FATTY ACIDS AND CHD

In 1952 Keys (1) hypothesized that serum cholesterol was a major determinant of CHD. At that time it was thought that dietary cholesterol and total fat were the major determinants of serum cholesterol (1,2). Later on it became clear that not dietary cholesterol and total fat but individual fatty acids, especially saturated fatty acids with 12–16 carbon atoms, were the most important determinants of serum cholesterol (3). These fatty acids have a strong cholesterol-elevating effect.

Recently a meta-analysis was published of 395 controlled dietary experiments among 129 groups of individuals with an average duration of 1 mon (4). In these metabolic ward studies, the effect of different dietary fatty acids and cholesterol on blood lipids and lipoproteins was studied using dietary carbohydrates as a reference. This meta-analysis confirmed the strong serum cholesterol-elevating effect of saturated fatty acids on serum total and LDL cholesterol. Monounsaturated fatty acids were neutral, and polyunsaturated fatty acids had a mild total and LDL cholesterol-lowering effect compared with carbohydrates. Dietary cholesterol had a slight total and LDL cholesterol-elevating effect. Dietary fatty acids also have an effect on high density lipoprotein (HDL) cholesterol. This lipoprotein fraction has in contrast to LDL cholesterol a protective effect on CHD occurrence. All major classes of fatty acids have an HDL cholesterol-elevating effect. The strongest effect is observed for saturated fatty acids. Thus saturated fatty acids elevate both LDL and HDL cholesterol. Because of their strong LDL, cholesterol-elevating effect, saturated fatty acids in the diet should be replaced by a mixture of unsaturated fatty acids and carbohydrates.

Clarke and coworkers (4) quantified the effect of changes in dietary fatty acid and cholesterol composition of the diet on total cholesterol. If 5% of the energy intake of saturated fat is replaced by polyunsaturated fat and another 5% by monounsaturated fat and the intake of dietary cholesterol is reduced by 200 mg, total cholesterol will be lowered by 0.76 mmol/L. If a Western population has an average serum cholesterol level of 6 mmol/L, the cholesterol level can be lowered by about 13% if this population changes to a low saturated fat–low cholesterol diet.

During the hardening process of polyunsaturated fat in margarine production, so-called *trans* fatty acids are formed. Controlled dietary experiments showed that especially the *trans* fatty acid elaidic acid has a total and LDL cholesterol-elevating effect and an HDL cholesterol-lowering effect (5). Presently, it is not clear whether there is a dose-response relationship between the intake of elaidic acid in the diet and total

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Abbreviations: AA, arachidonic acid; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; LDL, low density lipoprotein.

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and LDL cholesterol level in the blood. The HDL cholesterol-lowering effect of elaidic acid seems to be confined to high intake levels. More information is needed to make definite statements about the relationships between the intake of *trans* fatty acids and different lipoprotein fractions. However, it can be concluded that the *trans* fatty acid elaidic acid also has a total and LDL cholesterol-elevating effect at the lower levels of intake.

Serum total cholesterol-lowering is associated with a reduction of risk in the occurrence of CHD. Truswell (6) reviewed in a meta-analysis the results of 14 dietary intervention trials in which the effect of serum total cholesterol-lowering on the occurrence of CHD was studied. This included primary and secondary intervention trials and both uni- as well as multifactorial interventions. In all 14 trials an average serum total cholesterol reduction of 10% was obtained by replacing saturated fat by polyunsaturated fat. This was associated with a 13% reduction in CHD events and a 6% reduction in all-cause mortality. The five trials with the largest serum total cholesterol reduction showed on average a decrease in serum cholesterol of 13%. This decrease in serum total cholesterol was associated with a 30% decrease in CHD events and an 11% decrease in mortality from all causes. These results suggest that the stronger the reduction in serum total cholesterol, the larger the reduction in CHD events and all-cause mortality.

Polyunsaturated fatty acids are not a homogeneous category of fatty acids. They consist of two parent compounds: linoleic acid (LA), a fatty acid of the n-6 family with 18 carbon atoms and two double bonds, and α-linolenic acid, a fatty acid of the n-3 family with 18 carbon atoms and three double bonds. These fatty acids have different metabolic effects. LA can be elongated to arachidonic acid (AA), a fatty acid with 20 carbon atoms and four double bonds. α-Linolenic acid can be elongated to either eicosapentaenoic acid (EPA), a fatty acid with 20 carbon atoms and five double bonds, or docosahexaenoic acid (DHA), a fatty acid with 22 carbon atoms and six double bonds. The fatty acids with 20 carbon atoms, AA and EPA, play an important role in prostaglandin metabolism and may influence the thrombotic process.

In case-control studies carried out in Finland and Scotland in the 1980s, an inverse association was observed between the LA concentration in phospholipids or adipose tissue and CHD risk (7,8). At that time the LA intake in these population was low (<4% of energy). In cultures with a higher intake of LA, such an association was not observed.

In the case-control study from Finland an inverse relationship also was observed between the phospholipid concentration of α-linolenic acid and myocardial infarction (7). Also in an intervention trial among cardiac patients. a strong protective effect was observed of an α-linolenic acid-enriched Mediterranean diet on coronary and all-cause mortality (9). These data suggest that α-linolenic acid may play an important role in the prevention of CHD. However, more information is needed before definite statements can be made about the possible protective effect of α-linolenic acid.

Fish is an important source of the n-3 fatty acids EPA and DHA. We found in two prospective studies carried out in The Netherlands that a small amount of fish may be protective against CHD mortality (10,11). This association was observed in several prospective studies but not in all (12). The association was observed in cultures with a low fish intake especially if persons were compared who did not consume fish with those who consumed fish once a week. The association also depends on the end point. Associations are observed for hard end points, e.g., coronary mortality or cardiac arrest, but not for softer end points, e.g., CHD incidence.

The protective effect of fish, especially fatty fish, was shown in a secondary prevention trial carried out in Wales (13). About 2,000 cardiac patients were randomized, and members of the experimental group were advised to eat fatty fish three times a week. The average fish intake of the control group was about once a week. The difference in EPA intake between the two groups was about 200 mg/d. After 2 yr of follow-up, the cardiac mortality was reduced by 34% and the all-cause mortality by 29% among the patients who got the fish advice compared with the control group. Also in this trial no effect of the fish advice was found on the soft end point of CHD incidence. It can be concluded the fish is protective in relationship to CHD mortality.

ANTIOXIDANTS AND CHD

Research on antioxidants has traditionally focused on (pro-) vitamins with antioxidant properties, e.g., tocopherols (vitamin E), carotenoids (β-carotene), and vitamin C. Besides these so-called nutritive antioxidants, there are also nonnutritive antioxidants. Nonnutritive substances are compounds in foods without nutritional value. Recently it became clear that plant foods especially contain numerous compounds with strong antioxidant properties, e.g., polyphenols. Important examples are flavonoids, e.g., quercetin which is present in onions and apples, and catechins, e.g., epigallocatechin which is present in tea. The associations between (non)nutritive antioxidants and CHD will be summarized.

In large epidemiological studies, blood samples are stored for future analyses of blood parameters. In this context, associations are studied in so-called nested case-control studies. In these studies, antioxidant concentrations are measured retrospectively in stored blood samples of persons who developed CHD and in controls. This is an efficient study design because only the blood samples of the persons who developed the disease and a limited number of controls have to be analyzed.

In The Netherlands and Finland, three nested case-control studies were carried out studying the association between vitamin E levels in blood and cardiovascular diseases (14). No association was observed. In a case-control study carried out in Scotland, plasma vitamin E was inversely associated with angina pectoris in limited multivariate analyses (15). However, when the LA content of adipose tissue and the EPA content of platelets also were taken into account in the multivari-

ate model, the association between plasma vitamin E and angina pectoris was no longer statistically significant. Also no association was observed between the α-tocopherol concentration in adipose tissue and myocardial infarction in the multicenter EURAMIC case-control study (16).

In five prospective studies, inverse associations were observed between vitamin E intake and CHD risk (17–21). However, in three studies, this association was noted for supplement users (17–19) and in two studies for the vitamin E content in the diet (20,21). This means that the results of these cohort studies suggest a protective effect of vitamin E on CHD risk. However, it is unclear what level of intake provides protection.

In the CHAOS trial, the hypothesis was tested whether vitamin E supplementation among cardiac patients could prevent the risk of reinfarction and mortality (22). There was a reduction in nonfatal myocardial infarction in the patients supplemented with vitamin E. However, no association was observed with respect to hard end points, e.g., cardiovascular and all-cause mortality. Similar results in relationship to hard end points were observed in the ATBC trial carried out in Finland among 30,000 smokers (23). These results suggest that vitamin E supplementation is not effective in preventing hard end points, e.g., cardiovascular and all-cause mortality.

In the EURAMIC case-control study, an inverse association was found between β-carotene concentration in adipose tissue and myocardial infarction (16). This association was, however, confined to smokers. In four prospective studies, no association between β-carotene intake and CHD was observed (18,20,21). An exception was the inverse association between β-carotene intake and CHD in smokers as observed in the Health Professionals Follow-up Study (18).

Three large intervention trials were carried out, one in Finland and two in the United States, to study the effect of β-carotene supplementation on lung cancer and other causes of death (23,25,26). These trials took place among smokers, asbestos workers, and physicians. In spite of the hypothesized protective effect, these studies showed a detrimental effect of β-carotene supplementation on lung cancer. In the ATBC and CARET trials, β-carotene supplementation was also associated with a higher cardiovascular and all-cause mortality. In the Physical Health Trial, significant associations were not observed between β-carotene supplementation and cardiovascular and all-cause mortality. The data of the Physicians Health Trial suggest that the effect of β-carotene supplementation on myocardial infarction may be related to smoking status. An odds ratio of 0.88 [95% confidence interval (CI) 0.72–1.07] was observed among nonsmokers and of 1.08 (95% CI, 0.80–1.48) among smokers.

In six prospective studies the association between vitamin C intake and CHD was studied (18–21,24,27). No association was observed. An exception was the inverse association between vitamin C intake and CHD among nonsmokers noted in the Western Electric Study (24). Controlled trials on the association between vitamin C intake and CHD have not been carried out.

In 1993 we published a paper showing an inverse relationship between the intake of flavonols and the 5-yr mortality from CHD in the Zutphen Elderly Study (28). This association was also present when the follow-up period was extended to 10 yr (29). Besides in The Netherlands this association was also studied in three prospective studies carried out in Finland, the United States, and Wales (30–32). An inverse association was also observed in the study from Finland but not in those from the United States and Wales. It can be concluded that the association between flavonol intake and CHD is promising, but more data are needed before definite conclusions on this association can be drawn.

If we try to summarize the results on the associations between the intake of different antioxidants and CHD risk we can conclude that: (i) vitamin E is related to CHD risk in prospective cohort studies but not in case-control and intervention trials; (ii) β-carotene is related to CHD in case-control studies only in smokers but not in prospective cohort studies and intervention trials; (iii) vitamin C is not related to CHD risk in prospective cohort studies; and (iv) flavonols are related to CHD risk in some but not all prospective cohort studies.

FATTY ACIDS, ANTIOXIDANTS AND CHD FROM A PUBLIC HEALTH PERSPECTIVE

Within Europe large differences exist in mortality from CHD. Historically there was a marked North-South gradient with high rates in the North and low rates in the South. Over the last two decades, CHD mortality rates decreased in most northern European countries and the differences between northern and southern Europe became less pronounced (33). Nowadays, the highest CHD mortality rates are observed in eastern Europe. From a public health point of view, it is necessary to know what the major determinants of population CHD mortality rates are. If these determinants are known, preventive measures can be taken in order to reduce the burden of CHD mortality in populations.

The question why CHD mortality rates differ between population was asked in the 1950s by Professor Ancel Keys, University of Minnesota, Minneapolis, Minnesota. He carried out pilot studies in the United States, Europe, and Japan in the early 1950s (34). In 1958 he started the Seven Countries Study, a population-based survey in 16 cohorts of men originally aged 40–59 yr. Between 1958 and 1964, 12,763 men were examined. These men were re-examined after 5 and 10 yr of follow-up and were followed for mortality during 25 yr. During that period, about 6,000 men died and the underlying cause of death was adjudicated in a standardized way. About 1,500 men died from CHD.

Information on smoking habits was collected in all men in a standardized way in the baseline survey. Detailed dietary data were collected in small samples of the cohorts (35) The 7-d record was used as dietary survey method, and duplicate portions of the foods eaten were collected and chemically analyzed. In the 1960s it was possible to determine total fat and

the major classes of fatty acids, e.g., saturated, monounsaturated, and polyunsaturated fatty acids but not n-3 polyunsaturated and *trans* fatty acids. Therefore, it was decided in 1986 to recode the original dietary data of the 16 cohorts in a standardized way. These data were summarized in 16 major food groups, and the average consumption for each cohort was calculated (36). In 1987 food composites were collected representing the average food intake of the 16 cohorts in the 1960s. These food composites were chemically analyzed, and different nutritive and nonnutritive substances were determined.

Univariate analyses showed that the intake of saturated fatty acids was the strongest determinant of 25-yr CHD mortality rates of the 16 cohorts (37). Also the *trans* fatty acid elaidic acid was strongly related to CHD mortality, but this association was not independent from saturated fat intake. Therefore, only saturated fat was included in the multivariate model. In univariate models the dietary antioxidants vitamin E, β-carotene, and vitamin C were not related to long-term CHD occurrence (38). However, flavonoids were inversely related to 25-yr CHD mortality (39). No association was observed between cigarette smoking and CHD mortality, due to the high smoking and low CHD mortality rates in the two Japanese cohorts. However, after multivariate analysis, saturated fat intake and cigarette smoking were positively related with CHD mortality and flavonoid intake inversely. These results suggest that populations characterized by diets low in saturated fat and high in flavonoids and low smoking rates have low mortality rates from CHD. In general terms this means that a diet low in animal foods and hard margarines and high in plant foods is protective in relationship to CHD mortality.

These results from cross-cultural analyses do not prove that changes in diet and smoking habits are directly related to changes in the occurrence of CHD. In primary and secondary prevention trials, it is shown that changes in diet (e.g., lowering of saturated fat intake and increasing consumption of fish, vegetables, and fruits) and antismoking intervention lower not only the risk of (sudden) cardiac death but also of all-cause mortality (9,13,40,41). Together with the increasing evidence from basic research, showing the importance of oxidized LDL in the atherosclerotic process, it can be concluded that interventions on diet and smoking have a large impact on the rate of CHD occurrence in populations.

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Administration of Docosahexaenoic Acid Influences Behavior and Plasma Catecholamine Levels at Times of Psychological Stress

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ABSTRACT: The purpose of the present research was to clarify the effect of docosahexaenoic acid (DHA) intake on behavior and plasma catecholamines (CA). In Study 1, 42 students took either DHA-rich oil capsules containing 1.5–1.8 g DHA/d or control oil capsules containing 97% soybean oil plus 3% of another fish oil for 3 mon in a double-blind fashion. They took a psychological test (PF Study) at the start and end of the study. This study started at the end of summer vacation and ended just before the final exams. In the control group, external aggression (aggression against others) in PF Study was significantly increased at the end of the study as compared with that measured at the start (+8.9%), whereas it was not significantly changed in the DHA group (−1.0%). In a similar double-blind study (Study 2), we measured external aggression under nonstressful conditions. External aggression slightly decreased in the control group, whereas there were no significant changes in the DHA group. In Study 3 with 14 students, plasma CA were measured at the start and end of capsule administration period of 2 mon. Subjects were under continuous stress of the final exams that lasted throughout the whole study period. The ratio of plasma epinephrine to norepinephrine concentrations was significantly increased in the DHA group (78%), whereas it stayed at the same level in the control group. In Study 4, mice were fed either DHA-deficient diet or -sufficient diet for 4 wk, and their rearing frequency (an anxiety index) was measured. In the DHA-sufficient group, the rearing frequency was significantly less than in the other group. These effects of DHA intake may be applied to people in an attempt to ameliorate stress-related diseases.

Docosahexaenoic acid (DHA) is the major n-3 polyunsaturated fatty acid (PUFA) in the brain phospholipid fraction. The depletion of n-3 PUFA affects the brain functions of rats. Yamamoto *et al.* (1) fed rats either an n-3 PUFA-deficient diet or an n-3-sufficient diet for two generations and tested the higher functions of the brain. They found that the correct response ratio in a brightness-discrimination-learning test was better in the n-3-sufficient group than in the n-3-deficient group. However, the total responses, irrespective of their correctness, were 50% greater in the n-3-deficient group than in the n-3-sufficient group by the 25th session when rats were thoroughly trained for the test. The differences seen in the total responses were observed both in Wistar Kyoto rats and SHR (1). Mice fed an n-3-deficient diet and n-3-sufficient diet for two generations had different behavior and also different sensitivities to behavior-affecting medicines (2). These animal experiments suggest that DHA deficiency affects behavior of animals.

Investigating 6- to 12-yr-old boys, Stevens *et al.* (3) showed that boys with lower total n-3 PUFA concentrations in the plasma phospholipid fraction had a greater number of behavior problems, temper tantrums, and sleep problems. n-3 Fatty acid concentrations in blood are also associated with depression. Adams *et al.* (4) showed that the ratio of red blood cell (RBC) phospholipid arachidonic acid to eicosapentaenoic acid (EPA) correlates significantly with severity of depression. Furthermore, Hibbeln (5) recently noted that a high consumption of fish was significantly correlated with a lower annual prevalence of major depression.

From these studies we thought that it was necessary to test the effects of DHA on normal human behavior. In the present paper, we show the effects of DHA in nonsmoking students and also report on our related studies on mice.

MATERIALS AND METHODS

Study 1 (6): Subjects. Fifty-three students (19–30 yr of age) were recruited from two universities: one was a fourth-year class of Toyama Medical and Pharmaceutical University, and the other was a second-year class of Yokkaichi University. The subjects had been judged healthy by physical examination and blood tests 3–4 mon before the entry to the study.

Lipids 34, S33–S37 (1999).

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Abbreviations: CA, catecholamine; CHD, coronary heart disease; DHA, docosahexaenoic acid; EP, epinephrine; EPA, eicosapentaenoic acid; NE, norepinephrine; PUFA, polyunsaturated fatty acids; RBC, red blood cells.

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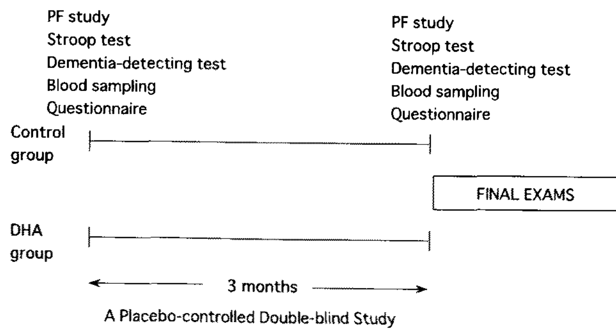

FIG. 1. Design of Study 1.

Study design and oils. The design of the study is shown in Figure 1. The study took place in the two universities with the same protocol. Subjects were asked to complete food frequency questionnaires and questionnaires about adverse effects of oil-containing capsules. At the end of the study another questionnaire was prepared to ask subjects to guess whether they had been taking DHA or control capsules. As shown in Figure 1, the final exams were scheduled a few days after the end of the study as a stressor component. Fasting blood was taken for fatty acid analysis. Mental and psychological tests were performed at the start and end of the study. All subjects were asked to keep their body weights and physical activity levels, and to consume their habitual diets during the study. They were also asked to take 10–12 capsules (according to subjects' body weights) containing either DHA-rich fish oil or control oil for 3 mon for the DHA and control groups, respectively. Each capsule contained 300 mg of oil and 0.3% α-tocopherol. The DHA-rich fish oil contained 49.3% DHA, 6.7% EPA, and 9.0% palmitic acid, 7.3% oleic acid and 3.3% arachidonic acid. Consequently, in the DHA group, subjects ingested 1.5–1.8 g DHA/d from capsules. The control oil was a mixture of 97% soybean oil and 3% of another fish oil. The purpose of the inclusion of another fish oil in the control oil was to camouflage fish odor of DHA capsules. The composition of the control oil was 54.1% linoleic acid, 22.3% oleic acid, 10.8% palmitic acid, 6.8% α-linolenic acid, 3.7% stearic acid, and 0.5% DHA.

PF Study, and Stroop and dementia-detecting tests. PF Study was originally developed by Rosenzweig (7). This psychological test consisted of 24 pictures illustrating frustration. Subjects were asked to look at pictures and describe their first reactions. Their reactions were analyzed according to their direction. If the direction of reactions was against others, the reactions were diagnosed external aggressive. Stroop test checked accuracy and speed of discrimination of color-meaning words written in different colors. Dementia-detecting test required two kinds of ability at a time, namely to pick out certain indicated letters (usually three kinds of letters) from a few sentences and put a circle on every indicated letter while understanding the sentences.

Study 2 (8). The protocol of Study 2 was essentially the same as Study 1. The only major difference was that there was no stressor component in Study 2. Minor differences were: (i) fatty acid composition was determined with the total phospholipid fraction of RBC; (ii) subjects ingested 10 DHA or placebo oil-containing capsules instead of 10–12 capsules/d. Another group of 59 nonsmoking volunteers was recruited from Toyama Medical and Pharmaceutical University and Kogakkan University.

Study 3. Plasma catecholamine (CA) concentrations were measured in a similar 2-mon study with another group of volunteers (medical students). In this study, subjects were asked to take 10 capsules of DHA-rich fish oil as in Study 2 or mixed plant oil. The major difference between Studies 1 and 3 was the presence of continuous psychological stress throughout the study period, during which subjects underwent more than 20 stressful final exams. The placebo oil was a mixture of 47% olive oil, 25% rapeseed oil, 25% soybean oil, and 3% of DHA-rich fish oil. At the start and end of the study, fasting subjects were asked to sit quietly with an indwelling needle in an antecubital vein for 30 min. Then blood was taken for plasma CA concentrations and for the fatty acid composition in red blood cells (RBC).

Study 4. Male ddY mice were group-fed either a DHA-rich diet (90% lipid-free diet + 8% lard + 2% DHA ethyl ester) or a control DHA-deficient diet (DHA was replaced with soybean oil) for 4 wk with the reversed day/night cycle (dark, 9:00–21:00; light 21:00–9:00). At the end of diets, locomotor activity (rearing) was measured with an animal movement analyzer (Scanet SV-10, MATYS, Toyama) during a dark cycle.

Statistical analysis. Data are expressed as means ± SD. Intragroup and intergroup differences were analyzed by paired and unpaired t-tests (comparing intragroup differences of the two groups), respectively.

RESULTS

Study 1. Noncompliant subjects such as those taking less than 70% of capsules, changing their body weights by more than 3 kg, smoking, and dropping out for personal reasons were excluded before the double-blind code was broken. Subjects did not complain of any serious adverse effects in either group. The questionnaires performed at the end of the study showed that subjects could not guess the kind of their own capsules more correctly than by chance. This was also the case for Studies 2 and 3.

Because two DHA subjects whose DHA concentrations in blood did not increase were excluded before analysis by one of the authors who did not know the results of PF Study, DHA concentrations in all the analyzed DHA subjects increased (3.1 ± 0.5 to 6.1 ± 1.6%, theoretically $p = 0$) in the total serum phospholipid fraction, whereas those in the control group did not change significantly (3.6 ± 1.3 to 3.5 ± 1.1%). The final numbers of subjects analyzed were 20 (7 males and 13 females) and 22 (5 males and 17 females) for the control group and DHA group, respectively. As shown in Figures 2 and 3, no significant differences were observed between the two groups in Stroop and dementia-detecting tests, respectively.

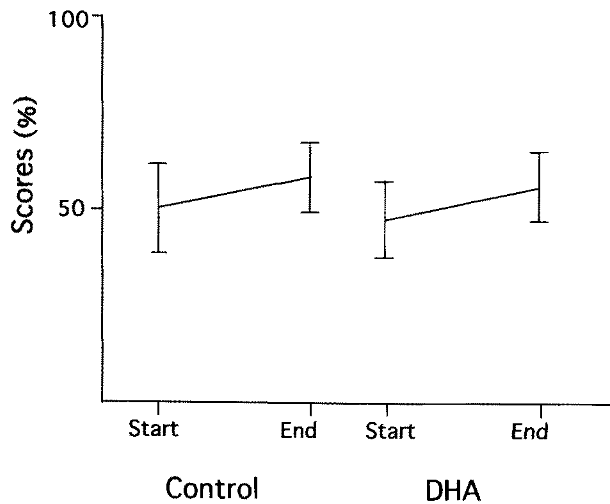

FIG. 2. Stroop Test (Study 1). Stroop test was performed at the start and end of 3 mon of capsule administration. There were no significant differences between the two groups. n = 19 for the control group and 22 for the DHA group.

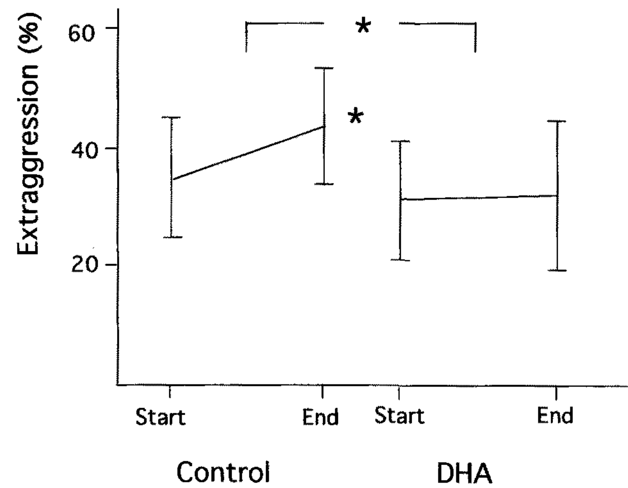

FIG. 4. Changes in external aggression (Study 1). External aggression was measured at the start and end of 3 mon of capsule administration. n = 19 for the control group and 22 for the DHA group. $^{*}P < 0.007$.

However, as shown in Figure 4, there were highly significant differences between the two groups in PF Study. In the control group, external aggression increased significantly, but in the DHA group, it stayed unchanged. The increase in external aggression in the control group was set off by a decrease in extraggression (aggression against nobody). The average daily intake of DHA from food was about 220 ± 90 mg in the control group and 230 ± 90 mg in the DHA group.

Study 2. Noncompliant subjects were excluded as in Study 1. Twenty-four control subjects (11 males and 13 females) and 22 DHA subjects (13 males and 9 females) were selected for analysis. DHA in the total phospholipid fraction of RBC in the %DHA group increased from 4.8 ± 0.9% to 7.0 ± 1.0% (theoretically $p = 0$), and arachidonic acid decreased from 9.4 ± 1.0 to 8.8 ± 0.6% ($P < 0.001$). In the control group, linoleic acid significantly increased from 8.3 ± 0.7 to 9.0 ± 0.9% ($P < 0.001$). As shown in Figure 5, external aggression decreased slightly but significantly ($P < 0.005$). However, the intergroup difference was only marginally significant ($p = 0.05$).

Study 3. DHA in the total phospholipid fraction of RBC increased in every DHA subjects from 5.8 ± 1.1 to 8.2 ± 1.1% ($P < 0.002$), whereas in the control group there were no significant changes in any fatty acids between the start and end of the study. The plasma norepinephrine (NE) decreased in the DHA group (2.3 ± 0.8 to 1.6 ± 0.5 nmol/L, −31%, $P < 0.03$), whereas it did not significantly change (2.0 ± 0.7 to 1.8 ± 0.7 nmol/L) in the control group. Epinephrine (EP) levels

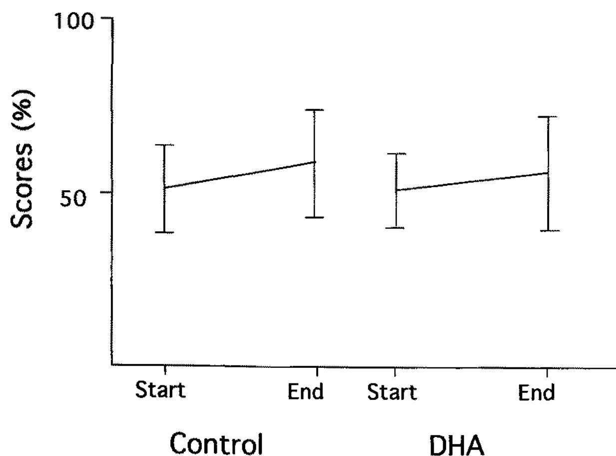

FIG. 3. Dementia-detecting test (Study 1). Dementia-detecting test was performed at the start and end of 3 mon of capsule administration. There were no significant differences between the two groups. n = 20 for the control group and 22 for the DHA group.

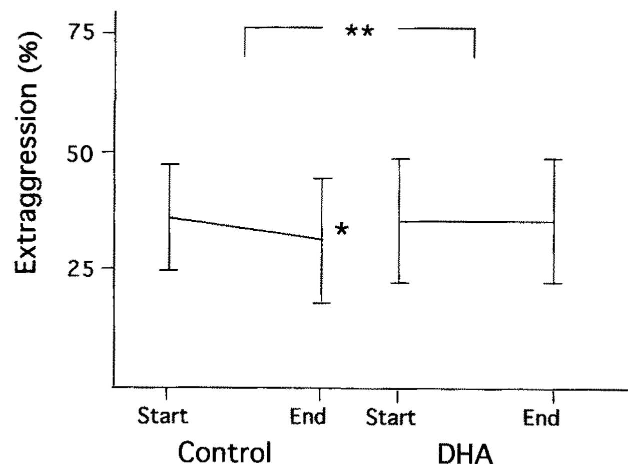

FIG. 5. Changes in external aggression (Study 2). External aggression was measured at the start and end of 3 mon of capsule administration. n = 24 for the control group and 22 for the DHA group. $^{*}P < 0.005$, $^{**}p = 0.05$.

decreased in the control group (187 ± 121 to 166 ± 177 pmol/L) and increased in the DHA group (197 ± 149 to 224 ± 156 pmol/L), although not significantly in either group. Consequently, the ratio of plasma EP to NE increased in all DHA subjects by 78% (Fig. 6).

Study 4. As shown in Figure 7, the locomotor activity, rearing frequency, was significantly higher in the control diet group than in the DHA group.

DISCUSSION

Study 1. We started this study on September 4, around the end of summer vacation, and terminated the study on December 4. In Toyama there were final exams in pathology for all subjects just a few days after the end of the study. These university exams were probably the toughest of all for Toyama subjects. In Yokkaichi, all subjects had to finish their graduation thesis (also the toughest trial for them) by the middle of December. Consequently, December 4 was one of the busiest and most frustrating days for both Toyama and Yokkaichi subjects. External aggression was naturally increased in the control group, considering the situations of subjects. Why, then, was external aggression in the DHA group stable? n-3 PUFA-depleted mice had different behavior (2), and n-3-depleted rats were more aggressive (1). Our control subjects might have suffered from DHA deficiency, while DHA subjects were not. With enough DHA intake, those final exams as stressor might not have been strong enough to provoke external aggression.

Enhanced aggression was once recognized as a risk factor of coronary heart disease (CHD) (9). But now one of the aggression components, hostility, is recognized as a more reliable risk factor for CHD and likely all-cause mortality as well (10).

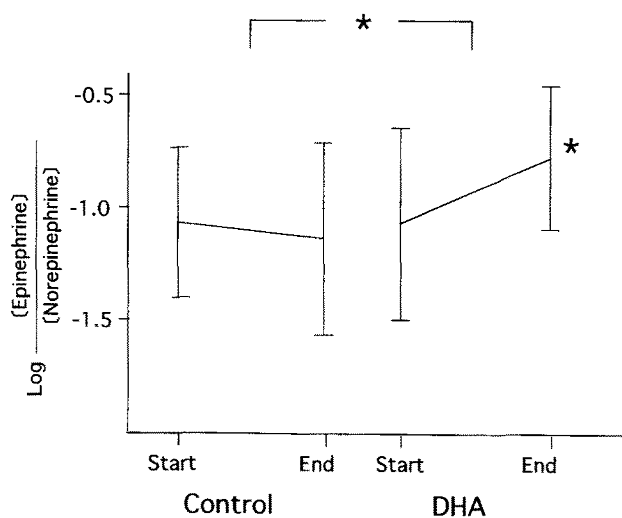

FIG. 6. Changes in the ratio of epinephrine to norepinephrine (Study 3). Catecholamines were measured at the start and end of 2 mon of capsule administration. Data were log-transformed for normalization. The averaged increase in the DHA group was 78%. $n = 7$ for each group; $^*P < 0.03$.

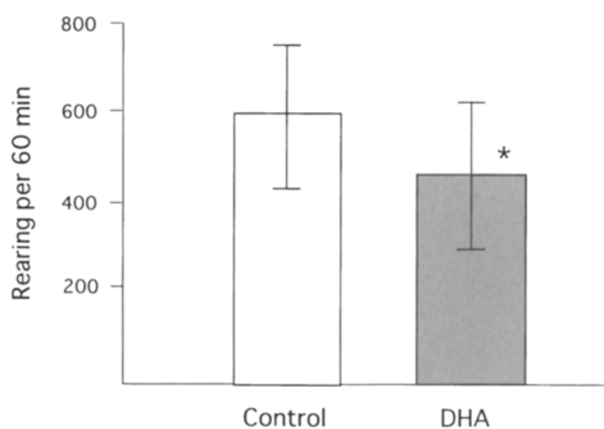

FIG. 7. The rearing frequency of rats (Study 4). The rearing frequency was measured after 4 wk of diets. $n = 20$ for each group. $^*P < 0.01$.

The preventive effects of fish oils on CHD death are well-known (11–13). In this context, the aggression-controlling effects of DHA may be one of the mechanisms of CHD prevention by fish oils.

Stroop and dementia-detecting tests did not show any differences at all. Performance at the final exams carried out a few days after the end of the study was essentially the same between the two groups (data not shown). These points indicate that capsules did not affect deep concentration and quick judgments.

Study 2. External aggression in the control group decreased contrary to Study 1, in which controls exhibited increased external aggression at the end of the study (Figs. 4 and 5). The major difference between these two studies consisted in the absence of extremely important university exams at the end of the study period in Study 2. Because we carefully planned the timing of psychological testing, any important university exams were not scheduled within 30 d from any checkpoints. It is likely that stress levels for students were alike between the two checking points. Then, why did measured external aggression decrease in the control group? Habituation to testing could account for such a decline, but this would not explain the failure of a drop in measured external aggression in the DHA group.

In general, the presence of a stressor increases the degree of aggression (14,15). Hostility scale measured with the Multiple Affect Adjective Checklist and aggression scale with the Adjective Rating Form of nondrinkers were higher than those of moderate drinkers when subjects were not provoked, whereas, after provocation, both scales increased in the two groups, those of moderate drinkers becoming higher than for nondrinkers (15). Thus, it is possible that the results of the psychological test without stressor as performed in Study 2 were different from Study 1. The results of Study 2 therefore do not necessarily contradict the finding of Study 1 that DHA reduced external aggression in a normal population under significant stress.

Study 3. What does an increase in the ratio of EP to NE mean? Christensen *et al.* (16) measured basal plasma CA lev-

els of 412 males of 70 yr of age and followed them for 7 yr. Analysis of plasma CA levels revealed that only 10% of the subjects with high plasma EP levels and low NE had died during the 7-yr follow-up period, but 50% of the subjects with low plasma EP and high NE had died during the same period. Psychological stress in patients with duodenal ulcer does not increase plasma EP levels but NE levels (17). Consequently, it is likely that subjects in the DHA group adapted with chronic psychological stress more favorably than for the control group in terms of CA levels.

Study 4. We still do not know how DHA works on human behavior. A simple animal model may be useful in answering this question. The models with rats (1) and mice (2) might be interesting. However, those models were definitely different from our human studies in the following two points: first, the dietary intervention period was too long compared with ours (two generations instead of 2–3 mon); second, in those studies (1,2), n-3 PUFA-deprived animals and n-3-sufficient animals were compared, whereas our human controls were not n-3 PUFA-deprived at all. In this context our animal model was very similar to our human study situations. Our control mice had some n-3 PUFA in their diet (soybean oil contained 6% α-linolenic acid); the experimental diet period was only 1 mon. Nevertheless, we were able to find a difference in behavior between the two groups. Mechanistic studies using this animal model are now underway.

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Evidence for the Unique Function of Docosahexaenoic Acid During the Evolution of the Modern Hominid Brain

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ABSTRACT: The African savanna ecosystem of the large mammals and primates was associated with a dramatic decline in relative brain capacity associated with little docosahexaenoic acid (DHA), which is required for brain structures and growth. The biochemistry implies that the expansion of the human brain required a plentiful source of preformed DHA. The richest source of DHA is the marine food chain, while the savanna environment offers very little of it. Consequently *Homo sapiens* could not have evolved on the savannas. Recent fossil evidence indicates that the lacustrine and marine food chain was being extensively exploited at the time cerebral expansion took place and suggests the alternative that the transition from the archaic to modern humans took place at the land/water interface. Contemporary data on tropical lakeshore dwellers reaffirm the above view with nutritional support for the vascular system, the development of which would have been a prerequisite for cerebral expansion. Both arachidonic acid and DHA would have been freely available from such habitats providing the double stimulus of preformed acyl components for the developing blood vessels and brain. The n-3 docosapentaenoic acid precursor (n-3 DPA) was the major n-3-metabolite in the savanna mammals. Despite this abundance, neither it nor the corresponding n-6 DPA was used for the photoreceptor nor the synapse. A substantial difference between DHA and other fatty acids is required to explain this high specificity. Studies on fluidity and other mechanical features of cell membranes did not reveal a difference of such magnitude between even α-linolenic acid and DHA sufficient to explain the exclusive use of DHA. We suggest that the evolution of the large human brain depended on a rich source of DHA from the land/water interface. We review a number of proposals for the possible influence of DHA on physical properties of the brain that are essential for its function.

THE INVERTEBRATES, REPTILES, AND MAMMALS

The origin of air-breathing animals. For the first 2.5 billion yr of life on the planet, the blue-green algae dominated the proto-oceans. The photosynthesis of the algae produced complex molecules, including proteins, carbohydrates, and lipids that were rich in n-3 fatty acids. Based on the explosion of the phyla in the fossil record, oxidative metabolism became predominant about 600 million yr ago. Thus animals, visual, nervous systems, and brains evolved in a docosahexaenoic acid (DHA)-rich environment (1).

Mammals. The dominance of n-3 fatty acids in the early oceans was associated with fish and reptiles requiring n-3 fatty acids for their reproduction. This dominance persisted until the end of the Cretaceous period, 70 million yr ago. In the wake of the extinction of the giant reptiles, cycads, ferns and their allies, and the flowering plants appear in the fossil record. They stored lipids, for energy during germination, containing seed oils rich in n-6 fatty acids. Then, a new set of species, the mammals, evolved: it may not be a coincidence that they required n-6 fatty acids for their reproduction.

Mammalian brain size is larger in relation to body size compared to the previous egg-laying amphibians, reptiles, and fish. The difference could be explained by the evolution of the placenta. The placenta enables nutrients and energy to be focused continuously on the development of one or a small number of progeny throughout the critical time of brain development. In the human, 70% of the calories used at this time are devoted to brain growth. The placenta is a rapidly growing vascular system with a high requirement for n-6 fatty acids, especially arachidonic acid (AA). In 42 species so far studied, AA and DHA are major acyl constituents of the brain, with the precursors being virtually absent. So the emergence of the n-6 fatty acids may have added the missing biochemical link, liberating genetic potentials for vascular development and hence the evolution of the placenta, mammary gland, and the larger brains of the mammals.

The difference between species is not the chemistry but the extent or size to which the brain is developed (1). The manner of these differences is so large as to imply that the availabilities of AA and DHA were limiting factors in the evolution of the brain (1,2). Indeed, the need for both n-6 and n-3 fatty acids for development and health of the vascular system and brain has long been recognized (3).

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; n-3 DPA, n-3 docosapentaenoic acid; LC-PUFA, long chain polyunsaturated fatty acid; PE, phosphatidylethanolamine.

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EVIDENCE FROM PALEONTOLOGY

The evolution of the modern human brain. The accepted dogma regarding the evolution of *Homo sapiens* is that he was originally a hunter and gatherer on the African savanna. A study of savanna and other African species shows that as they evolved larger and larger bodies, the relative size of the brain diminished logarithmically with increase in body weight (1,4). A cebus monkey of 0.9 kg body weight has 2.3% of its body weight as brain, a 60 kg chimpanzee 0.5%. The larger gorilla, at 110 kg, has only 0.25% brain, which is physically smaller than the chimpanzee's brain. At the extreme, the one-ton rhinoceros has <0.1% with its brain weighing only 350 g. It reaches that massive one-ton body weight at 4 yr of age.

Why do size and velocity of growth matter? The reason is that the biosynthesis of AA and DHA is relatively slow and may not be able to keep up with body growth in fast-growing animals. Rats and mice desaturate and chain-elongate the parent essential fatty acids to produce larger amounts of AA and DHA than their precursors. Stepping up in size from the guinea pig to the wild pig, the impact of velocity of growth results in a progressive decline in AA and DHA while the precursors linoleic and α-linolenic acids become more dominant in liver lipids (1). Instead of DHA, the n-3 docosapentaenoic acid (n-3 DPA) precursor is now the major metabolite of α-linolenic acid (5).

So the faster an animal grows, the larger it becomes and the greater is the constraint of the biosynthesis of AA and DHA. The large savanna mammals of Africa all shared the same fate: DHA and brain capacity declined as body size accelerated. The important issue is that desaturation and elongation in these large mammals peter out at the n-3 DPA with relatively little DHA being synthesized. What little DHA is synthesized is used in the brain and photoreceptor. The abundant n-3 DPA is not found here. Brain size was sacrificed, not brain DHA (1,6). This fact raises two issues: (i) The savanna food chain on which *H. sapiens* is supposed to have evolved is fairly devoid of DHA so how did *H.* evolve a large brain? (ii) Why was the more readily available n-3 DPA not used for the brain instead of DHA?

Modern human intellect and brain-specific nutrition. Australopithecus spp. are unremarkable in their apparent encephalization throughout their evolutionary history as far as can be deduced from the fossil record. No australopithecine has a cranial capacity much over 500 cm^3 (7), despite the existence of the genus for over 3 Myr. Contrast this to genus *Homo*, whose cranial capacity doubled from *H. erectus* to *H. sapiens* in a span of at most 1 Myr (Table 1). The *Homo* spp. fossil evidence and encephalization quotient values do not support a slow, linear Darwinian progression toward modern intelligence, but rather a sudden, exponential growth of relative brain size in the last 200,000 yr or so.

The earliest evidence for modern *H. sapiens* is found in Africa. *Homo* spp. in general are associated with lakeshore (lacustrine) environments in the East African Rift Valley, while australopithecines are associated more with forested areas (8,9). Thus far, evidence for precocious cultural development of *H. sapiens* is exclusively confined to lacustrine and coastal marine environments. Lakeshore sites in the Rift Valley yielded fairly sophisticated stone tools as old as 260 kyr associated with *H. sapiens* remains. The implications of this land/water habitat providing brain-specific nutrients have largely been overlooked.

TABLE 1
Mean Brain Volumes and Encephalization Quotients (EQ) for Selected Hominoid Species[a]

Species	Brain volume (cm^{-1})	EQ1	EQ2
Australopithecus afarensis	384	1.23	1.45
A. africanus	420	1.31	1.62
A. boisei	488	1.37	1.72
A. robustus	502	1.49	1.92
Homo habilis	579–597	1.74–1.79	2.10–2.29
H. rudolfensis	709	1.41	2.11
H. erectus	820–844	1.59–1.63	2.38–2.44
H. sapiens	1,250	3.05	4.26
Pan troglodytes	410	1.25	1.57

[a]EQ1 from the calculations of Martin (Ref. 4); EQ2 from the calculations of Harvey and Clutton-Brock (Ref. 47).

Fossils from coastal sites on the southern Cape of South Africa are widely regarded as the earliest modern human fossils (10–12). At numerous sites along the Cape, hominid occupation is evidenced dating from 120 to 60 kyr before now. Modern human fossils dating to about 100 kyr and recovered at Klasies River Mouth and Border Cave are found associated with incontrovertible evidence for the consumption of seafood dating to the time of rapid cerebral expansion (10,13–15).

Parkington (15) points out that in coastal hunter–gatherer cultures women are responsible for collecting shellfish. So Stone Age women could have easily provided themselves with a plentiful source of brain-specific nutrition, even when strength/mobility are compromised during pregnancy and lactation. Children would have naturally participated in exploitation of, at that time, this extremely rich resource. Early consumption of shellfish is also present in the archaeological record on the Mediterranean coast of Africa. It is likely to have occurred elsewhere as well; however, most possible coastal sites which could be investigated have been obliterated by the higher sea levels of the current interglacial era.

Successful early Homo *spp. were tropical coastal migrants.* Both *H. erectus* and *H. sapiens* successfully colonized areas outside of Africa. There is all but unanimous agreement among paleoanthropologists that *H. sapiens* originated in Africa and then spread throughout the world (16–19). Recently, stone tools of 0.8–0.9 Myr were found on the island of Flores, one of the Wallacean Islands lying between Java and Timor in Indonesia (20). The antiquity of the specimens suggests they were manufactured by *H. erectus*, not *H. sapiens*. Although Java and Bali were periodically connected to the mainland during the Pleistocene glaciation, even at times of lowest sea level, reaching Flores would have required a sea crossing of at least 19 km. This implies that at least in Indone-

sia, *H. erectus* had already reached the cognitive capability to build and use watercraft repeatedly.

Previous to the recent discovery by Morwood *et al.* (20), the earliest evidence for the use of watercraft dates from about only 40 kyr or slightly earlier with the migration of *H. sapiens* from the Wallacean Islands to Australia. That initial colonization of Australia, Tasmania, and New Guinea was accomplished by modern *H. sapiens*. Similar to the movements of *H. erectus*, these early migrants are considered to have followed a tropical coastal route. Therefore, both the earliest occurrence of modern *H. sapiens*, the earliest use of watercraft, and successful colonization of Southeast Asia were intimately associated with the utilization of food resources from the marine food chain.

We consider this association neither accidental nor coincidental, but a reflection of the dramatic influence of brain-specific nutrition on the evolutionary process. We do not accept the postulate that *H. sapiens a priori* evolved a large, complex brain, then began to hunt in order to maintain it—the brain must come first.

Our thesis is that there must have been enough long-chain polyunsaturated fatty acids (LC-PUFA) available in the diet to: (i) provide many generations of hominids with fuel for fetal/infant development as well as childhood and adult needs for the cardiovascular system and the brain, (ii) allow for substantial amounts of 18-carbon PUFA which would have been oxidized for energy requirements (21,22), and (iii) explain and allow for our inefficient conversion of linoleic acid to AA and linolenic acid to DHA [which is illustrated by preferential incorporation of DHA in the infant brain (23) and improved problem solving in infants fed DHA which persisted beyond the period of supplementation (24)].

THE STRUCTURE OF DHA VS. n-3 AND n-6 DPA

Global three-dimensional energy-minimized structures of DHA, n-3 DPA, n-6 DPA, and various phospholipids containing these LC-PUFA were constructed with MOPAC software (Alchemy 2000 v. 2.0; Tripos Inc., St. Louis, MO). MOPAC (molecular orbital package) calculates the steric energy and energy-minimized configuration of a given molecule by successive approximation and is considered to be reasonably accurate

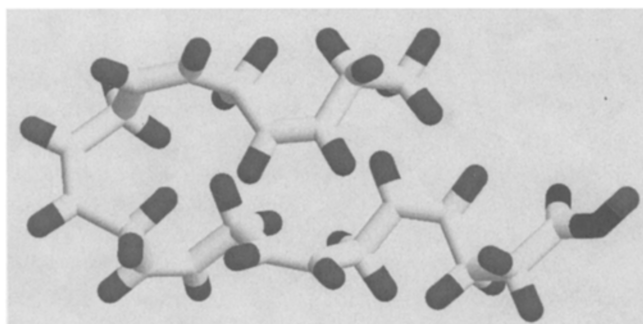


FIG. 1. 3-D energy-minimized structure of docosahexaenoic acid (DHA). This and following figures are energy minimized and drawn with molecular orbital package (MOPAC) software as described in the text.

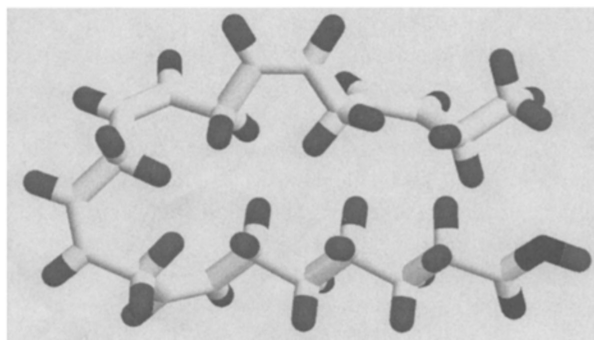


FIG. 2. n-3 Docosapentaenoic acid (n-3 DPA).

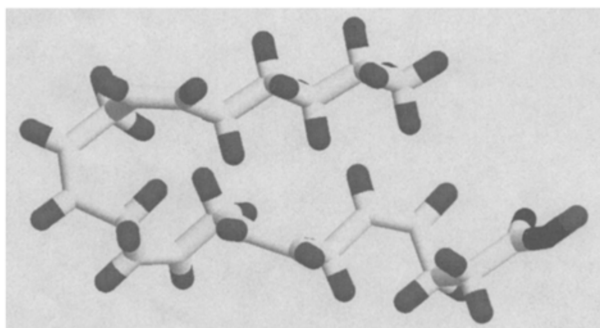
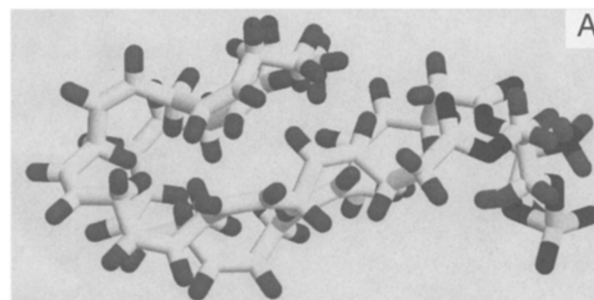


FIG. 3. n-6 Docosapentaenoic acid (n-6 DPA).



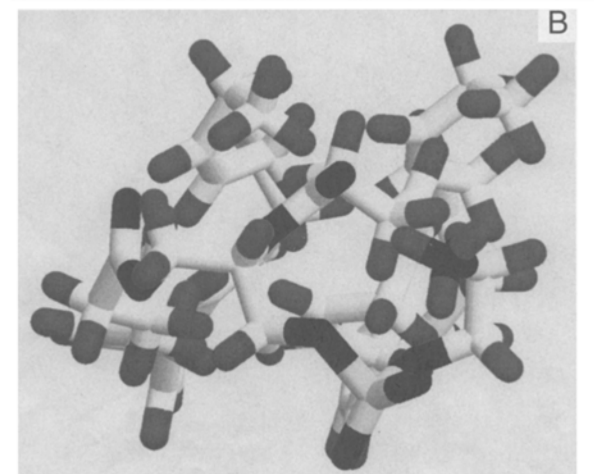


FIG. 4. (A) 3-D Energy-minimized structure of phospholipid with ethanolamine, DHA, DHA. Side view. (B) Ethanolamine, DHA, DHA. End view; note position of phosphate group. See Figure 1 for abbreviation.

as compared to known structures. The free fatty acids DHA, n-3 DPA, n-6 DPA are shown in Figures 1 to 3, respectively. The sixth ethylenic bond in DHA changes the character of the free fatty acids, completing the methylene-interrupted sequence along the carbon chain and conferring a folded, slightly spiral nature to the molecule. In n-3 DPA, the side of the chain closest to the terminal methyl is essentially ethylenic, while the other side is essentially saturated. The opposite is seen in the n-6 DPA, where the side of the chain closest to the methyl group is saturated, and the other side unsaturated. The DPA lack the full methylene-interrupted sequence of double bonds throughout the carbon chain, which could be the basis of why they apparently do not have the functionality required by retinal and brain tissue.

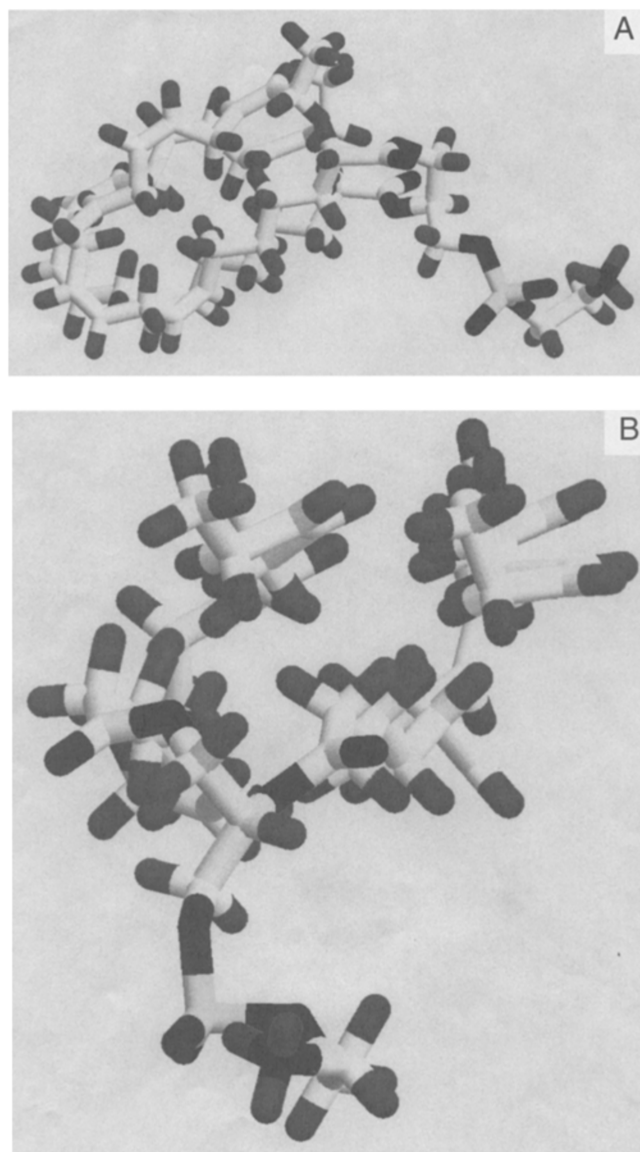


FIG. 5. (A) 3-D Energy-minimized structure of phospholipid with ethanolamine, n-3 DPA, n-3 DPA. Side view. (B) Ethanolamine, n-3 DPA, n-3 DPA. End view; note position of phosphate group. See Figure 2 for abbreviation.

The energy-minimized ethanolaminephosphoglyceride structures with DHA (Fig. 4) and n-3 DPA (Fig. 5) dramatically illustrate the significance of the missing double bond in DPA vs. DHA. The final (C_{19}) double bond in DHA constrains the position of the phosphoethanolamine headgroup, pulling it in and maintaining the spiral structure. This reduces the molecular volume and may facilitate communication between the headgroup and the esterified lipid chains. In contrast, the headgroup in n-3 DPA is far less constrained and in fact moves away from the lipid ester chains. This structure would be more typical of phospholipids in general since most fatty acids are less polyunsaturated than DHA. The cell membrane is in constant fluid motion so these structures only represent the preferred orientations of the molecules.

The evidence on the extensive coastal and lacustrine exploitation implies LC-PUFA were consistently abundant in the food supply as we evolved. *Homo* did not, however, go as far as the obligate carnivores in which the desaturation process is barely detectable (25). If *H. sapiens* had developed his intellect by evolving into a primate which can make heroically efficient use of 16- and 18-carbon PUFA from vegetarian sources, we would see an obvious signature in our current PUFA metabolism, since we are only a 300–100-kyr-old species. Instead we see the opposite. We might hypothesize that *Australopithecus* spp. could not mount this heroic metabolic effort either, which explains why their brain capacity was constrained by their land-based diet at 400–500 cc for 3 Myr and explains why exploitation of coastal foods fits with the rapid and recent cerebral expansion to 1.3 kg after 3 Myr of a static brain size (9,15).

CONTEMPORARY EVIDENCE

The human brain requires a rapidly developing heart and vascular system to meet the prodigious energy and nutrient demand during its development. The vascular system itself has a high requirement for AA (26). Hence the principles of vascular development were *sine qua non* vital for the final evolution of the large human brain.

If we now examine the contemporary evidence on cardiovascular disease, we find that land-based animal fats have been causally linked to heart disease since the Seven Countries Study of the 1950s and even earlier (27). Saturated fats and vascular degeneration would be incompatible with central nervous system expansion. Also, there is increasing evidence that cardiovascular disease has its origin in poor fetal nutrition (28), consistent with our hypothesis of long-term, multigeneration effects operating on vascular and central nervous system evolution. Those forces can act for expansion or degeneration.

Worldwide diets and cardiovascular risk factors show that marine fats, especially DHA, are cardioprotective (29–31). It is well known that people living on seafood have low cardiovascular risk factors. The diet of contemporary populations beside East African lakes (Lake Nyasa and Lake Turkana) is still largely based on fish rich in n-3 and n-6 LC-PUFA. From Table 2, calculated intakes of n-3 LC-PUFA are 1–4 g/d and AA 0.5–1.0 g/d, compared with n-3 LC-PUFA 0.2–0.3 g/d

and AA 0.1–0.2 g/d for populations consuming Western diets. Total dietary fat in the African populations was similar at 10–15% of the dietary energy (32).

We compared East African lakeshore, mainly vegetarian, and European cardiovascular risk factors (Table 3). Blood cholesterol, blood pressure, and lipoproteins [Lp(a)] are lower in the contemporary Africans living on lakeshores of Turkana and Nyasa compared to their vegetarian cousins and Europeans. Plasma AA, eicosapentaenoic acid, and DHA are highest in the fish-eating, lakeshore people and lowest in the vegetarian or omnivorous inland cousins. Furthermore, comparison of children from the lakeshore vs. European children living in East Africa showed that the two populations can be separated on the basis of blood cholesterol at the age of 6 yr! While the European children's blood pressure and cholesterol continue to rise, the Africans remain stable, illustrating the compatibility of the lacustrine diet with good cardiovascular performance compatible with the needs of fetal brain expansion. Interestingly, the Turkana have the highest mitochondrial DNA diversity of any ethnic group. In fact, 36 Turkana people have a higher diversity than the worldwide population database. The simplest explanation is that humans date back to the East African Rift Valley (33).

These unique conditions of the Rift Valley lakeshores replicate the contrast in the high mortality from vascular disease and high prevalence of mental disease among Americans and Europeans vs. the low risk for Japanese, Greenland, and Inuit Indians living on a rich fish and seafood diet (34). Similarly, comparison of fish-eating populations in the Faroes compared to their genetically similar mainland Denmark contemporaries shows that the high intake of fish and seafood results in higher birth weights and a lower proportion of preterm deliveries (35). The advantage of longer gestations is the greater exposure of the fetus and its developing vascular system and brain to the placental biomagnification of AA and DHA (36). The conclusion is that land-based diets led increasingly in this century to cardiovascular disease being the number one killer in Western consumers, and which would have made cerebrovascular expansion *in utero* difficult and incompatible with expansion of the hominid brain.

Experimental support for the above case came in an unexpected result from studies on diabetes by our colleagues, Professor Lucilla Poston and others at St. Thomas's Hospital Medical School. Pregnant rats were subjected to high saturated fat diets similar to those consumed in Western countries, and blood vessel function tested in mothers and newborn offspring. The results from small vessel myography described arterial dysfunction specifically associated with the high-fat Western-type diet. Vascular function tests on the 15-d-old pups from mothers on the high-fat (30%) diet exhibited reduced vascular endothelium-dependent relaxation to acetylcholine, with evidence of constrictor responses to noradrenaline and the thromboxane mimetic U46619. The vascular dysfunction was still observable at 120 d of age despite rearing on a normal diet. Thus the high-fat diet fed to the mother changed the intrauterine milieu, which caused persistent vascular dysfunction in the newborn animals without any genetic predisposition. Diabetes imposed on the high-fat diet made vascular function worse. Biochemical analysis of the tissues from the control low-fat and high saturated fat animals revealed the high-fat diet selectively depressed liver DHA of the pups. Here is experimental evidence of the negative influence of land-based animal fats fed to the mother on the next generation, emphasizing the importance of long-term nutritional forces (37).

TABLE 2
The Fatty Acid Composition Data of the Fish from Lakes Nyasa and Turkana (wt%)[a]

	Turkana Tilapia species	Turkana Tilapia? species 2	Nyasa Mfui	Nyasa Kambale	Nyasa Carp	Nyasa Mbelele catfish
Fat g (wt%)	2.3	2.6	1.1	1.8	4.9	10.3
20:4n-6	8.4	7.7	8.0	5.8	5.8	4.3
20:5n-3	2.8	1.8	3.1	2.2	1.8	4.2
22:5n-3	3.2	3.8	3.7	5.2	5.0	1.8
22:6n-3	15.7	18.1	19.1	13.3	7.8	8.6

THE SPECIFICITY OF DHA

The question which arises from this discussion is: What is so special about DHA? Why has DHA been chosen so overwhelmingly for photoreceptor and synaptic membranes, despite the availability of similar molecules which would be less difficult to obtain and are less vulnerable to oxidative damage (38,39)? In particular, what advantage does it convey relative to the very closely related n-3 and n-6 DPA, each of which differs from DHA only in the absence of one double bond (between carbons 4–5, and 19–20, respectively)?

As described above, nature's preference for DHA in the brain is strikingly demonstrated in large land mammals, in which DPA is the dominant n-3 metabolite, yet neural membranes still retain the DHA-rich composition observed in other species (possibly at the expense of gross brain size, since DHA is in such limited supply). Significant quantities of the n-6 form of DPA are observed only in situations of artificial n-3 deficiency, yet even here brain membranes are resistant to decreases in their DHA levels. Nature is thus highly sensitive to the slight difference between DHA and DPA molecules; the presence of DHA's full complement of six double bonds is for some reason an important priority in neural membranes.

What is the cause of such specificity in membrane composition? It is understood that biological membranes, while always having the form of a fluid lipid bilayer, have detailed distributions of lipid and protein molecules that reflect the interactions between lipids and integral membrane proteins (40). It seems that the one missing double bond in DPA species renders them unsuitable for whatever lipid–protein interaction favors DHA's inclusion in membranes of the brain.

Tight regulation of membrane lipid composition extends to differentiation between polyunsaturated species. We recently investigated the relationship between DHA and AA levels in plasma and red cell membranes of maternal and fetal

TABLE 3
Comparison of Cardiovascular Risk Fatty Factors, Plasma and Fish Fatty Acid Composition of Lakeshore, Fish-Eating Vegetarian and European, Communities in the Rift Valley and East Africa[a]

		Populations		
		Largely vegetarian	Lakeshore	significance of differences (P)
Plasma lipids (mg/dL)				
Plasma TC		136.3 ± 39.8 n = 686	121.6 ± 30.9 n = 622	<0.05
Plasma TG		105.3 ± 53.1	80.6 ± 40.7	<0.001
Lp(a)		32.3 ± 22.4	19.9 ± 17.7	<0.001
Blood pressure (mmHg)				
Nyasa Systolic		134.7 ± 20.4	120.4 ± 15.1	<0.001
Diastolic		77.6 ± 10.6	70.5 ± 8.9	<0.001
Turkana systolic		European	El Molo	
Age (yr)	0–3	82 ± 14 n = 15	85 ± 9.7 n = 6	ns
	6–10	98 ± 22 n = 16	87 ± 17 n = 16	ns
	16–20	90 ± 28 n = 24	119 ± 28 n = 14	<0.001
	25–45	131 ± 34 n = 265	105 ± 30 n = 24	<0.001
Cholesterol (mg/dL)				
Age (yr)	0–3	102 ± 24 n = 15	97 ± 18 n = 12	ns
	6–8	167 ± 35 n = 18	112 ± 32 n = 16	<0.01
	25+	228 ± 44 n = 145	147 ± 49 n = 24	<0.001
Plasma FA (wt%)				
Lake Nyasa				
LA		14.85 ± 4.30 n = 53	23.9 ± 4.37 n = 53	<0.002
LNA		0.60 ± 0.20	0.31 ± 0.14	<0.001
AA		8.26 ± 1.94	9.85 ± 2.68	<0.005
EPA		0.72 ± 0.22	2.48 ± 1.35	<0.001
DHA		1.48 ± 1.04	5.93 ± 1.77	<0.001
Lake Turkana		El Molo n = 32	Bantu n = 98	European n = 124
LA		9.3 ± 3.0	22.8 ± 4.8	19 ± 4.9
DHLA		1.9 ± 0.7	3.5 ± 1.3	2.4 ± 1.1
AA		12.2 ± 3.8	5.1 ± 2.7	7.0 ± 2.4
EPA		4.7 ± 1.3	1.6 ± 0.8	0.5 ± 0.2
DPA		2.6 ± 0.9	3.2 ± 1.2	2.1 ± 0.9
DHA		9.3 ± 3.3	3.5 ± 1.3	5.6 ± 2.2

[a]P < 0.001 for LA, AA, and DHA in El Molo compared to all others. n, number of subjects; TC, total cholesterol; TG, triglycerides; FA, fatty acids; LA, linoleic acid; LNA, α-linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid. Values are the average ± SD. Adapted from Ref. 32. El Molo live on a lava desert which runs down to the eastern shore of Lake Turkana, North Kenya (Ref. 48). The Bantu were Baganda and Bunyoro people of central Uganda. The Europeans were living in East Africa, mainly Kampala Uganda (data from Ref. 49). The slow conversion of linoleic to AA and α-linolenic to DHA is illustrated in the equilibrium of the higher linoleic content of the plasma lipids and the lower AA and DHA in the vegetarian and European plasmas compared to the fish-eating people where preformed AA and DHA are consumed in the diet and appear as higher levels in the plasma. Such data reflect the rate limitations of the conversion process, especially Δ6 desaturase which is involved twice in the synthesis of DHA (50). The higher circulating levels of AA and DHA would favor their incorporation into the developing brain, where their incorporation is an order of magnitude greater than their synthesis from precursors (51).

blood samples. While these studies revealed only a modest correlation in levels of the two PUFA species in plasma choline phosphoglycerides (r = 0.62, P < 0.001, n = 74), a strong positive correlation was revealed between DHA and AA in the maternal red cell membrane (r = 0.85, P < 0.0001, n = 74), and a still tighter relationship in the red cells of preterm infants (r = 0.88, P < 0.00005, n = 24) (41). Bearing in mind the very different dietary origins of these two PUFA, such significant correlations indicate that some powerful mechanism exists to regulate their relative abundances in the membrane.

Possibly, specific esterification processes could explain the correlations. The phosphatidylethanolamine (PE) and serine phosphoglycerides have the highest content of DHA. In the brain there is an active base-exchange reaction for serine and ethanolamine. Other fatty n-3 fatty acids do not esterify easily with PE and serine phosphoglycerides. So specificity of composition could be brought about by DHA–serine phosphoglycerides or DHA–PE formation. Nonetheless, the double bonds in positions 4–5 and 19–20 would still have to be relevant for the esterification, to explain why the n-6 and n-3 DPA might not match these conditions.

Nuclear magnetic resonance and fluorescence studies attempted to differentiate the membrane properties conferred by PUFA. In another paper (42), we discuss some of the constraints of such approaches and review the results obtained to date. Holte *et al.* (43) conducted a thorough nuclear magnetic resonance investigation of the effects of polyunsaturation on

lipid acyl chain orientational order which revealed significant changes as the number of double bonds increased from one to three, but little difference as further double bonds were introduced. Ehringer *et al.* (44) directly compared the effects of 18:3 and 22:6 on membrane physical properties and observed considerably higher permeability and perhaps vesicle fusibility in the samples containing DHA.

In summary, numerous studies were conducted on the physical effects of polyunsaturation on membranes, in which DHA was compared to a range of other unsaturated chains having from one to five double bonds. Thus far, however, all differences that were measured were matters of degree, and none provide a compelling explanation for the striking specificity with which DHA is selected for membranes of the eye and brain. In addition, to our knowledge, no study has compared DHA to either species of DPA to search for whatever property it is that causes neural membranes to discriminate so clearly between these seemingly similar molecules. The minimized energy structures presented here (see above) represent a preliminary step in this direction.

Where, then, can we hope to find an explanation of DHA's preferred status in neural membranes? An obvious starting point is in protein–lipid interactions: some way in which DHA favorably affects any of the myriad integral membrane proteins that are so important to neural membrane function. Such an effect could conceivably involve either a specific, molecular interaction between lipid and protein or some modulation of bulk properties of the bilayer which alters protein function. We believe that specific binding interactions between lipid and protein molecules in a biological membrane are unlikely, since the membrane's fluid state means that individual lipid molecules will be undergoing rapid translational diffusion within the bilary and thus will never be in prolonged contact with any one protein. Furthermore, Brown's studies (45) on the rod photoreceptor outer segment membrane revealed that specific chemical-type interactions could not be the cause of DHA's established role in supporting rhodopsin function. It was found that full rhodopsin efficiency could be obtained by substituting other lipid mixtures designed to mimic the bulk mechanical properties of the physiological, DHA-rich membrane. This gave rise to a model in which DHA's role was to promote mechanical conditions in the membrane suitable to stabilize certain critical conformational changes undergone by rhodopsin in the course of photoactivation. These models do not fully reconstitute the structure of the photoreceptor cell and its synaptic function, the ten thousand-fold adaptive capability of which is still unexplained. However, should this model be valid to conditions *in vivo*, it could be potentially be extended to other G-protein systems elsewhere in the central nervous system.

In applying this reasoning to the problem of distinguishing DHA from DPA, we must find a way in which the difference of one double bond might have a large enough impact on some bulk membrane property. The simulated structures shown in the figures are encouraging in this respect, as they show considerable differences between the minimized-energy conformations of di-DHA PE and di-DPA PE (perhaps the first results which show a difference of sufficient magnitude to account for nature's longstanding, clear discrimination between the two). It must be stated, though, that these simulations were carried out in vacuum and report only the lowest energy state; their applicability to lipid molecules in a fluid bilayer at physiological temperatures is thus open to question.

Another, more speculative, possibility is that DHA *in vivo* plays a more direct role in neuronal signaling, in which some special properties conferred on the membrane by DHA chains exert an influence on membrane electrical phenomena. These might include distinctive dielectric or polarizability properties arising from the unique periodic and symmetric arrangement of double bonds in the DHA chain (which would be disrupted in DPA). Conceivably, some polarization of π-electron clouds might occur and perhaps even be transmitted from one double bond to another (either within a given chain, or between neighboring chains in the membrane). It must be emphasized that this model is strictly speculative, and there is no evidence, experimental or theoretical, to support it. An experiment to measure the dielectric response of lipid systems at a broad range of applied frequencies is currently being developed. In a similar vein, Penrose (46) postulated that some brain functionality may arise due to quantum coherence in the microtubules of neurons (46); it may be worthwhile to look for a similar phenomenon in membranes containing DHA.

There is much still to be learned about the physical properties of membranes containing DHA. The extremely high degree of specificity with which it is selected for membranes of the brain (and has been, since very early evolutionary times) cannot be explained on the basis of the conventional measurements that have been made thus far. DHA's special role may relate either to undefined interactions with integral membrane proteins or, more speculatively, to some role in neuronal signaling arising from unusual electrical properties. Nature's sharp discrimination between DHA and the nearly identical DPA species may give guidance to further inquiries into DHA's putative role, by focusing attention on the importance of the full complement of six periodic double bonds.

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New Developments in Phospholipase A_2

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ABSTRACT: Some of the most recent data concerning various phospholipases A_2, with special emphasis on secretory, cytosolic, and calcium-independent phospholipases A_2 are summarized. Besides their contribution to the production of proinflammatory lipid mediators, the involvement of these enzymes in key cell responses such as apoptosis or tumor cell metastatic potential is also discussed, taking advantage of transgenic models based on gene invalidation by homologous recombination. The possible role of secretory and cytosolic platelet-activating factor acetyl hydrolases is also briefly mentioned. Finally, the ectopic expression in epididymis of an intestinal phospholipase B opens some novel issues as to the possible function of phospholipases in reproduction.

General interest for phospholipases A_2 (PLA_2) comes mainly from their role in the liberation of arachidonic acid, which is an obligatory and limiting step for the synthesis of eicosanoids (1). In this respect, other lipid mediators also deserve attention and include platelet-activating factor (PAF) produced by acetylation of the lysophospholipid 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (1), and lysophosphatidic acid, which is now considered as a novel phospholipid mediator with diverse biological activities (2,3). In recent years, the field of PLA_2 has rapidly grown, essentially because several enzymes were characterized at a molecular level following molecular cloning of the corresponding cDNA. Even though new and powerful tools were used to further explore the biological function of these enzymes, the complexity of the subject increased. Numerous recent reviews (1,4–7) shed some light on this complex field and cover the various types of PLA_2 which have been the object of a classification proposed by Dennis (8). The aim of this short review is not to cover exhaustively all the literature data presently available on PLA_2 but to present some recent concepts emerging in our understanding of PLA_2.

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Abbreviations: MAP kinase, mitogen-activated protein kinase; mPLB, membrane phospholipase B; PAF, platelet-activating factor; PLA_2, phospholipase A_2; mPLB, phospholipase B; $cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; $sPLA_2$, secretory PLA_2, TNFα, tumor necrosis factor α.

CLASSIFICATION OF PLA_2

The main families of PLA_2 are presented in Table 1, taking into account the classification of Dennis (8), assigning a specific number to each enzyme. The most abundant family is represented by secretory PLA_2 ($sPLA_2$). These include the digestive pancreatic PLA_2 (type IB PLA_2), snake and bee venom enzymes, and the type IIA $sPLA_2$, which has been the object of numerous studies based on its expression during inflammatory reactions. All of them are characterized by a low molecular mass, the presence of at least five disulfide bridges, and a requirement for calcium in the μM range, in agreement with the fact that these secreted enzymes are expected to exert their catalytic activity in an extracellular medium. Cytosolic PLA_2 ($cPLA_2$) displays a constitutive and ubiquitous expression and is activated by μM calcium concentrations achieved in the cytoplasm of cells stimulated with various agonists. In contrast, calcium-independent PLA_2 ($iPLA_2$) was initially recognized in heart cytosol as a 40 kDa protein able to associate with phosphofructokinase (9), whereas cDNA molecular cloning revealed that $iPLA_2$ are actually homotetramers of an 85-kDa protein (10). Despite an identical subcellular localization, $iPLA_2$ differs from $cPLA_2$ by the absolute lack of calcium requirement for its enzymatic activity. We will not describe in detail the properties of PAF acetyl hydrolases able to remove the acetyl group esterifying the *sn*-2 position of PAF and belonging, like $cPLA_2$ and $iPLA_2$, to the large group of serine esterases. For further information, the reader is referred to the review of Stafforini *et al.* (7) and to the recent cloning of intracellular PAF acetyl hydrolase II (11). We should also add to the list a 26-kDa lysosomal PLA_2 active at acidic pH (12). Finally, we have included in the classification of PLA_2 a peculiar enzyme initially purified from guinea pig intestine (13) and displaying, in addition to PLA_2, a high lysophospholipase activity allowing a total deacylation of glycerophospholipids, although the same hydrolytic activities were also reported against neutral glycerolipids (14). The cDNA coding for this brush-border hydrolase acting as an ectoenzyme has now been cloned from rabbit (15), rat (16), and guinea pig (17) intestine. Although all the available evidence indicates a digestive function for membrane phospholipase B (mPLB), we shall discuss at the end of this review novel ex-

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TABLE 1
Different Phospholipases A_2[a]

PLA_2	Type	Molecular mass	Disulfide bridges	Calcium requirement	Localization
$sPLA_2$	I, II, III, V, IX, X	13–15 kDa	5–8	mM	Secreted
$cPLA_2$	IV	85 kDa	0	μM	Cytosolic
$iPLA_2$	VI	4 × 85 kDa	0	0	Cytosolic
Secreted PAF acetyl hydrolase	VII	45 kDa	?	0	Secreted
Intracellular PAF acetyl hydrolase Ib	VIII	29, 30 kDa (+45 kDa)	0	0	Cytosolic
Intracellular PAF acetyl hydrolase II	?	40 kDa	0	0	Cytosolic and membrane-bound
Lysosomal PLA_2	?	26 kDa	?	0	Lysosomal
mPLB	?	170 kDa	Yes (?)	0	Membrane (extracellular)

[a]$cPLA_2$, cytosolic PLA_2; $iPLA_2$, calcium-independent PLA_2; PAF, platelet-activating factor; PLA_2, phospholipase A_2; mPLB, membrane phospholipase B; $sPLA_2$, secretory PLA_2.

pression data indicating a role of this enzyme in reproduction. But some of the specific points recently evolved in the fields of $sPLA_2$, $cPLA_2$, and $iPLA_2$ will be discussed first.

SECRETORY PLA_2

The main interest has been brought to type IIA $sPLA_2$, which appears as the major enzyme produced in response to proinflammatory cytokines and accumulates in inflammatory fluids as well as in the serum of patients suffering from septic shock (18). The situation might actually be more complex than previously anticipated, owing to the fact that novel $sPLA_2$ are involved as well, such as type V $sPLA_2$ in P388 macrophages (19) and mastocytes (20) or the recently cloned type × $sPLA_2$ expressed in a number of immune cells (21).

When considering type IIA $sPLA_2$ itself, at least five points deserve some attention: (i) the promoter of the human gene has been isolated, allowing investigation of the factors regulating enzyme expression, among which is single-strand DNA-binding proteins (22); (ii) combinatorial oligonucleotide synthesis led to the isolation of aptamers able to bind to type II $sPLA_2$ at nM concentrations, resulting in inhibition of the enzyme and opening interesting issues for the design of specific inhibitors with potential use as anti-inflammatory drugs (23); (iii) the enzyme displays potent bactericidal activity, even in the absence of cofactors such as bactericidal permeability-increasing protein when used against *Staphylococcus aureus*, for instance (24,25); (iv) a number of cells undergoing apoptosis were described as selective substrates of type II $sPLA_2$, in contrast to intact cells remaining insensitive to membrane attack by the enzyme (26); and (v) phosphatidic acid was found as the best substrate for the enzyme, site-directed mutagenesis giving some partial explanation for a behavior typical of type IIA $sPLA_2$ (27).

The latter two observations are interesting to discuss in the light of our previous study showing that type IIA $sPLA_2$ is only able to act on membranes whose transverse phospholipid asymmetry has been modified, allowing the exposure of phospholipids, such as phosphatidylethanolamine and phosphatidylserine, normally present in the internal leaflet (28). Since redistribution of phospholipids between the two halves of the plasma membrane is one of the earliest events occurring during apoptosis (29), this offers an explanation to the mechanism by which cell membranes could present a suitable substrate to $sPLA_2$. On the other hand, since phosphatidic acid is also translocated from the cytoplasmic to the extracellular side of the membrane, type II $sPLA_2$ could also be involved in the production of the novel phospholipid mediator lysophosphatidic acid (28), although other pathways should still be considered (3).

Another interesting field is the relationship between $sPLA_2$ and cancer. For instance, genetic studies provided evidence that $sPLA_2$ protected mice against the development of multiple intestinal neoplasia due to mutations in the adenomatous *polyposis coli* gene (30,31). Although we ignore whether the enzyme is able to selectively destroy malignant cells, those studies revealed that several mouse strains such as C57BL/6 actually carry a homozygous mutation-abolishing type IIA $sPLA_2$ expression (30). Further evidence that this enzyme could play a protective role in tumor development comes from the interesting observation that injection of phospholipase-activating protein induces regression of malignant gliomas (32). This has stimulated a number of genetic studies in man; however, no clear situation similar to that observed in the mouse has yet emerged (33–40). In contrast, there are a number of observations reporting an increased expression of type II $sPLA_2$ during cancer, reflecting an inflammatory reaction induced by the tumor as well as a consequence of cell malignancy (see Refs. 41 and 42 for an example).

To discuss whether type II $sPLA_2$ might modulate tumor cell proliferation or the reaction of surrounding tissues by promoting the formation of lipid mediators such as lysophosphatidic acid, as mentioned above, will be interesting. Another possible involvement of $sPLA_2$ in cancer might be *via* interactions with their specific membrane receptors, as recently shown for pancreatic PLA_2, which stimulates extracellular matrix invasion by normal and cancer cells (43). We will not discuss herein all the data accumulated on the characterization of $sPLA_2$ high-affinity receptor, which was the object of an important review (44). The molecular structure of $sPLA_2$ receptor, which displays a large extracellular domain containing carbohydrate recognition domains, is suitable to have the role of this membrane protein to be endocytic removal of $sPLA_2$ (45). Internalization of $sPLA_2$ might allow its nuclear translocation, opening some interesting issues as to the mechanism underlying its proliferative effects (46). Fi-

nally, a recent study reported a significantly decreased lethality of endotoxin shock in mice bearing an homologous disruption of $sPLA_2$ high-affinity receptor gene (47). Other membrane proteins such as heparan sulfates could also contribute to the interactions of $sPLA_2$, especially type IIA enzyme, with cell membrane (48). This will be discussed later on, when considering the complementarily observed between $sPLA_2$ and $cPLA_2$.

TYPE IV CYTOSOLIC PLA_2

As recently reviewed (5,49), a number of structural features revealed by molecular cloning of the cDNA coding for $cPLA_2$ have allowed us to understand the function and regulation of this enzyme. Three main points can be mentioned: (i) This serine esterase specific for arachidonic acid esterifying the *sn*-2 position of glycerophospholipids displays a nonclassical catalytic triad with apparently an arginine instead of an histidine residue; (ii) The N-terminal end is a calcium-phospholipid binding domain similar to the C2 domain present in protein kinase C; its crystal structure was recently resolved (50), and it is thought to direct association of the enzyme with its membrane phospholipid substrate in the presence of physiological μM calcium concentrations (51); (iii) the protein contains two serine residues (505 and 727) probably phosphorylated by various mitogen-activated protein kinases (MAP kinases) (see Ref. 52 for an up-to-date discussion). More recently, a pleckstrin homology domain was identified in the sequence of $cPLA_2$ and found to promote association of the protein to phosphatidylinositol 4,5-bisphosphate, resulting in a dramatic increase of its enzymatic activity (53).

Based on numerous studies including site-directed mutagenesis experiments, these features have promoted a rather broad consensus, indicating that $cPLA_2$ is activated by two complementary events, i.e., calcium-dependent translocation of the protein to the membrane, and phosphorylation *via* classical MAP kinases of Ser 505, which promotes increased activity of the enzyme (5,49). However, such a simple scheme should certainly be revisited for several reasons: (i) $cPLA_2$ phosphorylation might also involve stress-activated kinases such as p38, which does not result necessarily in enzyme activation, at least in platelets (54); and (ii) the site of $cPLA_2$ action might not be the plasma membrane, but instead the nuclear membrane, where lipoxygenase and cyclo-oxygenase 2 might also be located under conditions leading to eicosanoid production (55–58). In some cells, nuclear or cytosolic localization of $cPLA_2$ might even depend on cell density (59). Whether the lack of nucleus is in some way related to the apparently different regulation of $cPLA_2$ in platelets is unknown but would deserve some attention (54).

However, very new data from two independent groups might completely change our views about the so-called calcium-dependent translocation of $cPLA_2$, which appeared by immunocytochemical methods to display a punctuate labeling pattern all over the cytoplasm, without any evidence for a change in localization upon cell activation (60,61). These troubling observations should draw our attention to particular cytoplasmic structures called lipid bodies where both $cPLA_2$ and MAP kinases could be localized (61).

As to the most recent data concerning $cPLA_2$, five points justify a particular mention: (i) Gene invalidation was achieved by two independent groups, providing strong evidence for a nonredundant role of $cPLA_2$ in female reproduction, eicosanoid synthesis, allergic reactions, and postischemic brain injury (62,63); (ii) evidence was given for a direct involvement of $cPLA_2$ in apoptosis promoted by tumor necrosis factor α (TNFα) in a mechanism implicating enzyme activation *via* caspase-mediated proteolysis (64–68); (iii) $cPLA_2$ is an obligatory intermediate in the activation pathway of phagocyte NADPH oxidase (69); (iv) $cPLA_2$ displays an altered regulation in cystic fibrosis, suggesting a possible role of this enzyme in the pathophysiology of this disease (70); and (v) there is some genetic evidence for a possible involvement of $cPLA_2$ in the pathogenesis of schizophrenia, thus opening very fascinating issues on the role of $cPLA_2$ in brain (71,72). However, further studies are still required since a calcium-independent PLA_2 might also be involved (73).

Finally, as discussed above, the role of $cPLA_2$ cannot be considered without reference to $sPLA_2$, both enzymes acting in a complementary manner. For instance, numerous reports agree with the fact that $cPLA_2$ would be responsible for the immediate, rapid phase of arachidonate liberation, $sPLA_2$ being involved in a sustained and delayed phase of eicosanoid production. A recent study also showed that $cPLA_2$ is an obligatory intermediate for the cytokine-induced expression of $sPLA_2$ (74). However, the reverse order might be true as well, as elegantly shown by Hernandez *et al.* (75) in cultured astrocytes. Beside showing that type II $sPLA_2$ promotes MAP kinase and $cPLA_2$ activation probably *via* both specific-$sPLA_2$ receptor and membrane-bound heparan sulfates, the latter authors discuss available literature data illustrating the complementary nature of the two enzyme types. At the same time we finished this review, a remarkable study of Murakami *et al.* (76) appeared, fully clarifying the respective roles of $sPLA_2$ and $cPLA_2$, as well as $iPLA_2$, the latter one being discussed below.

TYPE VI CALCIUM-INDEPENDENT PLA_2

The last review devoted to $iPLA_2$ (4) described in detail the structure of this 85-kDa protein, characterized by the presence of eight ankyrin repeats possibly involved either in self-association of $iPLA_2$ as an homotetramer or in specific interactions with, for instance, cytoskeletal proteins. Balsinde and Dennis (4) also discussed very carefully the specificity of $iPLA_2$ inhibitors such as the bromoenol lactone currently used as a suicide substrate. The selectivity of the compound is not absolute, giving some doubt about a number of functions previously suspected for the enzyme. These include, for instance, arachidonic acid liberation (77), insulin secretion, as discussed in Reference 78, or long-term potentiation (79). Probably the use of antisense strategies will allow reevalua-

tion of some of the previous suggestions concerning the possible function of $iPLA_2$; this has indeed been the case for the proposal that $iPLA_2$ plays a main role in membrane phospholipid remodeling (80). Of course, clearer conclusions will certainly become available after gene targeting by homologous recombination, which does not seem to have been achieved.

When focusing on more recent findings on $iPLA_2$, molecular cloning of the cDNA coding for the human enzyme reveals a similar structure but also a great diversity in the gene expression pattern (81). For instance some splice variants containing the ankyrin motifs but lacking the catalytic triad are able to associate with and to inhibit the full-length enzyme (81). Another characteristic feature of the enzyme is its highest activity toward phosphatidic acid, as shown with the recombinant enzyme (10). Whether this could imply a contribution of $iPLA_2$ in the generation of lysophosphatidic acid still remains to be established (3). Also, the fact that $iPLA_2$ can be activated upon depletion of intracellular calcium pools might appear at first glance as a paradoxical observation but suggests that the enzyme could also be regulated in relation to calcium signaling (77). Finally, a very recent study indicated that Fas-mediated apoptosis is accompanied by an increased liberation of arachidonic and oleic acids by $iPLA_2$, $cPLA_2$ being inactivated by caspase-3-mediated cleavage (82). How this relates to the cleavage promoted by TNFα (66) will require special attention.

MEMBRANE ECTO-PHOSPHOLIPASE B

Molecular cloning of cDNA from three different species (15–17) revealed a good conservation of this enzyme, which is also apparently present in humans, as revealed from systematic search of sequence data (Gassama-Diagne, A., unpublished data). The protein is anchored in the membrane by a single hydrophobic segment followed by a short C-terminal cytoplasmic tail. Enzymatic activity is supported by the large heavily glycosylated (83) extracellular domain, which contains four homologous repeats. Despite the fact that two serine residues are included in a lipase consensus sequence present in domains II and IV (17), recent evidence indicates that the only active site is located in domain II and involves another serine residue (Table 1) (16,84,85).

The enzyme thus belongs to the group of brush-border hydrolases expressed upon enterocyte differentiation (13–17, 83–85) and is the first ecto-phospholipase so far characterized. By facing the intestinal lumen, mPLB thus appears obviously as a digestive enzyme able to fully deacylate glycerophospholipids as well as neutral glycerides such as diacylglycerol and monaoacylglycerol (14). However, additional sites of expression were recently identified, including testis (16) and epididymis (17). As discussed in our previous study (17), the latter observation agrees with the fact that spermatozoa lose up to 50% of their phospholipids upon their transit through epididymis, which is also the major source of glycerophosphocholine present in seminal plasma at mM concentrations. We thus suggest that mPLB might play a very critical role in sperm maturation. However, mPLB activation requires proteolytic cleavage by trypsin (17). This is easily achieved in the intestinal lumen (84) but still awaits further studies to identify which protease might be active in the male genital tract. Obviously, the precise role of mPLB in reproduction will be better defined after gene invalidation by homologous recombination.

CONCLUSION

This short review allowed us to summarize some of the most recent data available in the field of PLA_2. Molecular cloning of a number of cDNA provided novel and efficient tools to better understand the function of this complex family of proteins. However, as clearly illustrated for $cPLA_2$ (62,63), this might be only the first step of more-efficient strategies such as gene invalidation, which clearly appears as the most selective way to delineate PLA_2 function. In the case of $sPLA_2$, one can even more easily take advantage of the existence of spontaneous disruption of the gene, as recently illustrated for the role of $sPLA_2$ in gut infection by *Helicobacter felis* (86).

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Changes in Membrane Microdomains and Caveolae Constituents in Multidrug-Resistant Cancer Cells

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ABSTRACT: Cancer chemotherapy often fails because of the development of tumors which are resistant to most commonly used cytotoxic drugs. This phenomenon, multidrug resistance (MDR), is usually mediated by overexpression of P-glycoprotein (P-gp), an ATPase that pumps out the drugs used in chemotherapy, thereby preventing their accumulation in cancer cells and greatly reducing their cytotoxic efficacy. A large body of work indicates that MDR is associated also with marked changes in membrane lipid composition. Most notably, elevated levels of cholesterol, glycosphingolipids (e.g., glucosylceramide), and sphingomyelin have been reported. These lipids are enriched in caveolae and in membrane microdomains termed detergent-insoluble glycosphingolipid-enriched complexes (DIGs). Recently we demonstrated that in multidrug-resistant tumor cells there is a dramatic increase in the number of caveolae and in the level of caveolin-1, an essential structural constituent of caveolae. Another constituent of membrane microdomains, phospholipase D, is also elevated in MDR cells. These findings may be related to the fact that a significant fraction of cellular P-gp is associated with caveolin-rich membrane domains. The possible role of DIGs and caveolae in the acquisition and/or maintenance of the multidrug resistant phenotype is discussed.

MULTIDRUG RESISTANCE (MDR): DEFINITION, SIGNIFICANCE, CELLULAR MODELS

Anticancer drug resistance is the primary reason for the failure of chemotherapy in cancer patients (1,2). The resistance of human malignancy to chemotherapeutic intervention has a multifactorial etiology which falls into two categories: first, host-mediated resistance, with all the characteristics of tumor–host tissue interactions (e.g., tumor-immune system interactions); second, cellular resistance, which can be either intrinsic (inherent or natural) or acquired. It may be generally stated that as tumors progress in time and size, they become increasingly resistant to chemotherapy. Both of the above-mentioned forms of resistance are responsible for this development (2). Thus, clinical improvement of cancer therapy critically depends on understanding and controlling drug resistance.

One form of drug resistance, termed MDR, has been most studied, and was extensively characterized utilizing *ex vivo* models of multidrug resistant cancer cells in culture (3). Such model cell lines are generated by growing cancer cells under progressively higher concentrations of a given cytotoxic drug. Selection over many generations results in drug-resistant cell lines that can grow in the presence of drug concentration orders of magnitude higher than those tolerated by the parental cells (e.g., Ref. 4). The degree of resistance can be determined in different drug-sensitive and -resistant cell lines by using cell proliferation/survival assays like the MTT assay [based on conversion of 3(4,5-dimethylthiazol-2-yl)2,5-diphenyl tetrazolium bromide (MTT) to formazan by living cells] (5). EC_{50} values for cell killing can also provide a quantitative tool for comparing different cytotoxic drug potencies and for assessing the effectiveness of compounds that can reverse the MDR phenotype. It was repeatedly observed that selection for resistance to one particular drug is accompanied by resistance to a variety of other drugs. This general characteristic of cancer cells defines the MDR phenomenon. Drugs known to induce MDR include Vinca alkaloids (e.g., vincristine), anthracyclins (e.g., doxorubicine), actinomycin D, etopside, paclitaxel, and colchicine. Although, in general, these drugs are structurally and functionally unrelated, they have two common broad structural features, a planar hydrophobic ring and a tertiary amine (3). Presently many cell lines serve as models for MDR, including murine leukemia P388-Adr (adriamycin), and P388-VCR (vincristine) cells; chinese hamster ovary (CHO)-emetine cells; breast cancer MCF-7-AdrR; and MCF-7-VCR cells, and many more.

MOLECULAR MECHANISMS OF MDR

Many cellular changes have been described that accompany the multidrug-resistant phenotype. However, most studies have focused on the so-called "classical" MDR protein termed P-glycoprotein (P-gp), which is the product of the MDR1 gene (6,7). P-gp is a membrane-bound glycoprotein that belongs to the protein family of ABC transporters. P-gp acts as an ATP-dependent drug efflux pump and is believed to mediate drug resistance by actively extruding drugs from

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Abbreviations: ABC, ATP-binding cassette; DIGs, detergent-insoluble glycosphingolipid-enriched complex; GlcCer, glucosylceramide; MDR, multidrug resistance; P-gp, P-glycoprotein; PIP_2, phosphatidylinositol 4,5-bisphosphate; PLD, phospholipase D; SCRL, sphingolipid- and cholesterol-rich liposome; SREBP, sterol regulatory element-binding protein.

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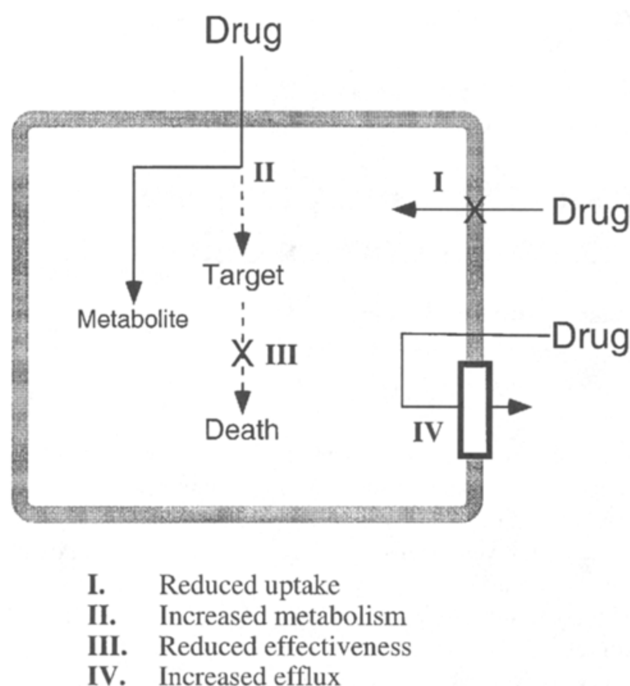

FIG. 1. Mechanisms of drug resistance in cancer cells. A schematic representation of a multidrug-resistant cell and four possible mechanisms that reduce a cytotoxic drug efficacy (and increase cellular drug resistance). Mechanisms I and IV occur at the plasma membrane and may be modulated by membrane lipid changes as detailed in the text.

the cells. A large body of work documents the modulation of P-gp activity by control mechanisms such as phosphorylation and the regulation of its expression on the transcriptional level (6). In additon to MDR1/P-gp, MDR1-like proteins were isolated that are overexpressed in various drug-resistant tumor cell lines. To date, the MDR1-like protein family includes multidrug resistance-associated protein (MRP) and its homologs (8). P-gp and MRP belong to the ATP-binding cassette (ABC) super-family of transporters, and despite having a differential substrate specificity, all operate by facilitating efflux of chemotherapeutic drugs.

Clearly, however, P-gp and related ABC transporters are not the sole determinants of drug resistance in MDR cells. Rather, cells seem to adopt multiple strategies in order to survive the lethal effects of chemotherapeutic drugs (2), and the various cellular mechanisms that contribute to the existence and degree of cross-resistance displayed by MDR cells are suggested to act simultaneously. Thus, in addition to the accelerated outward transport of drugs, mediated by overexpression of ABC transporters, there are additional cellular mechanisms for drug adaptation (for review see Ref. 2). These include: (i) altered cellular pharmacokinetics of drug uptake, that may be attributed to changes in membrane permeability, probably *via* changes in membrane lipid composition; (ii) increased inactivation of drugs, due either to overexpression of glutathione-*S*-transferase and elevated intracellular glutathione concentrations; or altered drug metabolism, e.g., by the cytochrome P-450 enzyme system (responsible for reducing quinone-containing anticancer drugs in tumor cells); or by aldehyde dehydrogenase, which detoxifies nitrogen mustard compounds; (iii) increased DNA repair *via* alterations in DNA topoisomerase-II enzyme activity. Together, these mechanisms result in a major decrease in intracellular drug accumulation and effectiveness (Fig. 1). In the present review we will focus on cellular and biochemical changes that occur at the level of the cell plasma membrane in multidrug-resistant cancer cells.

MEMBRANE LIPIDS CHANGES IN MULTIDRUG-RESISTANT CELLS

Studies on membrane alterations associated with anticancer drug resistance began to appear in the early 1980s. Cell resistance was suggested to be accompanied by changes in membrane microviscosity (lipid bilayer structural order or membrane fluidity). Whereas most studies reported that drug-resistant cells exhibit an increase in membrane fluidity, other studies suggested the opposite (see Ref. 9 and citations therein). An elegant study, dating to 1982, showed that adriamycin coupled to insoluble agarose matrix was almost as cytotoxic to L1210 cells as the unconjugated drug (10). It was concluded that the drug's cytotoxic action can solely be attributed to its interaction with the cell surface. Thus, it is safe to say that membrane composition and structure are important factors in drug action and cell resistance.

In this context, lipids are likely to fulfill multiple and complex functions in MDR, by influencing drug transport across the plasma membrane and by modulating the activity of drug transporters. Lipids determine cell membrane fluidity, which was shown to be a factor in the capacity of MDR cells to accumulate drugs (11). Higgins and Gottesman (12) proposed that P-gp substrates initially interact with the lipid bilayer, by partitioning into the lipid phase, before gaining access to P-gp. This view was corroborated by recent three-dimensional structural data showing a gap within the transmembrane region of P-gp through which drugs can enter the hydrophilic core of the protein (13). Additionally, a large body of data demonstrates the effect of the lipid environment of P-gp on its function. Like other membrane-bound ATPases, P-gp requires phospholipids for its ATPase activity. Using a partially purified preparation of P-gp, Doige *et al.* (14) showed that unsaturated phosphatidylcholine and phosphatidylserine are crucial for restoring P-gp ATPase activity, while saturated phosphatidylethanolamine is most effective in activating the ATPase. Urbatsch and Senior (15) suggested that in addition to the significant influence of the lipid environment on the characteristics of purified, reconstituted P-gp-ATPase activity, lipids may have a role in the coupling between drug-binding sites and catalytic sites of P-gp.

Indeed, chemical analyses of cell lipids and quantitative comparison of various lipid types in drug-sensitive and drug-resistant cells indicated that the acquisition of MDR is associated with significant changes in their membrane lipids. Such

changes can be attributed mainly to altered lipid uptake from the medium, or alterations in cell lipid synthesis and metabolism. Vrignaud *et al.* (16) have shown a twofold increase in fatty acid uptake by doxorubicine-resistant rat glioblastoma cells compared to the drug-sensitive wild-type clone. Other workers demonstrated a much higher amount of triglycerides in drug-resistant P388 leukemia cells (17). This work showed also that, in the resistant cells, the ratio of sphingomyelin to phosphatidylcholine is significantly higher (17). Another study showed that the incorporation of [^{3}H]palmitic acid into sphingomyelin was up to 3.5-fold higher in adriamycin-resistant MCF-7 cells in comparison with the drug-sensitive control cells (18). May *et al.* (19) utilized a purified plasma membrane preparation of leukemic T-lymphoblasts to show that upon development of drug resistance, there is a very significant elevation of cell ether-lipid content. In addition they demonstrated that lipid-to-protein ratio, in general, is increased. The role of fatty acids, triglycerides, or ether lipids in MDR cells is not specified; however, these elevations of lipid content might account for changes in MDR cell-membrane properties and may affect drug uptake, transport, and extrusion.

Patterns of glycosphingolipid expression are known to change profoundly upon oncogenic transformation of cells (20). Intriguingly, more recent work has indicated that such changes occur also in association with the MDR phenomenon. Lavie *et al.* (18) showed that the precursor of all glycosphingolipids, glucosylceramide (GlcCer), is markedly elevated in various MDR cell lines, including MCF-7-AdrR, KB-V, and NIH:OVCAR-3, as compared to their wild-type counterparts. It was further demonstrated that higher activity of GlcCer synthase is the principal cause of this alteration in MDR GlcCer level (18). These results are consistent with prior studies demonstrating a biosynthetic block at the point of gangliosides production, which was shown to accompany the acquisition of resistance to daunorubicine and vincristine in DC-F3 Chinese hamster lung cells (21). Together these results indicate that a general change of membrane glycosphingolipids occurs in MDR cells.

Mountford and Wright (22) compared several drug-sensitive and drug-resistant cell lines with respect to lipid composition of their purified plasma membrane. These authors found a significant elevation in free cholesterol level in vinblastine-resistant T lymphoblasts as compared with the vinblastine-sensitive parental cells. Similarly, human ovarian MDR cells exhibit higher membrane cholesterol levels compared with their wild-type counterpart, when cultured under conditions of cholesterol deprivation (23). These data indicate that cholesterol metabolism is modified in the MDR cells and suggest that MDR cells are better able to maintain high cholesterol levels. In conclusion, reports from studies carried over the last 30 yr in different MDR cells demonstrate significant changes in membrane lipid composition; in particular, fatty acids, ether lipids, triglycerides, glycosphingolipids, sphingomyelin, and cholesterol are affected. However, it is not known at this time how these alterations do contribute to the development or maintenance of MDR in cancer cells.

It is known that sphingolipids (sphingomyelin and glycosphingolipids) and cholesterol are highly enriched in distinct membrane microdomains termed detergent-insoluble glycosphingolipid-enriched complex (DIGs) that have received much attention in recent years (see below). The elevated levels of GlcCer, sphingomyelin and cholesterol, independently reported in different types of MDR cells, suggest that MDR might be associated with increased formation of these microdomains in the plasma membrane. In the following paragraphs we shall discuss the properties of membrane microdomains and review the evidence for their involvement in mediating MDR in cancer cells.

DIGs AND CAVEOLAE: MICRODOMAINS IN BIOLOGICAL MEMBRANES

There is growing evidence for the existence in biological membranes of microdomains that are laterally segregated in the plane of the bilayer and that are enriched in sphingolipids and in cholesterol (Fig. 2; Refs. 24,25). Such microdomains have been variously termed DIGs (26), GEMs (glycosphingolipid-enriched membranes; 27), LDTI (low density Triton-insoluble) membranes; 28), or rafts (29). As can be inferred from some of these terms, DIGs can be distinguished from the bulk of cellular membranes by their insolubility in certain nonionic detergents such as Triton X-100. Because of their low buoyant density, the detergent-insoluble microdomains can readily be separated from other cell constituents by flotation in a sucrose density gradient (30). Analysis of the lipid composition of DIGs, isolated in this manner from MDCK cells, revealed that they are highly enriched in cholesterol and sphin-

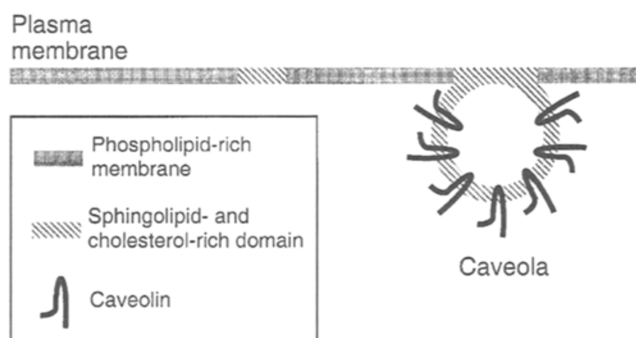

FIG. 2. Membrane microdomains and caveolae. Membrane microdomains detergent-insoluble glycosphingolipid-enriched complex [(DIGs); shaded areas] have a characteristic lipid composition, rich in sphingolipids and cholesterol, that is significantly different from the bulk phospholipid-rich membrane (solid areas). DIGs are laterally segregated in the plane of the membrane and exhibit a liquid-ordered (L_o) phase behavior, as opposed to the liquid crystalline phase of bulk membranes. Caveolae have a similar lipid composition and can be co-isolated with DIGs as detergent-insoluble particles with low buoyant density. Caveolae have a characteristic coat protein called caveolin—a 21-kDa integral membrane protein with a unique hairpin loop structure—and are present mainly in epithelial cells, adipocytes, and fibroblasts (see text for more details and references).

golipids. About 25% of cellular cholesterol was insoluble in Triton X-100, and cholesterol was nearly threefold enriched in the detergent-insoluble membranes as compared to the whole cell lysate (30). The sphingolipids sphingomyelin and lactosylceramide were virtually insoluble in Triton X-100, while other sphingolipids were only 50–60% soluble; as a class, sphingolipids were enriched nearly sevenfold in the DIGs (30). The results of this seminal study were confirmed by subsequent experimentation in other cell types (see Ref. 24 for review). The property of detergent insolubility is conferred upon DIGs by their specific lipid composition. This was demonstrated in experiments in which sphingolipid- and cholesterol-rich liposomes (SCRL) were largely resistant to detergent solubilization. Furthermore, detergent resistance was conferred upon glycosylphosphatidylinositol-anchored proteins incorporated into SCRL (31,32). Detergent insolubility correlates with the presence of liquid ordered (L_0) phase domains in either artificial or biological membranes (see Ref. 24 for review).

DIGs have been isolated from many cell types, and their biochemical analysis has provided important clues as to their biological functions. Numerous signaling proteins are enriched in DIGs (28,33), implicating these microdomains as platforms on which multimolecular signaling complexes are assembled and from which signal cascades aimed at intracellular effectors are launched. DIGs have been envisioned also as sphingolipid-cholesterol "rafts" which function in the sorting and subsequent transport of glycosylphosphatidylinositol-anchored and other membrane proteins destined for apical membranes of polarized cells (29). Finally, DIGs were hypothesized to serve as building blocks for assembly of caveolae (34).

DIGs are related, in their lipid composition and their resistance to detergent solubilization, to specific, morphologically and biochemically well-defined cellular structures called caveolae (35). Caveolae are nonclathrin-coated plasma membrane invaginations, 50–100 nm in size, that have a characteristic striated coat structure (36,37). A major caveolar coat protein is caveolin-1, a 21-kDa integral membrane protein (37,38). Caveolin-1 forms high molecular weight homo-oligomers and acts as a scaffolding protein for various other proteins (34). Because of their similar lipid composition, DIGs and caveolae are co-purified as low density, Triton-insoluble membrane particles on discontinuous sucrose density gradients (30,39). However, while DIGs are present in most if not all cell types, caveolae are found mostly (though not exclusively) in epithelial cells (38). Caveolae have been implicated in transport processes such as nonclathrin-mediated endocytosis (Ref. 40 and citations therein), as well as cholesterol efflux (41). In addition, recent work strongly suggests that, like DIGs, caveolae play an important role in cellular signal transduction (34,42).

COORDINATE UP-REGULATION OF CONSTITUENTS OF DIGs AND CAVEOLAE IN MDR CANCER CELLS

The functions assigned to DIGs and caveolae, namely, endocytosis, signaling, and cholesterol efflux, share a common cellular localization in the plasma membrane. Together, the data suggest that, depending on circumstances, both DIGs and caveolae microdomains act to sequester or recruit specific membrane and cytosolic proteins into large, multimolecular functional complexes. The MDR transporters are known also to be localized and function in the plasma membrane. It was therefore of interest that analysis of cell membranes from MDR cells showed marked changes in sphingolipids and cholesterol, both of which are characteristic constituents of DIGs and caveolae. The accumulation of GlcCer in various MDR cells seems to be of particular significance because it is associated with, and depends on, elevated GlcCer synthase activity (18). This suggests that MDR is associated with a regulated modification of membrane lipid biosynthetic pathways.

We have therefore examined whether other constituents of DIGs and caveolae are up-regulated in MDR cancer cells. DIGs and caveolae were prepared from HT-29 human colon carcinoma cells and from a colchicine-resistant HT-29-derived cell line (HT-29-MDR) by flotation of Triton X-100-insoluble membranes on a sucrose density gradient (30,39). Analysis of the gradient fractions by Western blotting with antibodies to caveolin-1 revealed a dramatic, over 10-fold increase in caveolin-1 levels in the MDR cells (43,44). To confirm the generality of this finding, we examined the expression of caveolin-1 in an adriamycin-resistant variant cell line derived from MCF-7 human breast adenocarcinoma cells (MCF-7-AdrR). Whereas in the parental MCF-7 cells caveolin-1 expression was nearly undetectable, the expression of caveolin-1 in MCF-7-AdrR cells was very high (43,44). The up-regulation of caveolin-1 expression in the HT-29-MDR cells was accompanied by a fivefold increase in the number of juxtamembrane, 50 to 100 nm noncoated invaginations, with the characteristic flask shape morphology of caveolae. Confirming previous results in other MDR cells (18), sphingolipid analysis revealed elevated levels of GlcCer in the HT-29-MDR cells cells, compared to the parental HT-29 cells; GlcCer levels returned to "normal" wild-type levels upon treatment of the MDR cells with the GlcCer synthase inhibitor 1-phenyl-2-decanoylamino-3-morpholino-1-propanol (PDMP) (43,44).

TABLE 1
Up-Regulation of Lipid and Protein Constituents of DIGs and Caveolae in MDR Cells[a]

Lipid/protein	Relative change	Reference
Cholesterol	↑	22, 23
Sphingomyelin	↑	17, 18
Glucosylceramide	↑↑	18
Caveolin-1	↑↑↑	44
Phospholipase D	↑↑	Fiucci, G., Czarny, M., Lavie, Y., Zhao, D., Berse, B., Blusztajn, J.K., and Liscovitch, M., unpublished data

[a]MDR, multidrug resistance; DIGs, detergent-insoluble glycosphingolipid-enriched complex.

DIGs and caveolae are enriched in phosphatidylinositol 4,5-bisphosphate (PIP_2) and in the lipid kinases that synthesize it (45–49). PIP_2 is a cofactor of phospholipase D (PLD) (50), and it is required for PLD activation by small GTPases such as ADP-ribosylation factor (51). More recent work has indicated that PLD directly interacts with PIP_2 in *in vitro* liposome-binding assays (52,53). The localization of PIP_2 in DIGs and caveolae raised the possibility that PLD might also be present there. Indeed, a PLD activity was found to be enriched in these domains in a variety of cell types, including HaCaT human keratinocytes, COS-7 cells, U937 promonocytes, and NIH-3T3 fibroblasts (54,55). The PLD activity found in DIGs and caveolae was not the ADP ribosylation factor-dependent PLD1 isoform, and likely represents a product of the PLD2 gene. These data suggest that PLD is a normal constituent of DIGs and caveolae. As MDR is accompanied with an up-regulation of other constituents of DIGs and caveolae, we compared the domain-associated PLD activity in parental and MDR cells. This analysis revealed that PLD activity in the DIGs and caveolae fraction was markedly elevated in both multidrug-resistant variants of HT-29 and MCF-7 cells, compared to their respective parental lines (54; Fiucci, G., Czarny, M., Lavie, Y., Zhao, D., Berse, B., Blusztajn, J.K., and Liscovitch, M., unpublished data).

In conclusion, our recent results indicate that caveolin-1 expression and PLD activity are greatly elevated in MDR cancer cells. Together with previous data showing increased levels of cholesterol and sphingolipids (Table 1), it may be concluded that acquisition of the multidrug-resistant phenotype by human cancer cells is accompanied with a coordinate up-regulation of plasma membrane DIGs and caveolae. The functional significance of this dramatic membrane modification is discussed below.

SUMMARY AND CONCLUSIONS: ARE MEMBRANE MICRODOMAINS INVOLVED IN MDR?

Acquisition of MDR is clearly a multifactorial process. Whereas expression of P-gp (and other ABC transporters) is a major factor, additional biochemical changes that are associated with MDR may make significant contributions to the drug-resistant phenotype. The results summarized above imply that there is a coordinated program for up-regulation of constituents of caveolae and related membrane microdomains in MDR cells. The lipids that seem to be involved in this phenomenon are GlcCer, sphingomyelin, and cholesterol. A structural protein of caveolae, caveolin-1, is also dramatically elevated, and so is a novel DIGs and caveolae-resident enzyme, PLD (Table 1). Indeed, the number of caveolae is greatly increased in MDR cells. Future studies may reveal that additional lipids (e.g., PIP_2) and proteins (e.g., flotillin), previously localized to DIGs and caveolae, are up-regulated in MDR cells. These results raise a number of interrelated and potentially important questions. First, how is the formation of DIGs and caveolae regulated? Second, is there a relationship between the expression of P-gp and these membrane changes? Third, do DIGs and caveolae contribute to MDR and, if yes, how?

A plausible mechanism for the coordinate up-regulation of the various lipid and protein components of caveolae may involve the greater number of caveolae found in MDR cells. If caveolae are required for, or contribute to drug resistance, cells that have more caveolae may be positively selected under the drug treatment regimen that results in establishment of an MDR cell line (see below). The elevated levels of lipid and protein constituents of caveolae may thus be a consequence of the fact that there are more caveolae in those cells. An alternative mechanism, i.e., that overexpression of P-gp somehow drives the up-regulation of caveolae, seems consistent with our preliminary results showing that P-gp is localized in part in DIGs/caveolae (43,44). The overexpression of P-gp in MDR cells may thus drive the increased synthesis of building blocks required for formation of these membrane microdomains. However, this mechanism is inconsistent with our findings, showing that there is no change in caveolin-1 expression in MCF-7 cells transfected with recombinant P-gp (44).

What might be the relationship between caveolae and MDR? Of the various functions assigned to caveolae, their proposed role in mediating cholesterol efflux seems most relevant in the context of MDR. Fielding and Fielding (56,57) showed that caveolae act as plasma membrane terminals for the outward transport of cellular cholesterol and thus act to reduce cellular cholesterol levels. Furthermore, caveolin-1 expression is under the control of free cholesterol, acting through the sterol regulatory element-binding protein (SREBP) pathway (58,59). The SREBP pathway has been implicated in the transcriptional activation of enzymes and proteins involved in biosynthesis or uptake of cholesterol (60). In contrast, and consistent with the proposed role of caveolae in free cholesterol efflux, SREBP has a suppressive effect on caveolin-1 expression (59). While the molecular mechanism of free cholesterol efflux through caveolae is not known, it is possible that caveolae may be capable of mediating or facilitating the export of certain lipophilic drugs (probably at relatively low efficiency). If such a capability does exist, during early stages of drug exposure cells may be under a selective pressure that favors cells with more caveolae and hence high expression of caveolin-1.

Interestingly, oncogenic transformation of cells is accompanied by decreased expression of caveolin-1 and a greatly reduced number of caveolae (61). Expression of caveolin-1 has a growth-inhibitory effect on transformed cells (62,63). Our results, showing an up-regulation of caveolin-1 in MDR cells, suggest that the acquisition of a multidrug-resistant phenotype is associated with a reversal of at least one aspect of oncogenic transformation. The slower rate of proliferation that must ensue may afford some protection to caveolin-expressing cells against the action of cytotoxic drugs, and thus possibly contribute to their selective growth advantage under chemotherapy.

In summary, a fairly large body of work indicates that significant membrane lipid alterations occur in MDR cells. Of

particular interest, several constituents of DIGs and caveolae—cholesterol, sphingolipids, caveolin-1, and PLD—are up-regulated in multidrug-resistant cancer cells. We propose that caveolae may be involved in drug efflux and thus be selected for during cell exposure to chemotherapeutic drugs. This hypothesis is amenable to experimental scrutiny and is currently under study in our laboratory.

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Activation of Mitogen-Activated Protein Kinase and Cytosolic Phospholipase A_2 by Hydrogen Peroxide in Fibroblasts

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The 85-kDa Ca^{2+}-dependent cytosolic phospholipase A_2 ($cPLA_2$) is of special interest since it selectively hydrolyzes glycerophospholipids at the *sn*-2 position, generating arachidonic acid, which plays an important role in various cellular processes. In many cell types, $cPLA_2$ can be activated by various stimuli, such as cytokines and growth factors. Growth factors stimulate $cPLA_2$ activity by activating the mitogen-activated protein kinase (MAPK) cascade, resulting in a phosphorylation of $cPLA_2$ by MAPK on Ser-505. $cPLA_2$ is fully activated when it is translocated to membranes in a Ca^{2+}-dependent manner. Localization studies of endogenous $cPLA_2$ revealed a punctate pattern randomly distributed throughout the cytoplasm. Recent evidence suggests that reactive oxygen species (ROS), such as superoxide and hydrogen peroxide (H_2O_2), function as intracellular second messengers. Furthermore, exogenously H_2O_2 could mimic growth factor-induced tyrosine phosphorylation in A431 cells. Recently we demonstrated that epidermal growth factor (EGF) induced a twofold $cPLA_2$ activation in Her 14 fibroblasts. This activation is mediated by the MAPK isoforms ERK-1 and ERK-2, which are phosphorylated and thus activated upon EGF stimulation. Because ROS are thought to function as intracellular second messengers, we investigated whether H_2O_2 is also able to activate MAPK and $cPLA_2$ in Her 14 fibroblasts. To this extent cells were stimulated for 30 min with 0.1, 0.5, 1, 2, or 5 mM H_2O_2, respectively, in phosphate-buffered saline containing 0.9 mM Ca^{2+}, 0.5 mM Mg^{2+}, and 5 mM glucose, whereafter the cells were scraped in buffer (50 mM HEPES pH 7.4; 0.25 M sucrose; 50 mM NaF; 250 μM Na_3VO_4; 1 mM EGTA; 10 μM leupeptin; 1 μM pepstatin; 1 mM phenyl methylsulfonyl fluoride). The proteins were separated on SDS-polyacrylamide gel electrophoresis and stained with a phospho-specific antibody for MAPK. H_2O_2 was found to phosphorylate ERK-1 and ERK-2 MAPK in a dose-dependent manner with a maximal effect at 5 mM. Under these conditions, no release of lactate dehydrogenase was detected, indicating that they are still viable. Subsequently, the time course of ERK-1 and ERK-2 phosphorylation induced by 5 mM H_2O_2 was examined, showing a rapid phosphorylation, with a maximal effect at 10 min. We then investigated the effect of H_2O_2 on $cPLA_2$ activity. $cPLA_2$ activity was measured by the release of radiolabeled arachidonic acid from the *sn*-2 position of 1-stearoyl-2-[1-^{14}C]arachidonoyl-glycero-3-phosphocholine *in vitro*. H_2O_2 stimulated $cPLA_2$ activity in a dose-dependent way with a maximum at 1 mM. $cPLA_2$ activity was already maximal after 5 min and remained increased up to 40 min. In EGF-induced signal transduction, MAPK is activated by MAPK kinase (MEK) which in turn is activated by Raf. In order to establish whether in H_2O_2-stimulated cells MAPK as well as $cPLA_2$ is activated through this pathway, we preincubated the cells with the MEK inhibitor PD98059 which inhibits the phosphorylation of MEK by Raf. Preliminary results using this inhibitor in H_2O_2-stimulated cells showed that ERK-1 and ERK-2 phosphorylations were significantly reduced as well as the H_2O_2-induced activity of $cPLA_2$. Here we show that H_2O_2 induces ERK-1 and ERK-2 phosphorylation dose- and time-dependently. In addition, $cPLA_2$ is also activated by H_2O_2 in a dose- and time-dependent manner. These phosphorylations of ERK-1, ERK-2, and $cPLA_2$ were inhibited by the MEK inhibitor, suggesting that MEK activates in part ERK-1 and ERK-2 and that these MAPK mediate $cPLA_2$ phosphorylation.

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Abbreviations: $cPLA_2$, cytosolic phospholipase A_2; EGF, epidermal growth factor; MAPK, mitogen-activated protein kinase; MEK, MAPK kinase; ROS, reactive oxygen species.

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Targeting of Cytosolic Phospholipase A_2 to Plasma Membrane Inhibits Its Activation by G-Protein Coupled Receptors

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The 85 kDa Ca^{2+}-sensitive cytosolic phospholipase A_2 ($cPLA_2$) specifically hydrolyzes arachidonic acid-containing phospholipids, thus initiating the biosynthesis of inflammatory mediators under the stimulation of membrane receptors. Two main mechanisms regulate $cPLA_2$ activation: its calcium-dependent translocation to membrane phospholipids and its phosphorylation by several Ser/Thr kinases, including MAPKinases. In order to investigate the respective importance of these two mechanisms in the activation of $cPLA_2$ by G protein-coupled receptors, we constitutively targeted $cPLA_2$ to plasma membrane, therefore bypassing calcium-dependent translocation.

We constructed a chimeric $cPLA_2$ constituted by the full-length $cPLA_2$ and containing at its C-terminal end the 18 last amino acids of K-Ras4B. This motif targeted cytosolic proteins to plasma membrane. Chinese Hamster ovary (CHO) cells permanently expressing α_{2B}-adrenergic receptor were stably transfected with the cDNA encoding for the chimeric $cPLA_2$-Ras. Immunofluorescence of one stable transfectant, the 2B7 clone, demonstrated that $cPLA_2$-Ras localizes to the plasma membrane. We demonstrated by gas chromatography/mass spectrometry that $cPLA_2$-Ras is enzymatically active on endogenous membrane phospholipids. Stimulation of the 2B7 clone with epinephrine induces a gel shift of $cPLA_2$-Ras, resulting from its phosphorylation on Ser505 by MAPKinases. However, treatment of 2B7 clone with epinephrine, calcium ionophore A23187, or both agonists leads to an inhibition of stimulation of arachidonic acid release in comparison with parental CHO-α_{2B} cells. In contrast with this inhibition, overexpression of wild-type $cPLA_2$ at the same level has no effect on arachidonic acid release induced by epinephrine and/or calcium ionophore.

These results suggest that the presence of $cPLA_2$-Ras at the plasma membrane inhibits the activation of endogenous $cPLA_2$ in CHO-α_{2B} cells.

In order to verify the level of activation of $cPLA_2$-Ras, we expressed the chimera in the baculovirus-*Sf9* cell system. Sf9 cells do not contain endogenous $cPLA_2$ and perform the same posttranslational modifications as in mammalian cells. We constructed two recombinant viruses, allowing the expression of either wild-type $cPLA_2$ or $cPLA_2$-Ras.

In these cells, the expression of wild-type $cPLA_2$ leads to an important stimulation of arachidonic acid release from prelabeled cells in response to A23187 or to okadaïc acid, a Ser/Thr phosphatase inhibitor. In contrast, the expression of $cPLA_2$-Ras leads to a dramatic decrease of arachidonic acid release in response to these agonists. This lack of activation of $cPLA_2$-Ras is not due to a lack of phosphorylation. Indeed, okadaïc acid induces the same increase of [^{32}P] incorporation in $cPLA_2$-Ras as compared to wild-type $cPLA_2$.

These results indicate that, in spite of constitutive membrane localization and agonist-induced phosphorylation, the chimeric $cPLA_2$-Ras is enzymatically active but is not activated in response to stimuli. Moreover its plasma membrane targeting inhibits endogenous $cPLA_2$ activation in CHO-α_{2B} cells.

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Abbreviations: CHO, Chinese Hamster ovary; $cPLA_2$, cytosolic phospholipase A_2.

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Inhibition of Ca^{2+}-Independent Phospholipase A_2 Affects Arachidonate-, Not Docosahexaenoate-, Phospholipid Remodeling in U_{III} Cells

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The phospholipase A_2 (PLA_2) enzymes are now recognized to play a central role in cellular signaling mechanisms by regulating the synthesis of lipids mediators and the remodeling of phospholipid (PL) fatty acyl chains. We previously showed in rat uterine stromal cells (U_{III}) a very specific pattern in arachidonic acid (AA) and docosahexaenoic acid (DHA) distribution between PL classes. $[^3H]$ AA was mainly incorporated in the diacyl subclasses of phosphatidylcholine (PC) and phosphatidylethanolamine (PE) and in phosphatidylinositol, while $[^3H]$ DHA was principally recovered in PE plasmalogens. At low concentrations, AA appears to be incorporated in selected PL classes *via* the deacylation/reacylation cycle. Thus, in resting cells, availability of lysophospholipid acceptor provided by a PLA_2 activity is a limiting factor for incorporation of AA into PL. In this report, we describe the involvement of PLA_2 enzymes involved in the esterification of AA and DHA in unstimulated U_{III} cells. The extent of $[^3H]$ AA and $[^3H]$ DHA esterification into PL of cells incubated in Ca^{2+}-free medium supplemented with EGTA and BAPTA, the latter being an intracellular Ca^{2+} chelator, was the same as that in the presence of Ca^{2+}, suggesting that the PLA_2 responsible for supplying the lysophospholipid acceptor for AA and DHA esterification was Ca^{2+}-independent. A Ca^{2+}-independent PLA_2 activity was detected in U_{III} cell homogenates with 1,2-dipalmitoyl-glycerophosphocholine as substrate. One common feature of the best characterized Ca^{2+}-independent PLA_2 is their irreversible inhibition by a selective inhibitor bromoenol-lactone (BEL). Preincubation of U_{III} cells with increasing amounts of BEL resulted in a dose-dependent inhibition of Ca^{2+}-independent PLA_2 activity with an apparent IC_{50} of about 7 μM. Cells treated with BEL for 30 min and then exposed to exogenous $[^3H]$ AA for 30 min showed a partial inhibition (about 20%) of $[^3H]$ AA esterification into PL, affecting mainly arachidonate-containing PC. The cellular amount of lysophosphatidylcholine (lysoPC) isolated by thin-layer chromatography in cells prelabeled with $[^3H]$ choline was also reduced by BEL treatment. Maximal inhibition of lysoPC levels never exceeded 20%, even with BEL at doses higher than 50 μM. This decrease is in accordance with inhibition by BEL of AA esterification into PL and demonstrates that a PLA_2 activity is involved. By contrast, $[^3H]$ DHA esterification was not affected by BEL treatment. These results suggest that the pathways for AA and DHA esterification into U_{III} cells PL are at least partially different. We also studied the effects of BEL on the remodeling of PL fatty acyl chains. Cells were prelabeled with $[^3H]$ AA or $[^3H]$ DHA, and the transfer of radioactive fatty acid between PL classes was analyzed after an additional incubation of 4 h without the label, in the absence or presence of BEL. In control cells, the transfer of AA occurs mainly from diacyl-glycerophosphocholine (diacyl-GPC) to alkenylacyl-glycerophosphoethanolamine (alkenyl-acyl-GPE) and at a lesser extent from diacyl-GPC to diacyl-GPE. In BEL-treated cells, the redistribution of AA between diacyl-GPC and -GPE was completely inhibited, indicating that a BEL sensitive Ca^{2+}-independent PLA_2 controls this pathway. By contrast, BEL treatment did not affect the transfer of AA from diacyl-GPC to alkenylacyl-GPE as well as the remodeling of DHA. These last results suggest that the esterification of AA and DHA into plasmalogens involves another PLA_2, which is Ca^{2+}-independent but BEL-insensitive.

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Abbreviations: AA, arachidonic acid; alkenylacyl-GPE, alkenylacyl-glycerophosphoethanolamine; BEL, bromoenol-lactone; DHA, docosahexaenoic acid; diacyl-GPC, diacyl-glycerophosphocholine; PC, phosphatidylcholine; PE, phophatidylethanolamine; PL, phospholipid; PLA_2, phospholipase A_2; U_{III}, rat uterine stromal cells.

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Hormonal Regulation of Phosphatidylcholine Metabolism and Transport

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Phosphatidylcholine (PC), a major membrane phospholipid, is involved in a wide variety of cell functions. Involvement of PC in signal transduction [i.e., phospholipase D (PLD)-mediated PC hydrolysis] is a well-characterized process, but very little is known about its metabolism after hormonal stimulus. We investigated this process in a well-characterized experimental system, consisting of L6 myogenic cells stimulated by Arg^8-vasopressin (AVP), which rapidly activates PLD, inducing measurable PC hydrolysis in the plasma membrane (PM) (1).

Pre-loaded L6 cells were stimulated with 30 nM AVP, PM were purified by centrifugation in a sucrose discontinuous gradient, and [^{3}H-choline/^{14}C-arachidonate]-labeled PC was separated by thin-layer chromatography. Vesicle traffic was investigated by transmission electron microscopy and fluorescence measurements of trimethylammonium diphenylhexatriene (TMA-DPH) and acridine orange (AO).

A marked decrease of [^{3}H-choline]-PC (87 ± 4% of control) after 1 min of AVP stimulation was measured in PM isolated from L6 cells, followed by a return to the pre-stimulus level (98 ± 8% of control) within the following 4 min. Preincubating the cells with hexadecylphosphocholine (HePC) did not affect PC hydrolysis but selectively inhibited its *de novo* synthesis and the restoration of labeled PC in PM. In other experiments, unlabeled cells were incubated with ^{14}C-arachidonate along with AVP (or vehicle). In these conditions, AVP stimulated incorporation of the label into PC separated from purified PM (300 ± 12% of control after 1 min, and 174 ± 6% of control after 3 min).

Ultrastructural quantitative analysis revealed a significant vesicle traffic, rapidly stimulated after AVP treatment. Hormone-induced vesicle transport consisted mainly of exocytosis, as evidenced by the AVP-induced release of AO. The kinetics of this traffic (measurable after 1 min of hormonal treatment and returning to the basal level within a few minutes) is consistent with the previous data and confirmed also by TMA-DPH fluorescence measurements of vesicle traffic. The latter measurements allowed us to better characterize AVP-induced vesicle traffic: this depended both on PC hydrolysis (since it was sensitive to Zn^{2+} inhibition of PLD) and on PC neosynthesis (since it was inhibited by HePC). Moreover, vesicle traffic was on a direct route from the endoplasmic reticulum to the PM, since it was not inhibited by Brefeldin A, and it required the integrity of the microtubule-microfilament network. We observed also a hormone-induced vesicle traffic with the same features in a different experimental system, namely in endothelin-1 (ET1)-stimulated rat peritubular myoid cells.

These data demonstrate that hormone-induced plasma membrane PC hydrolysis is immediately followed by stimulation of its neosynthesis and by its return to the PM, thus restoring the pre-stimulus level. These experimental systems allow us to distinguish which one of the known PC biosynthetic pathways (2) is involved in restoring the plasma membrane PC pool after PLD activation. It is worth noting that both AVP-stimulated myogenic cells and ETl-stimulated myoid cells are not specialized secretory cells. This excludes the involvement of the membrane cycling process characteristic of secretory cells in the observed phenomenon and it allows us to assign a role to hormone-stimulated exocytosis in nonsecretory cells.

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Abbreviations: AO, acridine orange; AVP, Ang^8-vasopressin, ET1, endothelin-1, HePC, hexadecylphosphocholine; PC, phosphatidylcholine; PLD, phospholipase D; PM, plasma membrane; TMA-DPH, trimethylammonium diphenylhexatriene.

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Abnormalities in Sarcolemmal Phospholipase D and Phospholipase C Isoenzymes and in Their Interactions in Post-Infarcted Failing Hearts

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Phospholipase D (PLD) activity associated with the myocardial cell membrane (sarcolemma, SL) specifically hydrolyzes phosphatidylcholine to form phosphatidic acid (PtdOH). As reported for other cells, SL PLD is coupled to a PtdOH phosphohydrolase for the coordinate production of a *sn*-1,2-diacylglycerol (DAG) pool originating from phosphatidylcholine (1). PLD is part of the SL signaling system (2). Its importance for the heart function is shown by PtdOH-induced changes in cellular Ca^{2+} movements and contractile performance, as well as by its potential involvement in the development of cardiac hypertrophy (2). We reported biochemical evidence for the presence, at the SL level, of a *cis*-unsaturated fatty acid (UFA)-sensitive PLD activity (*cis* UFA-PLD); arachidonic, oleic and docosahexaenoic acids being the most efficient stimulants among the fatty acids tested) (3,4) which is affected by oxidants and thiol modifiers (5). SL phosphoinositide-phospholipase C (PLC), which participates in many different physiological processes of the cardiac cell (6), is also inhibited by the oxidative stress (7). Oxidants generation, which alters functional protein thiols, is an important feature of congestive heart failure (CHF) following myocardial infarction (8). Therefore, *cis* UFA-PLD and PLC β_1, γ_1 and δ_1, which are the most relevant and well-characterized variants of PLC in mammalian cells, were studied in SL membranes purified from the surviving left ventricle of rats at 8 wk after occlusion of the left-anterior descending coronary artery, when the animals were in a moderate stage of CHF (9).

A significant reduction of the SL level of signaling PtdOH was observed in CHF, due to the imbalance between increased PtdOH synthesis (124% of controls) by *cis* UFA-PLD and comparatively higher PtdOH dephosphorylation (135% of controls) by PtdOH phosphohydrolase. As expected, the specific DAG pool derived from *cis* UFA-PLD/PtdOH phosphohydrolase combined activities was significantly augmented in CHF.

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Abbreviations: CHF, congestive heart failure; DAG, diacylglycerol; PLC, phospholipase C; PLD, phospholipase D; PtdOH, phosphatidic acid; SL, sarcolemma; UFA, unsaturated fatty acid.

The rank order of PLC isoenzymes hydrolytic activity toward phosphatidylinositol 4,5-bisphosphate in control SL was PLC δ_1 > PLC γ_1 > PLC β_1, while that of the protein mass was PLC γ_1 > PLC δ_1 > PLC β_1. CHF resulted in a drastic reduction of PLC γ_1 and δ_1 activity (11 and 14% of controls, respectively) and protein mass (7 and 18% of controls, respectively), in direct contrast with a significant increase of both PLC β_1 parameters. *In vivo* treatment with the angiotensin-converting enzyme inhibitor imidapril, instituted 4 wk post-infarct, significantly improved the contractile function of the 8-wk postinfarcted failing hearts with partial correction of the PLC isoenzymes parameters and normalization of *cis* UFA-PLD and PtdOH phosphohydrolase activities.

PtdOH stimulates the total SL PLC activity in normal hearts (2). As the level of *cis* UFA-PLD-derived PtdOH was diminished in CHF, we investigated whether or not the response of PLC isoenzymes to PtdOH was also altered in this disease state. In controls, *in vitro* stimulation by exogenous PtdOH (30 μM) was limited to the γ_1 and δ_1 variants. In CHF, these PtdOH-stimulated PLC activities were significantly lower than the correspondent control values. We also showed that PLC is one of the mechanisms for PtdOH-induced increase in intracellular Ca^{2+} ($[Ca^{2+}]_i$) and contractile performance of the normal heart (10). In CHF, PtdOH-mediated rise of cardiomyocytal $[Ca^{2+}]_i$ was significantly reduced; this might have been caused, at least partly, by the attenuated response of PLC γ_1 and δ_1 to PtdOH.

Our results indicate that, in post-MI CHF: (i) the bioprocesses mediated by the SL PtdOH and DAG pool originating from the *cis* UFA-PLD/PtdOH phosphohydrolase pathway may be impaired; (ii) SL PLC β_1-dependent function is amplified with almost complete loss of the PLC γ_1 and δ_1-related signaling; this event would impact negatively on the complex second-messenger response of PLC-linked receptors; and (iii) the possibility of valid interactions between SL *cis* UFA-PLD and PLC signaling pathways may be hampered by the diminished level of SL PtdOH as well as by the striking decrease of PLC γ_1 and δ_1 and their insufficient response to PtdOH, with possible consequences for $[Ca^{2+}]_i$ homeostasis and contractile performance of the post-infarcted failing

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heart. Moreover, the reversibility of PLC, *cis* UFA-PLD, and PtdOH phosphohydrolase pathological changes by the imidapril therapy may confer pathophysiological significance to these enzymes and may be related to the mechanism of the beneficial action of this angiotensin-converting enzyme inhibitor.

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Role of Sphingosine in Induced Apoptosis

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Apoptosis is a programmed cell death shown to play a key role in normal development, differentiation, glandular atrophy following hormonal withdrawal, and maturation of the immune system. Its hallmark biochemical feature is endonuclease activation, giving rise to internucleosomal DNA fragmentation. Internucleosomal cleavage of DNA is a result of a apoptotic-specific signal transduction which involves protein kinases, protein phosphatases, proteases, oncoproteins, and lipid second messengers. Utilization of the sphingomyelin (SM) pathway for induction of the apoptotic response has now been demonstrated in a large number of mammalian cells. The role of ceramide in apoptotic signal transduction is widely presented in literature, while the highly toxic product of its hydrolysis sphingosine does not get the appropriate attention. Our investigation was devoted to more precise study of the role of sphingosine in the apoptotic signal transduction, induced by both tumor necrosis factor-α (TNF-α) and other agents, including protein synthesis inhibitor cycloheximide (CHI) and mycotoxin fumonisin B_1.

Relation of sphingosine accumulation in murine liver with biological activity of mutant forms of recombinant human TNF-α. The inflammatory cytokine TNF-α has been shown to initiate apoptotic cell death and DNA fragmentation in several mammalian cell lines. Biological action of TNF has been associated in part with enhanced SM turnover. To determine the role of sphingosine in TNF cytotoxicity, TNF mutants differing in their toxicity to L929 cells were produced and studied. We showed that TNF-α administration to mice produced a rapid (<30 min) increase in free sphingosine level. Wild-type TNF-α and the mutant with deleted residues 67–70 exhibited the highest toxicity and caused accumulation of sphingosine exceeding its control level 5.5 times in murine liver. TNF-α variants with single-point mutation (E127Q, I155L, or V150I) and double mutations (V150I and I155L) caused moderate increases in sphingosine content and were significantly less toxic. The observed relationship between toxicity of TNF-α mutants and the extent of sphingosine accumulation in murine liver provides evidence to suggest that sphingosine may be mediator of TNF-α -induced cell damage and death.

Apoptosis in rat liver induced by CHI. CHI is a well-known inhibitor of protein synthesis and synergistically increases TNF-α cytotoxicity. Regulation of apoptosis differs with cell type and stage of differentiation and does not always require protein synthesis. Herein we demonstrate that CHI in sublethal doses (0.3 mg/100 g) causes an apoptosis in liver cells *in vivo.* Inhibition (95%) of protein synthesis by sublethal dose of CHI induced short-term superexpression of *c-myc, c-fos, c-jun,* and *p53* genes which are involved directly in programmed cell death detected by electron microscopy and gel electrophoresis of DNA. We examined changes in free sphingosine level in liver cell and nuclei during treatment of rats by different doses of CHI (0.01, 0.05, and 0.3 mg/100 g). Sphingosine level in the liver cells increased concomitantly with an increasing of *c-myc* and *c-fos* genes expression after injection of sublethal dose of CHI. On the other hand, the level of free sphingosine in liver isolated from rats after injection of CHI at doses which did not sharply increase gene expression did not show any increase. Sphingosine functions as an inhibitor of protein kinase C (PKC). Other PKC inhibitors strongly induce apoptosis in certain cell types. Our results suggest that sphingosine may function as an endogenous mediator of apoptotic signaling.

Fumonisin B1-induced apoptosis in vivo. Fumonisin B1, a mycotoxin, is an inhibitor of ceramide synthase, a key enzyme in *de novo* sphingolipid biosynthesis and reacylation of free sphingosine. We found correlation between accumulation of intracellular free sphingoid basis and DNA degradation in liver cells after injection of fumonisin B1 in doses of 5 and 20 μg per mouse.

Sphingosine- induced apoptosis in vivo. Injection of sphingosine to mice in doses of 20 and 50 μg per mouse induced large-scale fragmentation of DNA in liver, spleen, and thymus comparable with TNF action in doses of 20 μg per animal. Sphingosine-enchanced TNF-α-induced DNA degradation in thymus to oligonucleosomal fragmentation. Sphingosine can

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Abbreviations: CHI, cycloheximide; PKC, protein kinase C; SM, sphingomyelin; TNF, tumor necrosis factor.

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dramatically potentiate apoptotic events induced by TNF even if the cytokine is used in subtoxic doses. Thus this natural compound, along with TNF-α, might fill a prominent place in contemporary pharmacology. The details of the apoptotic pathway downstream of sphingosine are not known, although several targets for sphingosine action were identified, including PKC, casein kinase II, Rb, and bcl-2 proteins which play the key role in transmission of apoptotic signal.

Sphingosine Kinase: Assay Conditions, Tissue Distribution in Rat, and Subcellular Localization in Rat Kidney and Liver

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The phosphorylation of sphingoid bases is the penultimate step in the breakdown of sphingolipids. Sphingenine-1-phosphate (SeP), a product of this reaction, however is also bioactive and was proposed as a second messenger (1). Sphingosine kinase, the enzyme catalyzing the formation of SeP, is poorly characterized. Often it was reported to be cytosolic, but evidence for a membrane-bound activity also exists and it is not known whether catabolic SeP and bioactive SeP are formed by the same enzyme. The aim of this study was therefore to get a better insight in the subcellular distribution of sphingosine kinase(s).

Using D-erythro-[^{3}H-4,5]sphinganine as substrate, assay conditions were optimized in rat liver homogenates, taking care of inhibiting sphingosine-phosphate lyase (2) and sphingosine-phosphatase (3), two membrane-bound activities present in biological samples that destroy the reaction product.

A tissue distribution study in rat revealed the highest kinase activities in testis, followed by kidney and intestine.

Based on subcellular fractionation of rat liver and kidney homogenates, the presence of (at least) two kinase activities is deduced, a membrane-bound and a cytosolic one. The latter one accounts only for about 20% of the total tissue activity. Further separation of the membrane vesicles by means of density gradient centrifugation showed that a portion of the kinase activity is associated with the plasma membrane.

It is tempting to speculate that the cytosolic sphingosine kinase is responsible for the formation of catabolic SeP that subsequently will be cleaved at the cytosolic site of endoplasmic reticulum by the sphingosine-phosphate lyase (2). The plasma membrane-bound sphingosine-kinase would act on sphingenine formed in or near the plasma membrane and be involved in the generation of bioactive SeP.

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Abbreviations: SeP, sphingenine-1-phosphate.

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α2-Adrenergic Receptor-Mediated Release of Lysophosphatidic Acid by Adipocytes: A Paracrine Signal for Preadipocyte Growth

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Lysophosphatidic acid (LPA) is a bioactive phospholipid able to generate several cellular responses (proliferation, rearrangement of the cytoskeleton, ion exchanges) by interaction with specific G protein-coupled receptors. LPA is produced by aggregating platelets constituting the main source of serum LPA. Our study brings evidence for a local production of LPA in white adipose tissue (WAT) exposed to α2-adrenergic agonists. This α2-adrenergic-dependent production of LPA could play an important role in the control of adipogenesis.

WAT is composed of nonproliferating mature adipocytes storing triglycerides, and of preadipocytes which can proliferate and differentiate into new fat cells. An excess of adipose tissue is due to adipose cell hypertrophy accompanied, in most severe obesities, by adipose tissue hyperplasia. Formation of mature fat cells from preadipocytes (adipogenesis) can take place throughout life in rodents and humans. It can be regulated not only by circulating factors but also by autocrine/paracrine factors produced locally in WAT. Determining the identity of the factors regulating adipogenesis is a major point in understanding normal and pathologic growth of adipose tissue.

α2-Adrenergic receptors are highly expressed in adipocytes, particularly human adipocytes, and mediate some specific biological responses induced by circulating catecholamines (epinephrine and norepinephrine) in WAT such as antilipolysis in mature adipocytes, and growth and cytoskeletal rearrangements in preadipocytes.

We report that conditioned media from isolated adipocytes increase spreading (reflecting regulation of actin cytoskeleton) and proliferation of 3T3F442A preadipocytes. These effects were amplified when an α2-adrenergic agonist was present during the obtention of the conditioned media. This α2-adrenergic-dependent trophic activity was completely abolished by pretreatment of the conditioned media with a lysophospholipase (phospholipase B), or a specific desensitization of LPA-responsiveness of 3T3F442A preadipocytes (generated by previous chronic treatment with high concentration of LPA). Among the different lysophospholipids tested, only LPA was able to induce spreading and proliferation of 3T3F442A preadipocytes. α2-Adrenergic stimulation also leads to a rapid, sustained, and pertussis-toxin-dependent release of [^{32}P]LPA from [^{32}P]-labeled adipocytes. Identity of LPA was confirmed by mass spectrometry after derivatization of the phospholipids of the conditioned media with 1-pyrenyldiazomethane.

α2-Adrenergic-dependent release of LPA was also demonstrated *in vivo* by *in situ* microdialysis. This technique allows analysis of the extracellular fluid composition of intact adipose tissue. Microdialysis probes were implanted in human subcutaneous adipose tissue and perfused with physiological solution supplemented or not with an α2-adrenergic agonist. Microdialysates were collected and tested for their ability to generate LPA-like responses on preadipocytes. Microdialysates obtained in the presence of the α2-adrenergic agonist exhibited a higher spreading effect as compared to microdialysates obtained in the absence of agonist. The additional spreading effect was completely abolished by pretreatment with phospholipase B and by previous desensitization of LPA-receptors. Based upon these results, it was proposed that stimulation of α2-adrenergic receptors provokes the extracellular release of LPA from adipocytes, both *in vitro* and *in vivo*.

Because of the close proximity of adipocytes and preadipocytes in the adipose tissue, and because of the ability of LPA to regulate preadipocyte growth, the α2-adrenergic-dependent local production of LPA could play an important role in the process of adipogenesis.

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Abbreviations: LPA, lysophosphatidic acid; WAT, white adipose tissue.

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Phosphatidic Acid-Dependent Activation of Adenosine-3′,5′-cyclic-monophosphate-phosphodiesterase Is Necessary for Arg-Vasopressin Induction of Myogenesis

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Arg^8-Vasopressin (AVP) stimulates differentiation of L6 myoblasts (1) likely through activation of phospholipase D (PLD) and generation of phosphatidic acid (PA) (2). Conversely, high intracellular adenosine-3′,5′-cyclic monophosphate (cAMP) levels and protein kinase A (PKA) activation inhibit myoblast differentiation (3). Intracellular cAMP level is not only regulated by activation of adenylyl cyclase but also by its hydrolysis due to phosphodiesterase (PDE) activity. We reported that PA can activate specific isoforms of type IV cAMP-phosphodiesterase (PDE4) *in vitro* (4). Here, we investigate whether AVP-induced differentiation of L6 myoblasts involves activation of PDE4 isoforms by PA.

PLD activity was assessed measuring [^{3}H]-myristate phosphatidylethanol production (2). PDE4 expression and activity in L6 cells were investigated by RNase-protection assay, immunoprecipitation, Western blot (WB), and PDE assay. PKA activity was evaluated by measuring kemptide phosphorylation in the presence of protein kinase inhibitor. L6 cell differentiation was assessed by creatin kinase activity and nuclear localization of the specific muscle transcription factor myogenin by immunofluorescence (1).

RNase-protection assay, immunoprecipitation, and Western blot analyses indicated that only one isoform of PDE4 with apparent M.W. of 100 kDa is expressed in L6 myoblasts, and it is responsible for 75% of the cAMP hydrolyzing activity of these cells. Incubation of L6 cells with 12-*O*-tetradecanoylphorbol 13-acetate (a potent activator of PLD) stimulated a significant and sustained increase of PDE4 activity of about 50%, similar to the activation observed after direct addition of exogenous PA to L6 cell homogenate. AVP stimulation of L6 cells induced a biphasic increase of PDE activity with a first rapid peak occurring after 2 min (150% over controls), followed by a slower increase, reaching a maximum after 15 min (150% over controls) and subsequently declining. On the contrary, insulin-like growth factor I [a cofactor—in our experimental conditions—of L6 cell differentiation which is unable by itself to induce differentiation (1)] did not stimulate PLD nor PDE activity. Treatment of L6 cells with propranolol (an inhibitor of PA-phosphohydrolase) before AVP stimulation led to an increase of PA accumulation and inhibition of diacylglycerol formation. These effects led to parallel modifications of AVP-induced PDE4 activation with an increase of the first phase and a decrease of the late phase. This suggests that the two phases of AVP-dependent PDE activation are mediated by PA and, respectively, by its conversion product, diacylglycerol. Protein kinase C downregulation obtained with long-term 12-*O*-tetradecanoylphorbol 13-acetate treatment (24 h) selectively abolished AVP-dependent PLD activation and PDE activation. Preliminary results indicate that PKA activity is lowered by L6 stimulation with AVP by about 50% after 3 h of hormonal treatment. Experiments performed with selective inhibitors for different PDE families (rolipram, RS 25344 for PDE4, milrinone for type III PDE, and zaprinast for type V PDE) demonstrated that only inhibition of PDE4 results in complete suppression of the AVP-induced differentiation. This result is confirmed by immunofluorescence experiments which showed inhibition of myogenin translocation into the cell nucleus. Taken together, these results suggest that PA can act as a cross-talk molecule between the PLD and the cAMP pathways by stimulating PDE4 activity in L6 cells and decreasing PKA activity. This event may play a crucial role in the control of the physiological process of myoblast differentiation induced by AVP.

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Abbreviations: AVP, arg^8-vasopressin; cAMP, adenosine-3′,5′-cyclic monophosphate; PA, phosphatidic acid; PDE, phosphodiesterase; PDE4, type IV cAMP-PDE; PKA, protein kinase A; PLD, phospholipase D.

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Regulation of cAMP-Phosphodiesterases by Phosphatidic Acid Binding

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Many hormones and growth factors stimulate the production of the putative second messenger phosphatidic acid (PA) in various cell types (fibroblasts, lymphocytes, myoblasts, etc.). In turn, this phospholipid alters the function of several effector proteins playing major roles in cell signal transduction, such as protein kinases, phosphatases and phospholipases. We previously showed that PA selectively stimulates in an acellular system some isoforms of cyclic AMP phosphodiesterases (PDE) belonging to the type 4 family (1), i.e., the enzymes hydrolyzing specifically cAMP and inhibited by the reference inhibitor rolipram. This family of enzymes plays a critical role in controlling the cAMP levels in several cell systems, in particular lymphocytes and myoblasts. The PA-sensitive PDE 4 isoforms are characterized by the presence of conserved regulatory sequences in the N-terminal region (1). We also observed that only acidic phospholipids are able to activate type 4 PDE, and among them PA, especially the stearoyl-arachidonoyl species, is the most potent activator (2). Since this PA species is preferentially synthesized in thymic lymphocytes in response to a mitogenic stimulation (2), we have proposed the hypothesis that the regulation of PDE 4 activity by PA may have a physiological relevance.

Methods and Results. To demonstrate the physiological relevance of PDE 4 regulation by PA in intact cells, we used transiently transfected MA10 Leydig tumor cells overexpressing different isoforms of PDE 4 and studied the influence of an accumulation of endogenous PA induced by propranolol, an inhibitor of the PA-degradating enzyme PA-phosphohydrolase. In MA10 cells overexpressing PDE4D3, an isoform of PDE 4 sensitive to PA-activation, PA accumulation induced a strong stimulation of PDE activity (threefold), peaking at 45 min. By contrast, when the overexpressed PDE was PDE4D1, a PA-insensitive isoform, PA accumulation did not modify PDE activity. Furthermore, the treatment of PDE4D3-transfected cells by propranolol induced significant decreases of both intracellular cAMP levels and cAMP-dependent protein kinase activity.

To determine the mechanism of PDE stimulation by PA, MA10 cells overexpressing PDE4D3 were preincubated with ^{32}P-orthophosphate, to label phospholipids. After treatment by propranolol, the cells were homogenized, and PDE was immunoprecipitated by a specific antibody. Lipids present in the immunopellet were extracted and analyzed by thin-layer chromatography. Autoradiography of the thin-layer chromatography plates showed that PA was the major phospholipid bound to PDE4D3, which supports the hypothesis that endogenous PA activates PDE4D3 by means of direct binding to the protein.

A preliminary characterization of the PA binding site on PDE4D3 was performed by engineering deletions of selected regions in the regulatory domain. Three regions particularly rich in basic and/or hydrophobic aminoacid residues were deleted. Deletion of amino acids 2 to 19 had no effect, while deletion of amino acids 31 to 59, or amino acids 99 to 130 suppressed PA-activating effect, suggesting that these last regions might be involved in the binding of PA to PDE4D3.

Conclusions. These results show that intracellular PA can regulate PDE 4 activity in intact cells by direct binding of the phospholipid to some PDE 4 isoforms, and thus modulate cAMP levels. This conclusion reinforces the concept that PA has a role of second messenger, and defines type 4 PDE as new effector enzymes for its intracellular actions. The observed cross-talk between the cAMP pathway and phospholipid metabolism is likely to be of importance in various physiological processes submitted to regulation by cAMP, such as lymphocyte proliferation and differentiation of skeletal myoblasts.

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Abbreviations: PA, phosphatidic acid; PDE, phosphodiesterases.

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Tumor Necrosis Factor-α and Ceramides in Insulin Resistance

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ABSTRACT: The present studies tested the hypothesis that some effects of tumor necrosis factor-α (TNF-α) are mediated by activation of sphingomyelinases and the production of ceramides. Differentiated 3T3-L1 adipocytes were incubated with short-chain ceramide analogs, (C_2- and C_6-ceramides: *N*-acetyl- and *N*-hexanoyl-sphingosines, respectively), and this treatment increased 2-deoxyglucose uptake in the absence of insulin progressively from 2–24 h. This effect was inhibited by blocking the activations of mitogen-activated protein kinase, phosphatidylinositol 3-kinase (PI 3-kinase), and ribosomal S6 kinase which mediated an increase in GLUT1 concentrations. Long-term increases in PI 3-kinase activity associated with insulin receptor substrate-1 (IRS-1) increased the proportion of GLUT1 and GLUT4 in plasma membranes. These events explain the increases in noninsulin-dependent glucose uptake and incorporation of this glucose into the fatty acid and glycerol moieties of triacylglycerol. The mechanisms by which TNF-α and ceramides increase PI 3-kinase activity were investigated further by using rat2 fibroblasts. Incubation for 20 min with TNF-α, bacterial sphingomyelinase, or C_2-ceramides increased PI 3-kinase activity by about fivefold, and this effect depended upon a stimulation of tyrosine kinase activity and an increase in Ras-GTP. This demonstrates the existence of a novel signaling pathway for TNF-α that could contribute to the effects of this cytokine in stimulating basal glucose uptake. By contrast, treating the 3T3-L1 adipocytes for 2–24 h with C_2-ceramide diminished insulin-stimulated glucose uptake by decreasing the insulin-induced translocation of GLUT1 and GLUT4 to plasma membranes. This inhibition was observed when there was no increase in basal glucose uptake, and it occurred downstream of PI 3-kinase. Our work provides further mechanisms whereby TNF-α and ceramides produce insulin resistance and decrease the effectiveness of insulin in stimulating glucose disposal from the blood. Conversely, TNF-α and ceramides increase the ability of adipocytes to take up glucose and store triacylglycerol in the absence of insulin.

Insulin resistance (defined as decreased ability of insulin to stimulate glucose uptake) is an important component of Type 2 diabetes (1). It is caused by a postreceptor defect in insulin signaling which normally leads to glucose transport into cells. This involves activation of the insulin receptor (IR) by phosphorylation of tyrosine residues, which then causes the tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1). Activated IRS-1 then binds phosphatidylinositol 3-kinase (PI 3-kinase) which itself becomes activated and stimulates the movement of glucose transporters (mainly GLUT4) to the cell surface. The increase in GLUT4 at the plasma membrane is then responsible for insulin-dependent glucose uptake. Insulin resistance results from a decreased efficiency to stimulate this cascade. It can occur in sepsis, advanced cancer, and trauma, partly as a result of tumor necrosis factor-α (TNF-α) action. TNF-α is also secreted by adipose tissue and is implicated in insulin resistance associated with obesity (2,3).

Incubation of 3T3-L1 adipocytes (4) or hepatoma cells (5) with TNF-α was reported to decrease the insulin-induced tyrosine phosphorylation of IR and IRS-1. TNF-α increases the serine phosphorylation of IRS-1, which inhibits the tyrosine kinase activity of IR (6) and impairs the association of IRS-1 with PI 3-kinase (7). In rat hepatoma Fao cells, changes in IRS-1 were seen after 1 h (7), whereas incubations of 2 to 4 d were used in 3T3-F442A adipocytes (6). By contrast to these results, incubation of 3T3-L1 adipocytes with TNF-α for 30–60 min had the opposite effect of increasing the tyrosine phosphorylation of IRS-1 and its binding of PI 3-kinase (8). Other work with 3T3-L1 adipocytes ascribed the long-term effects of TNF-α in producing insulin resistance to a decreased expression of IRS-1 and GLUT4 rather than decreased tyrosine phosphorylation of IRS-1 (9). TNF-α activates several signaling cascades, including a stimulation of sphingomyelinases and consequent ceramide production (10). Exogenous sphingomyelinase and cell-permeable (short-chain) ceramides were shown to mimic some effects of TNF-α in decreasing the insulin-dependent tyrosine phosphorylation of IRS-1 (11,12) and activation of phosphoprotein phosphatase-1 (13). The present studies investigated further the nature of the insulin resistance that is produced by TNF-α and determined which effects of TNF-α were mediated by ceramides.

METHODS

3T3-L1 fibroblasts were cultured in Dulbecco's minimum essential medium (DMEM) supplemented with 10% fetal

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Abbreviations: C_2-, acetyl-; C_6-, hexanoyl; DMEM, Dulbecco's minimum essential medium; GLUT4, glucose transporter 4; IRS-1, insulin receptor substrate-1; PI 3-kinase, phosphatidylinositol 3-kinase; TNF-α, tumor necrosis factor-α.

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bovine serum, differentiated and maintained in DMEM containing 10% fetal bovine serum (14). Experiments were performed 9–10 d after induction of differentiation, when greater than 90% of the cells expressed the adipocyte phenotype. Glucose transport was determined over 10 min after adding [^{3}H]2-deoxyglucose. Lipid synthesis from [^{14}C]glucose was determined by extraction of the triacylglycerol, saponification, and separation of the fatty acid and glycerol moieties. Western blot analysis was performed by the enhanced chemiluminescence method (14). In other work, rat2 fibroblasts were grown to confluence and were used to study the mechanisms by which TNF-α, bacterial sphingomyelinase, or C_2-ceramide stimulates PI 3-kinase activity.

RESULTS AND DISCUSSION

Treatment of 3T3-L1 adipocytes with 100 μM C_2-ceramide for 2, 6, and 24 h increased basal glucose transport by 1.6, 4.3- and 7.6-fold, respectively (Table 1). C_2-ceramide decreased insulin-stimulated glucose uptake by 53 to 60% after 2, 6, and 24 h. C_2-ceramide also decreased the subsequent insulin-dependent incorporation of glucose into the fatty acid and glycerol of triacylglycerols by about 80 and 33% after 2 h, respectively. However, C_2-ceramide increased these incorporations in the absence of insulin by about 4- and 12-fold, respectively, after 24 h (Brindley, D.N., Wang, C.-N., Mei, J., Xu, J., and Hanna, A.N., unpublished). Cells incubated for 6 and 24 h with 100 μM dihydro-C_2-ceramide, which is unable to mimic several biological effects of C_2-ceramide, showed relatively little increase in basal glucose transport or incorporation into lipids. The effects of C_2-ceramide on glucose transport were therefore relatively specific.

Incubation with C_2-ceramide for 24 h increased (P < 0.001) the total amount of GLUT1 by fivefold compared to control cells (Fig. 1A). C_2-ceramide treatment for 24 h increased GLUT1 concentrations by ninefold in the plasma membrane fraction (14). Insulin increased the amount of GLUT1 in plasma membranes of control and C_2-ceramide treated cells by about 260 (P < 0.001) and 10%, respectively (14). C_2-ceramide treatment therefore blocked the insulin-in-

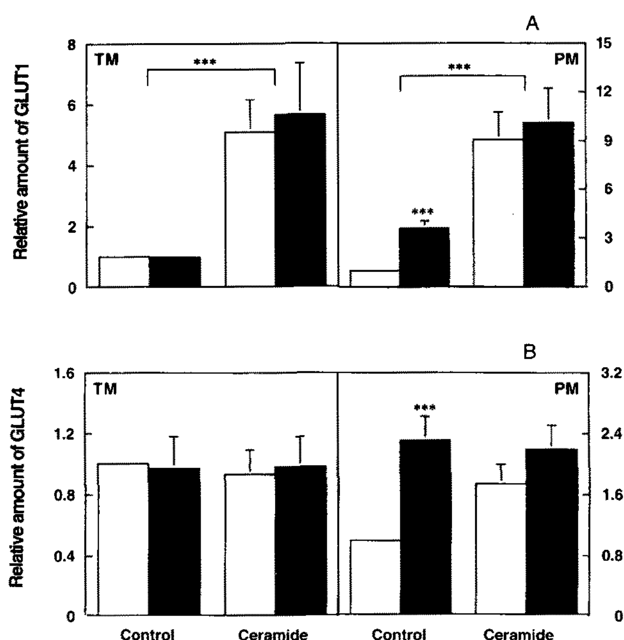

FIG. 1. Effect of C_2-ceramide on the distribution of glucose transporter 1 (GLUT1) and GLUT4 in subcellular membrane fractions. 3T3-L1 adipocytes were incubated with 100 μM C_2-ceramide for 24 h. Cells were then treated with or without 20 nM insulin for 30 min as indicated and subjected to subcellular fractionation. Results are shown for the total membrane (TM) and plasma membranes (PM) fractions. The figure shows the relative level of GLUT1 in membrane fractions prepared from unstimulated cells (open bars) or cells stimulated with 20 nM insulin (solid bars). Results are means ± SD (where large enough to be shown) from five independent experiments and are expressed relative to control cells under basal condition in each fraction. Significant differences between control and ceramide-treated cells or between control and insulin treatment are indicated by ***, $P \leq 0.001$.

TABLE 1
Effects of C_2-Ceramide and Dihydro-C_2-Ceramide on the Basal and Insulin-Induced 2-Deoxyglucose Transport[a]

Treatment	Insulin absent	Insulin present	Insulin-stimulated transport
	(pmol of 2-deoxyglucose/min per 2 × 10^6 cells)		
Procedure 1			
Control	99 ± 15	715 ± 217	616 ± 232
C_2-ceramide (6 h)	423 ± 61*	712 ± 156	289 ± 95*
Dihydro-C_2-ceramide (6 h)	155 ± 36	645 ± 80	490 ± 82
C_2-ceramide (24 h)	753 ± 164***	1010 ± 237**	258 ± 91*
Dihydro-C_2-ceramide (24 h)	234 ± 30	880 ± 169	646 ± 141
Procedure 2			
Control	200 ± 4	1516 ± 71	1315 ± 72
C_2-ceramide (2 h)	320 ± 20*	848 ± 152***	526 ± 139***

[a]In Procedure 1, cells were incubated for 24 h or for the final 6 h in the absence or presence of 100 μM C_2-ceramide or dihydro-C_2-ceramide. Basal 2-deoxyglucose transport and that induced by 20 nM insulin were measured, and the difference between these values gave insulin-stimulated glucose transport. In Procedure 2, a different series of cells were treated with C_2-ceramide for 2 h. Glucose uptake was measured as above except that the Krebs'-Ringer buffer contained 0.5% bovine serum albumin. Results are means ± SD from three independent experiments. Significant differences between control and treated cells are indicated by *, $P \leq 0.01$; **, $P \leq 0.05$; and ***, $P \leq 0.001$. The results are reproduced from Reference 14, with permission.

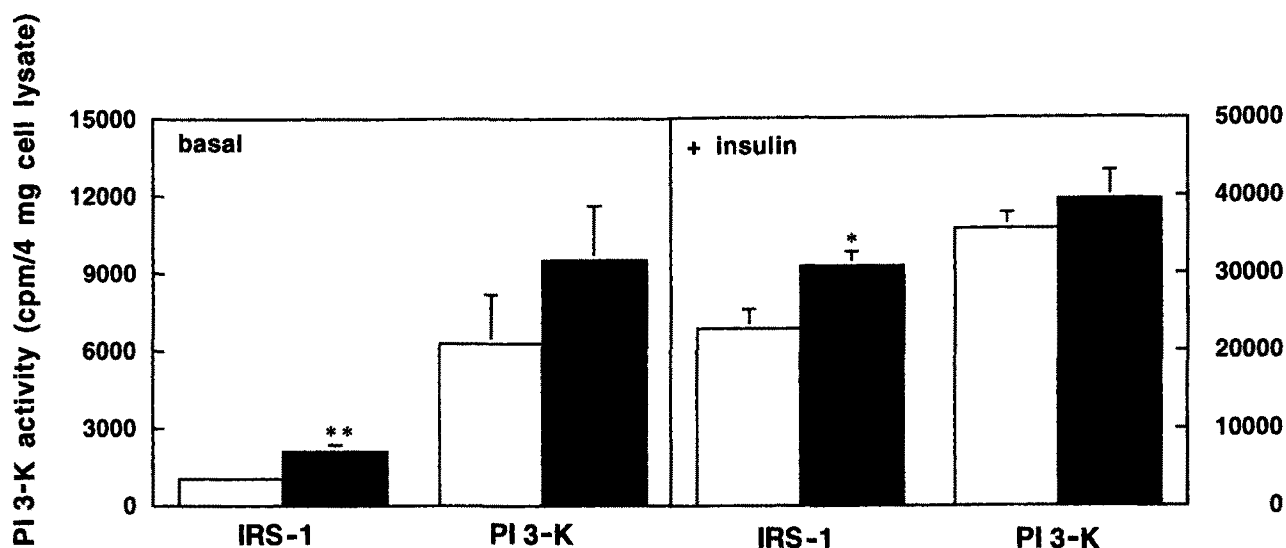

FIG. 2. Effects of C_2-ceramide on the activity and association of phosphatidylinositol 3-kinase (PI 3-K) with insulin receptor substrate-1 (IRS-1). Differentiated 3T3-L1 adipocytes were treated with 100 μM C_2-ceramide for 12 h and the cells were then stimulated with 100 nM insulin for 10 min as indicated. PI 3-K was then precipitated from cell lysates with antibodies against IRS-1 or the 85 kDa subunit to measure activity associated with IRS-1 or total activity, respectively. PI 3-K activity was determined by the phosphorylation of PI in control cells (open bars), or ceramide-treated cells (solid bars). Results are means ± SD from three independent experiments. Significant differences between control and ceramide-treated cells are indicated by: *, $P \leq 0.05$ and **, $P \leq 0.01$. The results are reproduced from Reference 14 with permission.

duced translocation of GLUT1 to plasma membranes after incubation for 2 or 24 h.

Treatment with C_2-ceramide (Fig. 1B) for 2 or 24 h did not affect GLUT4 levels in the total membrane fraction significantly. However, C_2-ceramide increased the level of GLUT4 in plasma membranes by 1.7-fold. Insulin increased GLUT4 levels in plasma membranes by 2.3-fold in control cells. However, the increase was only 1.26-fold in C_2-ceramide-treated cells. Consequently, C_2-ceramide also inhibited the insulin-induced increase in the translocation of GLUT4 to plasma membranes.

Incubation of 3T3-L1 adipocytes for 24 h with C_2-ceramide did not alter significantly the masses of IR or IRS-1, nor their tyrosine phosphorylations after insulin stimulation (14). The amount of PI 3K (85 kDa subunit) that was coimmunoprecipitated with IRS-1 in the presence or absence of insulin was also not significantly decreased after incubation with C_2-ceramide. By contrast, incubation for 24 h with 10 ng/mL TNF-α decreased the mass of IR by about 20%, and there was an equivalent decrease in its tyrosine phosphorylation. The mass, tyrosine phosphorylation, and association of IRS-1 with PI 3-kinase were decreased by about 60, 75, and 60%, respectively. These results indicate that TNF-α decreases the activation of IRS-1 by pathways that are independent of ceramide action. The effects of C_2-ceramide on PI 3-kinase were investigated in greater detail by determining its activity after coimmunoprecipitation with IRS-1, or by using an anti-p85 antibody to assess the total PI 3-kinase activity. C_2-ceramide treatment for 12 h did not change the total PI 3-kinase activity significantly in the absence of insulin, or the stimulation of activity by insulin (Fig. 2). However, C_2-ceramide had an insulin-like effect in increasing the PI 3-kinase activity associated with IRS-1 in the presence or absence of insulin.

In order to determine further the mechanism for this increase in PI 3-kinase activity, we treated rat2 fibroblasts with 10 ng of TNF-α/mL, 0.1 units of bacterial sphingomyelinase/mL, or 40 μM C_2-ceramide. These treatments increased the activity of PI 3-kinase by about five- to sixfold after 20 min (15). The increases in PI 3-kinase activity produced by these agonists involved a stimulation of tyrosine kinase activity since the effects were blocked by the tyrosine kinase inhibitors, genistein and PP1. Also the stimulation of PI 3-kinase activity was dependent upon an increase in the concentration of Ras-GTP after 5 min. which interacted with the p110 subunit of PI 3-kinase. Furthermore, the stimulation of PI 3-kinase activity by TNF-α, sphingomyelinase, and C_2-ceramide did not occur in fibroblasts expressing dominant/negative (N17) Ras (15). The increase in PI 3-kinase activity in the rat2 fibroblasts resulted in the stimulation of MAP kinase activity since this effect was blocked by compound, Ly 290024. The activation of PI 3-kinase and MAP kinase activities by ceramides and TNF-α was much more rapid than that observed in 3T3-L1 adipocytes.

Treatment of 3T3-L1 adipocytes with insulin stimulates both MAP kinases, and IRS-1-associated PI 3-K, which in turn activates pp70 S6 kinase (1). These events increase GLUT1 synthesis (16,17) and thus contribute to increased basal glucose transport. Our results show that the increase in GLUT1 concentrations and the stimulation of glucose uptake that is produced by C_2-ceramide is inhibited by PD 98059, Ly294002, or with rapamycin (14). These compounds block the activity of MAP kinase, PI-3K, and the stimulation of

pp70 S6 kinase, respectively. Cell-permeable ceramides therefore mimic some effects of TNF-α, and ceramides have insulin-like effects especially in stimulating basal glucose uptake. We identified a novel inhibition of insulin-stimulated glucose uptake that is downstream of PI 3-kinase which may result from an effect of ceramides in inhibiting the movement of GLUT4-containing vesicles to the cell surface.

The observations that TNF-α increased GLUT1 concentrations and basal glucose uptake (Refs. 18–20 and our unpublished work) are compatible with the effect of TNF-α in stimulating the peripheral uptake of glucose *in vivo* (21). Furthermore, GLUT1 expression in plasma membranes prepared from skeletal muscle of obese diabetic SHR rats is increased by 40% compared to the lean genotype (22). The idea that TNF-α and ceramides exacerbate insulin resistance may seem paradoxical if these agents, on their own, stimulate glucose uptake and lipid synthesis. However, the problem with TNF-α is that despite an increase in noninsulin-stimulated (basal) glucose uptake, the insulin-resistant tissue is unable to respond with sufficient glucose uptake to dispose of a post-prandial glucose load effectively. This may predispose to hyperglycemia and hyperinsulinemia after meals. It is interesting to speculate that the increased glucose uptake in adipocytes that is produced by TNF-α is designed to enable the cells to store lipids effectively in conditions of semistarvation when insulin concentrations are low. This may have provided an evolutionary advantage when food is in limited supply during many seasons of the year. However, this condition with its attendant insulin resistance becomes a health risk in obesity and type 2 diabetes in affluent societies. Our work provides further mechanisms for explaining the effects of TNF-α and ceramides in decreasing the importance of insulin as a regulator of metabolic balance in adipose tissue.

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Magnetic Resonance Imaging-Based Balance Analysis of Linoleate Utilization During Weight Loss in Obese Humans

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Fatty acid balance analysis in animals demonstrates that, despite apparently adequate linoleate intake (>2% of energy), impaired weight gain due to energy deficit selectively impairs whole-body accumulation of linoleate by increasing its β-oxidation (1). No methods have been described by which linoleate balance could be measured in living humans, but this would be helpful in determining how energy balance affects metabolism and β-oxidation of polyunsaturates. Exercise increases fat oxidation (2) and reduces skeletal muscle phospholipid content of linoleate (3), but weight loss does not appear to affect the adipose tissue content of linoleate (4). In this study, we assessed the potential application of magnetic resonance imaging (MRI) to indirectly measure whole-body changes in linoleate levels in humans in order to estimate whole body β-oxidation of linoleate during weight loss.

Adipose tissue needle biopsies and whole-body MRI scans were obtained before and after voluntary weight loss averaging 12 kg over 16 wk in obese humans (starting body mass indexes of 33–34). Weight loss was achieved by diet only (n = 11) or diet plus exercise (n = 9). Linoleate intakes were determined from food records maintained throughout the study. Whole body linoleate content was determined from the percentage of linoleate in the biopsies plus literature values for linoleate in lean tissue multiplied by actual whole-body lean and adipose tissue weights obtained by MRI (5). Linoleate excretion and the density and water content of adipose tissue were also measured.

Average linoleate intake across both groups was 6 g/d or 3.2% of energy intake. Linoleate excretion was 4% of intake. During weight loss, whole-body linoleate depletion exceeded linoleate intake by about 60% in both groups leading to net β-oxidation of linoleate (Table 1). Despite similar weight loss in both groups, the percentage of linoleate in adipose tissue was significantly lower after weight loss only in the diet plus exercise group (12.0 vs. 12.8% before weight loss; $P < 0.05$), leading to 35% higher β-oxidation of linoleate in the diet plus exercise group compared to the diet only group (Table 1). The percentage of n-6 long chain polyunsaturates in adipose tissue did not change during weight loss. Linoleate intake during weight loss did not predict adipose tissue linoleate values (percentage or kg) or β-oxidation of linoleate. Linoleate in adipose tissue (final percentage or change in percentage during weight loss) also did not predict the measured change in body linoleate content during weight loss.

TABLE 1
Whole-Body Linoleate Balance (kg) (over 16 wk) in Obese Humans Undergoing Weight Loss by Diet Alone or by Diet plus Exercise[a]

	Diet only (kg)	Diet plus exercise (kg)
Intake	0.54 ± 0.20	0.76 ± 0.20
Excretion	0.02 ± 0.01	0.03 ± 0.02
Accumulation[b]	−0.90 ± 0.46	−1.18 ± 0.76
β-Oxidation[c]	−1.42 ± 0.44	−1.91 ± 0.69

[a]Values are mean ± SD kg linoleate; n = 11 (Diet only), n = 9 (Diet plus exercise); no statistically differences between groups.
[b]The negative values indicate a loss from body stores. These measurements assume negligible desaturation of linoleate to longer chain n-6 polyunsaturates during energy deficiency (6).
[c]Calculated by difference (intake −[excretion + accumulation]).

Thus, β-oxidation of linoleate during weight loss in obese humans was not related to linoleate intake but was directly proportional to weight loss if accompanied by exercise. Adipose tissue levels of linoleate were reduced only if exercise was a component of energy deficiency. These results suggest that exercise contributes to selective linoleate depletion from adipose tissue during weight loss. This methodology involving a combination fatty acid analysis of fat biopsies and MRI scanning of whole-body changes in total lean and adipose tissue permits analysis of the utilization of linoleate in living humans.

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Abbreviation: MRI, magnetic resonance imaging.

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Diabetes Puts Myocardial n-3 Fatty Acid Status at Risk in the Absence of Supplementation in the Rat

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Diabetes is associated with high cardiovascular disease morbidity and mortality. Excess incidence of myocardial infarction and heart failure includes not only components of vascular disease and hypertension but also an independent diabetic cardiomyopathy (1). Polyunsaturated fatty acid (PUFA) metabolism is also perturbed in diabetes with inhibition of desaturase activity (2), resulting in reduced desaturation of linoleic acid (18:2n-6) to its long-chain derivative arachidonic acid (20:4n-6). The long-chain n-3 PUFA eicosapentaenoic acid (20:5n-3, EPA) and docosahexaenoic acid (22:6n-3, DHA) are increasingly being associated with reduced cardiovascular disease mortality (3). The n-3 PUFA are subject to the same metabolic pathways as the n-6 PUFA in a competitive manner and since the provision of adequate long-chain n-3 PUFA from shorter-chain precursors is only poorly met under normal conditions, diabetes may impact adversely upon n-3 PUFA composition of the heart, thereby putting the heart and heart function at risk (4).

This study investigates the effect of diabetes on myocardial membrane fatty acids and the ability of purified PUFA to modify incorporation and influence ischemia-induced cardiac arrhythmias, a physiological endpoint that varies with myocardial membrane composition (4).

METHODS

Male rats (Hooded Wistar, 10-wk-old) made diabetic with streptozotocin (50 mg/kg i.p.) were fed diets with 5% total fat as olive oil (OO) or 4.5% OO plus 0.5% (1.1% energy) of purified PUFA ethyl esters (18:3n-6, γ-linolenic acid; EPA; or DHA; or a mix containing 26% EPA + 37% DHA (n-3 Mix). Ischemia was produced by acute coronary artery ligation (4)

RESULTS

After 5 wk of diabetes, little change was seen in membrane levels of arachidonic acid or linoleic acid, but DHA fell to almost half its control level ($P < 0.0001$, analysis of variance, Bonferroni) in OO-fed rats (Table 1). The ratio of n-6/n-3 PUFA was significantly elevated in diabetes to almost double that of nondiabetic OO-fed animals.

All n-3 diets lowered arachidonic acid, raised EPA levels, and markedly raised DHA levels in myocardial membranes of nondiabetic animals, and the n-6/n-3 ratio was reduced to less than half the level found in OO-fed animals (all $P < 0.0001$). DHA levels were significantly higher with the DHA and n-3 Mix diets than with purified EPA ($P < 0.001$).

TABLE 1
Content of Major Polyunsaturated Fatty Acids in Myocardial Phospholipid of Nondiabetic and Diabetic Rats Fed Different Fats[a]

	Dietary group									
	OO	OO/d[b]	GLA	GLA/d	EPA	EPA/d	DHA	DHA/d	Mix	Mix/d
Fatty acid	(% of total fatty acids)									
18:2n-6	11.90 ± 0.47	13.01 ± 0.72	8.19 ± 0.64	10.48 ± 1.11	14.86 ± 0.67	13.16 ± 1.22	13.97 ± 1.04	11.46 ± 1.08	11.91 ± 1.39	12.68 ± 1.68
20:4n-6	24.20 ± 0.49	22.68 ± 1.27	25.50 ± 0.51	27.63 ± 0.53	16.10 ± 0.61	18.06 ± 0.81	13.32 ± 0.94	15.72 ± 0.88	19.04 ± 0.90	18.04 ± 0.94
20:5n-3	0.08 ± 0.08	0.12 ± 0.12	0.18 ± 0.11	0.13 ± 0.11	1.95 ± 0.06	1.16 ± 0.14	0.48 ± 0.13	0.09 ± 0.09	0.72 ± 0.06	0.68 ± 0.18
22:6n-3	9.66 ± 0.80	4.94 ± 0.20	8.15 ± 0.60	4.36 ± 0.50	14.65 ± 0.40	9.19 ± 1.32	18.00 ± 0.75	20.01 ± 0.91	18.08 ± 0.86	13.79 ± 1.20

[a]The results are presented as the mean of five hearts ± SEM.
[b]d, diabetic; EPA, eicosapentaenoic acid; DHA, docosahexaenoic acid; OO, olive oil; GLA, γ-linolenic acid.

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Abbreviations: DHA, docosahexaenoic acid; EPA, licosapentaenoic acid; OO, olive oil; PUFA, polyunsaturated fatty acid.

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Arrhythmias were significantly inhibited by DHA (arrhythmia score 2.08 ± 0.62, $n = 12$) and n-3 Mix diet (2.25 ± 0.58, $n = 12$) compared with the OO diet (4.77 ± 0.52, $n = 13$) in control animals ($P < 0.01$).

In diabetes, the level of DHA incorporation into myocardial membranes fell in the EPA group to the level seen in nondiabetic OO-fed animals, and DHA also fell significantly with the n-3 Mix diet ($P < 0.0001$). The DHA diet maintained high myocardial DHA levels in diabetes. Arrhythmia severity was depressed during acute myocardial ischemia in diabetic animals (OO: 3.00 ± 0.39, $n = 12$), and arrhythmia severity was further reduced only with the DHA diet (1.09 ± 0.58, $n = 11$, $P < 0.01$).

CONCLUSIONS

The changes in fatty acid incorporation into myocardial membranes that occurred with acute diabetes in this study were indicative of depressed PUFA desaturation. However this was largely confined to the long-chain n-3 PUFA, DHA. Anti-arrhythmic effects of n-3 PUFA diets were associated with elevated DHA levels. It is concluded that perturbed PUFA metabolism in diabetes puts myocardial n-3 PUFA status at high risk which may have important implications for heart function and that this may be prevented by low-level supplementation with purified DHA.

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Neuroprotective Effect of Fish Oil in Diabetic Neuropathy

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Diabetic neuropathy is a degenerative complication of diabetes accompanied by an alteration of nerve conduction velocity and Na,K-ATPase activity. This decrease in Na,K-ATPase activity has been implicated in the pathogenesis of electrophysiological abnormalities characterizing neuropathic complication in different animal models and in humans (1). Na,K-ATPase is a membrane-bound enzyme consisting of three catalytic isoenzymes ($\alpha 1$, $\alpha 2$, $\alpha 3$) and a large lipid core (2). It plays a fundamental role in Na^+ and K^+ transport by maintaining the ionic gradients necessary for nervous excitability. A decrease in this process leads to an increase in the intracellular concentration of sodium and a corresponding decrease in membrane sodium potential. The mechanism responsible for impairment of Na,K-ATPase during hyperglycemia is still unclear, but alterations of the kinetic parameters of the enzyme and/or of subunit expression are likely (3,4). In recent years there has been great interest in the effect of n-3 essential fatty acids, found particularly in fish oil, on the prevention of atherosclerosis and hypertension in animal models and patients with vascular disease (5). Fish oil treatment as MaxEPA was recently reported to improve vascular function in diabetic patients (6), and prevent $\alpha 2$ isoenzyme activity of Na,K-ATPase in diabetic cardiomyopathy (7).

Aim of the study. The present study in rats was designed, first, to measure diabetes-induced abnormalities in Na,K-ATPase acyivity, isoenzyme expression, fatty acid content in sciatic membranes, and nerve conduction velocity and second, to assess the preventive ability of a fish-oil rich diet (rich in n-3 fatty acids) on these abnormalities.

Methods. Diabetes was induced by intravenous streptozotocin injection. Diabetic animals (D) and nondiabetic control animals (C) were fed the standard rat chow either without supplementation or supplemented with either fish oil (DM, CM) or olive oil (DO, CO) at a daily dose of 0.5 g/kg by gavage during 8 wk.

Results. Analysis of the fatty acid composition of purified sciatic nerve membranes from diabetic animals showed a decreased incorporation of 16:1n-7 fatty acid and arachidonic acid. Fish oil supplementation changed the fatty acid content of sciatic nerve membranes, decreasing 18:2n-6 fatty acid and preventing the decrease of arachidonic acid and 18:1n-9 fatty acid. Protein expression of Na,K-ATPase α-subunits, Na,K-ATPase activity, and ouabain affinity were assayed in purified sciatic nerve membranes from CO, DO, and DM. Na,K-ATPase activity was significantly lower in sciatic nerve membranes of diabetic rats and significantly restored in diabetic animals that received fish oil supplementation. The Na,K-ATPase activity correlated with the nerve conduction velocity. Diabetes induced a specific decrease of $\alpha 1$ and $\alpha 3$ isoform activity and protein expression in sciatic nerve membranes. Fish oil supplementation restored partial activity and expression to varying degrees depending on the isoenzyme. These effects were associated with a significant beneficial effect on nerve conduction velocity.

Conclusion. These findings suggest that the lipid metabolism which is affected by diabetes and fish oil supplementation modulates the activity and level of the $\alpha 1$ and $\alpha 3$ isoenzymes. This study indicates that fish oil has beneficial effects on diabetes-induced alterations in sciatic nerve Na,K-ATPase activity and function.

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Abbreviations: C, control animals; CM, C with fish oil; CO, C with olive oil; D, diabetic animals; DM, D with fish oil; DO, D with olive oil.

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Hyperglycemia Inhibits Liver Fatty Acid Oxidation and Increases Triacylglycerol Secretion in Humans

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Hypertriglyceridemia is the most common abnormality associated with insulin resistance and a strong predictor of coronary heart disease mortality in NIDDM patients. Other pathological responses, such as alterations of the coagulation system and pancreatitis, have also been attributed to hypertriglyceridemia. However, the mechanisms responsible for the development of hypertriglyceridemia in insulin-resistant states remain unclear. The goal of the present study was to investigate the role of moderate hyperglycemia in the development of hypertriglyceridemia.

METHODS

Five healthy volunteers were studied in the post-absorptive state (basal) and, on a different occasion, during a 15-h dextrose infusion (hyperglycemic clamp). Catheters were inserted in a peripheral vein for tracer infusion and a femoral artery and a hepatic vein for determination of splanchnic fatty acid kinetics and oxidation. [U-^{13}C]-fatty acids were infused to measure fatty acid appearance and oxidation, whereas *in vivo* labeled [^{13}C] very low density lipoprotein–triglycerides (VLDL–TG) were infused to measure the rate of TG secretion. The VLDL–TG tracer was produced 3–4 d prior to the infusion studies by ingestion of [U-^{13}C]-glycerol followed by plasmapheresis 4 h later. VLDL–TG were isolated out of ~450 cc of plasma, under sterile conditions, and were stored until the day of the study. Lipids and heparin were infused in the clamp experiments only to maintain plasma fatty acid concentration constant. Splanchnic blood flow was measured by infusion of Indocyanin Green.

RESULTS

Plasma glucose concentration increased from 4.8 ± 0.2 in the basal to 8.8 ± 0.5 mM during the clamp ($P < 0.05$), whereas insulin increased from 5.5 ± 1.1 to 35.8 ± 11.4 μU/mL ($P < 0.05$). Glucose balance across the splanchnic region switched from net release (basal; 9 ± 1 mg/dL) to net uptake (clamp; 36 ± 12 mg/dL, $P < 0.05$). The rate of appearance of fatty acids was maintained constant in the basal and clamp experiments (4.8 ± 0.6 and 4.4 ± 0.6 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively). This is crucial, as the primary goal of the present study was to isolate the effects of hyperglycemia on splanchnic fatty acid kinetics and oxidation and the rate of TG secretion. Splanchnic fatty acid oxidation decreased significantly during the clamp (0.38 ± 0.04 and 0.2 ± 0.05 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ during basal and clamp, respectively; $P < 0.05$). The decrease in splanchnic fatty acid oxidation was not related to a decline in free fatty acid (FFA) availability, as the absolute FFA uptake by the splanchnic region was similar in the basal and clamp experiments (0.7 ± 0.3 and 0.7 ± 0.5 $\mu mol \cdot kg^{-1} \cdot min^{-1}$, respectively). Ketone production was 1.5 ± 0.3 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ in the basal state but dropped to undetectable levels during the clamp, suggesting complete inhibition of ketone body production during hyperglycemia. Interestingly, ketone body production was nearly completely inhibited during hyperglycemia even though splanchnic fatty acid oxidation to CO_2 was proceeding at a significant rate.

The rate of VLDL–TG appearance in plasma increased from 0.35 ± 0.07 in the basal state to 0.53 ± 0.11 $\mu mo \cdot kg^{-1} \cdot min^{-1}$ during the clamp ($P < 0.05$). Plasma-derived fatty acids contributed 91 ± 10% of the fatty acids for VLDL–TG formation in the basal state and 70 ± 22% during the clamp ($P < 0.05$). Nonplasma-derived fatty acids contributed the remaining FFA. Plasma VLDL–TG concentration increased from 0.28 ± 0.06 to 0.43 ± 0.05 mM ($P < 0.05$). The increase in plasma VLDL–TG concentration during the clamp appeared to be mostly due to the increased VLDL–TG secretion as the clearance of VLDL–TG did not change significantly.

DISCUSSION

It is concluded that moderate hyperglycemia increases glucose uptake and oxidation across the splanchnic region. Increased glucose oxidation, in turn, inhibits hepatic fatty acid oxidation even in the setting of constant fatty acid uptake. Inhibition of fatty acid oxidation increases the availability of fatty acids for TG synthesis, resulting in accelerated TG production rate.

These data provide a potential mechanism for the observed hypertriglyceridemia in conditions where glucose uptake and oxidation by the liver are enhanced (e.g., upper body obesity, NIDDM, and high-carbohydrate diets). In all of the above-mentioned conditions, increased availability of glucose for oxidation is coupled with normal or elevated systemic fatty acid availability, thereby creating a metabolic profile favoring enhanced incorporation of fatty acids into TG.

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Abbreviations: FFA, free fatty acid; VLDL–TG, very low density lipoprotein–triglyceride.

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Effects of 3-Thia Fatty Acids on β-Oxidation and Carnitine Palmitoyltransferase-I Activity in Cultured Hepatocytes

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3-Thia fatty acids are fatty acid analogs in which an S atom replaces the β-methylene group in the chain. Consequently, they cannot be β-oxidized. When fed to rats, they induce a decrease in plasma triglycerides and cholesterol already after 3 h, mainly by stimulating mitochondrial β-oxidation and decreasing triacylglycerol secretion, followed by an increase in activity of peroxisomal β-oxidation enzymes after ~12 h.

When 3-thia fatty acids are added to isolated hepatocytes, they are shown to stimulate fatty acid oxidation and to inhibit fatty acid synthesis. The strength of this effect depends on the chain length of 3-thia fatty acid used. Dodecylthioacetic acid (DTA) and tetradecylthioacetic acid are most potent, shorter and longer 3-thia fatty acids having reduced effects. Hepatocytes from both fasted and fasted-carbohydrate refed rats are responsive toward 3-thia fatty acids, although the effect is more pronounced in the latter.

When hepatocytes are transferred to cell culture, both their palmitic acid oxidation rate and carnitine palmitoyltransferase-I (CPT-I) activity (the rate-limiting enzyme in β-oxidation pathway) decrease rapidly. Addition of DTA increases the oxidation rate of palmitate, but not of shorter fatty acids like octanoate. CPT-I activity is stimulated by DTA in a similar manner as palmitate oxidation. Normal fatty acids, e.g., palmitic acid, have some effect on CPT-I activity, but only when added in high concentration. This activation is most likely caused by free DTA, and not by its CoA ester. The activation of CPT-I is very fast (maximum is reached after 5–10 min), and if the thia fatty acid is removed from the medium the CPT-I activity returns to the low level within 2 h.

We conclude that the 3-thia fatty acids activate a latent CPT-I enzyme since the activation is too fast to be explained by enzyme synthesis (gene transcription). Apparently we can exclude the involvement of malonyl-CoA, because of the similarity between hepatocytes from fasted and fasted-refed animals.

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Abbreviations: CPT-I, carnitine palmitoyltransferase-I; DTA, dodecylthioacetic acid.

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Polyunsaturated Fatty Acids and Breast Cancer

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To address the role of dietary lipids in mammary carcinogenesis and in breast cancer and metastases development, we studied the lipid composition of adipose breast tissue as an indicator of past dietary fatty acid intake in patients with benign or malignant breast tumors. Using a case-control approach, we found α-linolenic acid (18:3n-3) to be inversely related to the risk of breast cancer. We confronted the lipid profile to breast cancer clinical presentation and posttreatment evolution. We found that the level of 18:3n-3 was inversely related to risk of developing metastases in breast cancer patients. The potential role of conjugated isomers of 18:2n-6 (CLA) in breast cancer is currently being evaluated with similar observational method. Preliminary data indicate a strong protective effect of CLA against breast cancer.

Using an animal system of *N*-methylnitrosourea-induced mammary tumors, we found that dietary 18:3n-3 (rapeseed oil) or n-3 polyunsaturated acid (PUFA) (fish oil) inhibited tumor development and growth only in the absence of the antioxidant vitamin E. This inhibition was even more pronounced in the presence of prooxidants. Such effects were not found when lipid diet was low in PUFA. Tumor cell loss may be involved, because tumor cell proliferation was not affected. These data suggest that PUFA inhibit tumor growth because they are lipid substrates to peroxidation, an intermediate step toward cell death.

Similar studies were made to address the effect of PUFA on tumor response to anticancer agents. We found that the responsiveness of breast malignant tumors to induction chemotherapy was high when the level of docosahexaenoic acid (DHA, 22:6n-3) in stored lipids of patients was elevated. Using cultured breast cancer cell lines, we observed that addition of DHA to medium increased the toxicity of anticancer drugs that generate an oxidative stress and also increased cell lipid peroxides. The effect was lost when antioxidant was added. *In vivo*, rat mammary tumors were sensitized to epirubicin when fish oil and oxidants were provided in the rat diet. We believe that tumor growth-inhibiting lipoperoxides are generated during drug action, provided that DHA substrate is available to tumor cells, thus leading to increased drug efficacy.

These studies demonstrate the importance of dietary components in the genesis and development of breast cancer. Precise identification of their role should permit their use as nutritional targets in therapy and prevention of cancers.

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Abbreviations: CLA, conjugated linoleic acids; DHA, docosahexaenoic acid; PUFA, polyunsaturated fatty acid.

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Altered Lipid Metabolism Associated with the Progression of Premalignant Lesions in Rat Liver

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Altered lipid metabolism has been associated with cellular growth, altered cell function, and the development of premalignant lesions in rat liver. Fatty acid (FA) changes have a wide range of effects regarding the integrity of cellular membranes that are known to affect the membrane structure, the activity of membranal enzymes, and the affinity of growth factor receptors. Membranal FA also play a role as second messengers during signal transduction and cell regulation *via* the activation of protein kinase C (PKC), mitogen-activated protein kinase (MAP kinase), and generation of ceramide. FA also play a determining role in the regulation of prostaglandin production and decreased lipid peroxidative activity generally observed in tumor tissue. In the present study, alterations in lipid metabolism were monitored in rat hepatocyte nodules by comparing the lipid profiles of nodular tissue with surrounding and control tissue as a function of time by harvesting the nodules (1, 3, 6, and 9 mon after induction) as well as the surrounding and control tissue. As cellular proliferation is an important determinant in cancer development, tissue fractions obtained from hepatectomized rats were included as a control for normal liver regeneration. In the nodular tissue, the concentrations of phosphatidylethanolamine (PE) and cholesterol increased significantly compared to control and surrounding tissue. Phosphatidylcholine (PC) was also increased, but only significantly at 1 mon. This resulted in a decrease of the PC/PE ratio. Similar changes were observed in the regenerating liver, with the exception of PC which tended to decrease. The nodular membrane fluidity was significantly decreased at months 1, 3 and 6, but increased at month 9. In the regenerating liver, the membrane fluidity was significantly increased at days 1, 2, and 7. The levels of the different n-6 and n-3 FA in PC and PE were analyzed quantitatively and qualitatively. In the nodular tissue, 18:1n-9 and 18:2n-6 increased in PE and PC while 20:4n-6 decreased in PC and increased quantitatively in PE only over the 9-mon period. 22:5n-6 and 22:6n-3, the end products of the n-6 and n-3 metabolic pathways, respectively, decreased qualitatively and quantitatively in PE and PC. The FA profiles of PC and PE of the surrounding tissue reflected the pattern of the untreated control rat livers. In the regenerating liver, the FA profiles of PC and PE showed the same pattern as described for the hepatocyte nodules, except for a qualitative and quantitative decrease of 18:1n-9 in PC and PE. These results indicated that the lipid parameters associated with increased cellular proliferation in hepatocyte nodules closely mimic normal regeneration in the liver. However, a determining event is the persistence of the lipid changes associated with the nodules compared to the reversion of these changes in regenerating liver. This implies that nodular lipid metabolism escapes the normal regulatory mechanisms required for controlled regeneration and homeostasis. The above changes in the nodule lipid parameters suggest various paths in which FA can play a role in the persistent cellular proliferation known to prevail in these lesions. The increase in nodular PE led to an increase in the level of 20:4n-6 which, in its capacity as second messenger, can influence cellular proliferation *via* increased levels of prostaglandin E_2, cAMP, activation of PKC and phosphorylation of phospholipase A_2 *via* MAP kinase. 20:4n-6 can also play a role in apoptosis *via* ceramide generation and indirectly through the deregulation of c-myc expression; however, in hepatocyte nodules the FA appears to be one of the major players in the progression of these lesions into neoplasia. The increased level of 18:1n-9, a FA with antioxidative properties, as well as the decreased levels of the PUFA have been associated with the decreased lipid peroxidation level in hepatocyte nodules. The resultant decrease in peroxidative metabolites, which are known to induce apoptosis, could affect the apoptotic process in the nodular environment. The altered phospholipid and FA profiles in the hepatocyte nodules could provide a unique opportunity to alter the lipid parameters by dietary manipulation in order to modulate the increased cellular proliferation and the progression of these lesions into neoplasia.

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Abbreviations: FA, fatty acid; MAP, mitogen-activated protein; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PKC, protein kinase.

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Reversal of Tumor Cell Drug Resistance by Essential Fatty Acids

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Tumor cell drug resistance is a major problem in cancer chemotherapy. Several tumors initially respond favorably to chemotherapy, but subsequently develop multidrug resistance (MDR). The development of this MDR state is due to increased efflux and decreased influx of anticancer drugs into the tumor cells. This leads to a decrease in the accumulation of anticancer drugs inside the tumor cells and hence decreased response to their cytotoxic action. Further, the antioxidant status of the tumor cells and the efflux and influx of anticancer drugs which depends on the cell membrane properties can also be related to tumor cell drug resistance. It is known that cell membrane properties depend on their lipid composition.

It is known that essential fatty acids and their metabolites are cytotoxic to a variety of tumor cells *in vitro* and *in vivo* (1–5). But the effect of these fatty acids on tumor cell drug resistance is not well documented.

In our study, it was noted that γ-linolenic acid (GLA) and eicosapentaenoic acid (EPA) potentiated the cytotoxicity of anticancer drugs vincristine, *cis*-platinum, and doxorubicin on human cervical carcinoma cells *in vitro* by enhancing the uptake of anticancer drugs. In addition, docosahexaenoic acid (DHA), EPA, GLA, and dihomo γ-linolenic acid were found to by cytotoxic to both vincristine-sensitive and -resistant human cervical carcinoma cells *in vitro*. These fatty acids enhanced the uptake and inhibited the efflux of vincristine and thus enhanced the intracellular concentration of the drug. Further, these fatty acids could decrease the levels of various antioxidants in the tumor cells. In an extension of this study, it was also noted that pre-incubation of vincristine-resistant cells with suboptimal doses of fatty acids enhanced the cytotoxic action of vincristine. Fatty acid analysis showed that vincristine-resistant tumor cells have low amounts of α-linolenic acid, GLA, and DHA in comparison to the sensitive cells. These results suggest that essential fatty acids and their metabolites can reverse and/or inhibit tumor cell drug resistance at least *in vitro*.

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Abbreviations: DHA, docosafexaenoic acid; EPA, eicosapentaenoic acid; GLA, γ-linolenic acid; MDR, multidrug resistance.

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Protection from Chemotherapy-Induced Alopecia by Docosahexaenoic Acid

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Chemotherapy-induced alopecia is a common side effect of many chemotherapeutic drugs used for the treatment of cancer. Several methods currently utilized to prevent chemotherapy-induced alopecia are unsatisfactory. Research of new treatments has been hampered, in part, by the lack of a suitable and reproducible experimental animal model to determine the cellular target of anticancer drugs in the hair follicle and how the interaction of these drugs with that target leads to hair loss. Recently made observations in the young rat model (1) have provided a new insight into this problem and opened new avenues for further investigation. Using this model, Cece *et al.* (2) reported that chemotherapy treatment induced apoptosis in a hair follicle cell. Thus we examined whether docosahexaenoic acid (DHA), which had been found to have a modulator effect on neural apoptosis, could prevent chemotherapy-induced alopecia in this model.

Lactating Sprague-Dawley rats with 10 newborns/mother were purchased. In these experiments, 8-d-old rats were used for cytosine arabinoside (Ara-C)-induced alopecia, and 11-d-old rats were used for etoposide (VP-16)-induced alopecia. All chemotherapy was given i.p., and DHA was given p.o. Degree of alopecia was assessed on the 17-d-old (Ara-C) or 20-d-old (VP-16) animals.

The results are as follows: (i) all rats in the chemotherapy-treated group became totally alopecia; (ii) in these alopecia-induced groups, apoptosis was detected by TUNEL staining method in skin sections; (iii) in contrast, most rats in the DHA-treated group were protected from chemotherapy-induced alopecia; (iv) recombinant human interleukin1α (rhIL1α) or intrinsic IL1 inducer, lipopolysaccharide, also could strongly protect alopecia; and (v) the effect of DHA could not be neutralized by IL1-receptor antagonist.

In conclusion, it is suggested that DHA protection against chemotherapy-induced alopecia could be mediated either directly or indirectly through apoptosis modulation without involving IL1 systems.

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Abbreviation: DHA, docosahexaenoic acid; IL, interleukin.

Proliferation and Types of Killing of Leukemia Cell Lines By Very Long Chain Polyunsaturated Fatty Acids

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Polyunsaturated fatty acids (PUFA) may reduce cell multiplication in cultures of normal (1) as well as transformed (2,3) white blood cells. We assessed the sensitivity of 14 different leukemia cell lines to PUFA by measuring cell number after 3 d of incubation. Ten of the examined cell lines were sensitive to 30, 60, and/or 120 mM of arachidonic, eicosapentaenoic (EPA), and docosahexaenoic acid (DHA), whereas four cell lines were resistant. The sensitivity to PUFA was not associated with any particular cell lineage, clinical origin, or specific mRNA pattern of bcl-2 and c-myc. Effects on cell viability were assessed by studying cell membrane integrity, DNA fragmentation, and cell morphology. The sensitive cell lines Raji and Ramos died by necrosis and apoptosis, respectively, during incubation with EPA, whereas the viability of the resistant U-698 cell line was unaffected. The effects of EPA on Raji cells were counteracted by vitamin E, indicating that lipid peroxidation may be involved. However, apoptosis induced by EPA in Ramos cells was unaffected by vitamin E as well as eicosanoid synthesis inhibitors. Thus, our results indicate that a majority of leukemia cell lines are sensitive to PUFA. This sensitivity may be caused by induction of apoptosis or necrosis by very long-chain PUFA.

For further analyses the monoblastic cell line U-937-1 was cultured in the presence of EPA or oleic acid (OA, 18:1n-9). EPA caused a dose-dependent inhibition of cell growth, whereas OA was not inhibitory at any concentration tested. At the highest EPA concentrations used, 120 and 240 mM, inhibition of cell growth was accompanied by initiation of apoptosis, as shown by microscopy after staining with propidium iodide and Hoechst 33342. At 60 mM concentration, EPA caused a 35% reduction in cell proliferation without inducing apoptosis and was therefore used for further studies. The addition of antioxidants or inhibitors of eicosanoid synthesis had no influence on the reduced cell growth with EPA. Furthermore, the inhibition required the continuous presence of EPA in the incubation medium. The cells resumed a normal proliferation rate when they were placed in EPA-free medium. The inhibition of proliferation was not accompanied by differentiation of the cells into macrophage-like cells, as the expression of serglycin and the ability to perform respiratory burst were not affected by EPA. The expression of mRNA for CD23 increased with EPA, but to a smaller extent than after incubation with retinoic acid or phorbol myristate acetate. Furthermore, the cell surface expression of the antigens CD36 and CD68 was lower in cells treated with EPA or OA compared to untreated cells. The cell cycle distribution of U-937-1 cells was similar in cells treated with EPA and phorbol myristate acetate, whereas retinoic acid-treated cells accumulated in the G1 phase of the cell cycle. However, when the cells were subjected to side scatter and forward scatter analyses during flow cytometry, those treated with EPA and OA showed an increased side scatter. Accumulation of lipid droplets could be demonstrated by electron microscopy, and the number of droplets per cell was higher in cells exposed to EPA than OA. The cells showed 5.5- and 15.5-fold increases in the content of triacylglycerol after OA and EPA treatment, respectively, whereas there was no difference in the cellular content of cholesterol. The triacylglycerol fraction in EPA-treated cells contained high amounts of EPA, and OA-treated cells had high levels of OA. No inhibition of cell growth or accumulation of lipid droplets was observed in U-937-1 cells treated with a sulfur-substituted EPA. The accumulation of EPA-rich triacylglycerols with 60 mM EPA was correlated to inhibition of cell growth and was unaffected by antioxidants or eicosanoid inhibitors, and without any initiation of apoptosis. This may indicate the existence of other mechanisms for regulation of cell behavior than the well-established lipid peroxide and eicosanoid pathways.

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Abbreviations: EPA, eicosapentaenoic acid; OA, oleic acid; PUFA, polyunsaturated acid.

Cytotoxic Drug Efficacy Correlates with Adipose Tissue Docosahexaenoic Acid Level in Locally Advanced Breast Carcinoma

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Experimental studies indicated that long chain n-3 polyunsaturated fatty acids (PUFA) may increase sensitivity of mammary tumors to several cytotoxic drugs. No data in humans are available concerning the potential influence of dietary fatty acids on chemosensitivity of tumors to cytotoxic drugs. In a previous study in breast cancer patients, we found that a decreased level of α-linolenic acid in adipose tissue, used as a biomarker of past qualitative dietary fatty acids, was associated with subsequent development of visceral metastases, suggesting that the type of fatty acids available to tumor cells may have influenced the outcome of breast cancer, by alterating the response of cancer cells to the initial treatment. The aim of the study was to examine the association between the level of individual fatty acids in the adipose breast tissue and the response of the breast tumor to primary chemotherapy, in 56 patients with locally advanced breast carcinoma.

Adipose breast tissues were obtained at the time of biopsy, and individual fatty acids were measured as a percentage of total fatty acids, using capillary gas chromatography. Patients then received primary chemotherapy, combining mitoxantrone, vindesine, cyclophosphamide, and 5-fluorouracil every 4 wk. Tumor size was reassessed after three cycles of chemotherapy. Tumor response was evaluated according to World Heath Organization criteria.

Complete or partial response to chemotherapy was achieved in 26 patients. Level of n-3 PUFA was higher in the group of patients with complete or partial response to chemotherapy than in patients with no response or with tumor progression ($P < 0.004$). Among n-3 PUFA, only docosahexaenoic acid (22:6n-3) was significantly associated with tumor response ($P < 0.005$). In a logistic regression model taking into account age, body mass index, and tumor size, 22:6n-3 level proved to be an independent predictor for chemosensitivity ($P = 0.03$).

These results suggest that, in breast cancer, 22:6n-3 may increase the response of the tumor to the cytotoxic agents used. Thus, dietary intervention could provide an effective means to increase 22:6n-3 availability in tumor tissues and thereby may affect chemosensitivity of tumors.

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Abbreviation: PUFA, polyunsaturated fatty acid.

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Effects of Eicosapentaenoic and Docosahexaenoic Acids Dietary Supplementation on Cell Proliferation and Apoptosis in Rat Colonic Mucosa

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n-3 Polyunsaturated fatty acids (PUFA) have been shown to inhibit chemical-induced carcinogenesis of colon in rats (Reddy, B.S., and Maruyama, H., *Cancer Res. 46*:3367–3370, 1986) and to normalize altered proliferative patterns of the colonic mucosa in human subjects at high risk for colon cancer (Anti, M., *Gastroenterology 103*, 883–891, 1992 and *Gastroenterology 107*, 1709–1715, 1994). To determine whether n-3 PUFA also influence the normal cellular turnover, we investigated the effects of dietary supplementation with eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), the two major n-3 PUFA, on cell proliferation, apoptosis, and crypt morphology in normal colonic mucosa. Moreover we analyzed some metabolic pathways such as fatty acid incorporation into membrane phospholipids and peroxisomal β-oxidation involved in the effects of n-3 PUFA. Sixty male inbred ACI/T rats were randomly divided into three groups of 20 each, receiving oleic acid (control), EPA, or DHA (1 g/kg body wt/d) as ethyl esters by gastric gavage for 10 d. Cell proliferation and apoptosis were examined by bromodeoxyuridine and by *in situ* nick end labeling staining, respectively; fatty acid composition of phospholipid (PL) classes by thin-layer chromatography and gas–liquid chromatography and the activity of palmitoyl-CoA oxidase, the limiting enzyme of peroxisomal β-oxidation, were measured fluorimetrically by a coupled peroxidase reaction in mucosal homogenate. Both EPA and DHA inhibited cell proliferation and induced apoptosis in normal colonic mucosa of rats, but they did not modify crypt morphology and the total number of cells per crypt. EPA and DHA treatment decreased arachidonic acid content in phosphatidylethanolamine (PE) and increased EPA in all PL classes. After DHA treatment, DHA itself increased in phosphatidylcholine (PC) and PE but not in phosphatidylinositol (PI). As a consequence of the modifications in fatty acid composition, n-6/n-3 PUFA ratio decreased in total PL, in PC and PE fractions of both EPA and DHA groups. On the contrary, n-6/n-3 PUFA ratio did not change in the PI fraction. Unsaturation index was not modified in total PL as well as in PL classes. A significant increase in peroxisomal β-oxidation (140% in EPA and 170% in DHA rats) was observed after both EPA and DHA supplementation. The increase in this metabolic pathway resulted in an overproduction of hydrogen peroxide. The changes in cell proliferation and apoptosis were related to changes in the composition of membrane PL and in fatty acid peroxisomal metabolism. The observation that arachidonic acid level is not affected in PC and PI suggests that eicosanoids are not involved in n-3 PUFA effects. Moreover, the induction of peroxisomal β-oxidation with a consequent increase in hydrogen peroxide production suggests that a mild increase in reactive oxygen species could be implicated in the inhibition of cell proliferation and in the induction of apoptosis by n-3 PUFA. Our finding that EPA and DHA did not modify crypt morphology and the total number of cells per crypt, although they altered cell proliferation and apoptosis in normal colonic mucosa, suggests a possible use of these fatty acids as dietary chemopreventive agents.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PL, phospholipids; PUFA, polyunsaturated fatty acids.

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Fatty Acid Composition in Serum Phospholipids and Risk of Breast Cancer: A Prospective Cohort Study in Northern Sweden

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Data derived from animal experiments indicated that diets rich in n-3 polyunsaturated fatty acids (PUFA) from marine origin inhibited tumor growth and metastasis. Prospective cohort studies generally provided weak support for the hypothesis that dietary intake of n-3 PUFA might protect against breast cancer. Conclusive evidence for a role of individual fatty acids in breast cancer risk may be precluded by the many methodological limitations in measurements of dietary intake of fatty acids. In this regard, the use of reliable markers of dietary fatty acids would be of major interest. Serum phospholipid fatty acid composition is known to reflect dietary concentrations for PUFA. Thus, the aim of the study was to evaluate the hypothesis of a possible protection of n-3 fatty acids against breast cancer in women, using fatty acid concentrations in serum phospholipids as a marker of dietary intake of PUFA, in a prospective cohort study in Umeå, Sweden.

Patients were selected from three cohort studies in the town of Umeå and its surroundings in northern Sweden. We examined the fatty acid composition of phospholipids in prediagnostic sera of 196 women who developed breast cancer, and of 388 referrents matched for age at recruitment, age of blood sample, and sampling center. Individual fatty acids were measured as a percentage of total fatty acids, using capillary gas chromatography.

Conditional logistic regression model was performed to obtain odds ratio and 95% confidence intervals, while adjusting for weight and height. Quartile cut points for fatty acids were determined based on the referrents, and the lowest quartile was used as the reference category.

We found no association between n-3 PUFA in serum phospholipids and risk of breast cancer. In the subgroup of postmenopausal women, we found an inverse association between the ratio stearic acid (18:0)/oleic acid (18:1n-9), which reflects Δ-9 desaturase activity, and risk of breast cancer. The adjusted relative risk of breast cancer for postmenopausal women in the highest quartile of the ratio 18:0/18:1n-9 was 0.38 compared to women in the lowest quartile (*P* trend = 0.019).

Our data do not support the hypothesis that dietary intake of n-3 PUFA might protect against breast cancer. The inverse association between the ratio 18:0/18:1n-9 and risk of breast cancer in postmenopausal women may suggest an increase in breast cancer in women with high activity of the enzyme Δ-9 desaturase, which may reflect an underlying metabolic profile, characterized by insulin resistance and chronic hyperinsulinemia.

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Abbreviation: PUFA, polyunsaturated fatty acid.

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Essentiality of Docosahexaenoic Acid in Retina Photoreceptor Cell Development

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Unless docosahexaenoic acid (DHA, 22:6n-3) is added, retina photoreceptor cells in culture undergo a degeneration process that leads to their massive death in 2 wk (1). We recently showed that this occurs by the activation of an apoptotic pathway and that DHA provides against such event, postponing the onset of apoptosis and slowing down its progression (2). Our objective in this project is to investigate the factors associated with this action of DHA. Here we present the effect of diverse fatty acids including DHA on lipid composition and metabolism and on the apoptosis and differentiation of photoreceptors.

Rat retina neuronal cultures prepared from 1–2-d-old rats (1,3) were incubated in a chemically defined serum-free medium, in the absence and in the presence of DHA and other fatty acids (20:4, 18:1, 16:0), unlabeled and radioactive. Cells were identified by morphological and immunocytochemical techniques, and apoptosis was determined using nuclear markers (2).

In cells incubated with DHA, its proportion in lipids increased four- to sixfold, in contrast to other fatty acids whose addition did not affect the cell lipid composition. However, all labeled fatty acids were actively incorporated and esterified in neuronal lipids, particularly in phosphatidylcholine, showing that efficient mechanisms exist for the uptake of each. Apart from this common feature the distribution of radioactivity among lipids varied, showing a characteristic profile for each fatty acid. The addition of unlabeled DHA to cells did not affect the incorporation of labeled 16:0 or 18:1 in phosphatidylcholine but significantly decreased that of 20:4. Retinal neurons thus have specific mechanisms for handling fatty acids of different length and unsaturation, the incorporation of DHA taking priority in this phase of development. This may contribute to the high proportion of DHA-enriched phospholipids found in retinal neurons. Furthermore, neurons may have specific mechanisms for handling DHA. While there was a net uptake of all fatty acids tested when added as radioactive substrates, only DHA was able to modify the lipid acyl chain composition when supplied as unlabeled fatty acid. This suggests that, whereas other fatty acids are incorporated by deacylation–reacylation of preexisting phospholipid molecules, DHA is also used for the synthesis of new molecular species of phospholipids.

Of all fatty acids tested, DHA was not only the only one effective in preventing apoptosis but also the most competent in promoting photoreceptor cell differentiation. In control cultures, photoreceptors have a small round body, a single neurite and some of them develop a *cilium*, but none show the characteristic outer segment these cells have *in vivo*. DHA supplementation led to the formation of new apical processes: after 6–7 d, over one-third of the photoreceptors showed these processes at the end of their cilium, looking like the first steps in the assembly of their outer segment. DHA also led to an increase in the expression of opsin: over 40% more photoreceptors expressed this protein in DHA-treated than in control cultures. Even more striking was the redistribution of opsin induced by DHA. Immunocytochemical studies using Rho4-D2, a monoclonal antibody against rhodopsin, showed that without DHA opsin was uniformly distributed over the entire neurolemma, as is found in immature photoreceptors. Addition of DHA stimulated the correct localization of the protein in the newly formed apical processes, as it occurs in mature photoreceptors. Accretion of DHA entails the formation of the DHA-containing phospholipid molecules that are necessary for the assembly of the disk membranes where (rhod)opsin is to be embedded. The availability of these molecules may be essential for a timely formation of photoreceptor outer segments with proper localization of the newly formed opsin, this enhanced differentiation in turn, delaying photoreceptor apoptosis.

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Abbreviations: DHA, docosahexaenoic acid.

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A Role for Cerebral and Retinal Endothelial Cells in the Supply of Docosahexaenoic Acid to the Brain and the Retina?

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Docosahexaenoic acid (DHA, 22:6n-3), the most highly polyunsaturated n-3 fatty acid in animals, is derived from a series of elongation and desaturation reactions originating from linolenic acid (18:3n-3). DHA is found in abundant proportion in neuronal tissues such as brain and retina. The reason for such high levels of DHA is not clearly understood although changes in cerebral and retinal functions were reported in animals and humans fed diets deficient in n-3 fatty acids. Because of its functional importance, more information is needed about the delivery of DHA to these structures. DHA is primarily synthesized in the liver from dietary linolenic acid and distributed to target organs *via* the circulation. The brain and the retina can also form DHA; astrocytes and retinal pigment epithelium were respectively involved. In the present study, we examined whether cerebral and retinal microvascular endothelial cells could produce DHA.

Confluent bovine retinal and rat cerebral endothelial cells (EC) were incubated with 1 μCi of [3-^{14}C]22:5n-3 for 24 h. At the end of incubation, media and cells were harvested. Total lipids were extracted; fatty acid phenacyl esters were prepared and analyzed by high-performance liquid chromatography (HPLC), and radioactivity measured by continuous scintillation counting. In both cerebral and retinal EC, the primary product of 22:5n-3 metabolism, which includes retroconversion, elongation and desaturation reactions, was 20:5n-3, followed by 22:6n-3, 24:5n-3, and 22:6n-3. The formation of 24-carbon metabolites is consistent with the proposal of Voss *et al.* (1) that the conversion of 22:5n-3 to 22:6n-3 is independent of a δ4-desaturase. Interestingly, all of these metabolites were recovered in the medium. Thin-layer chromatography analyses revealed that 80–90% of the radioactivity found in the medium was associated with the free fatty acid fraction. The HPLC profiles obtained from the free fatty acid fractions did not differ from that obtained from media total lipids, indicating that the metabolites were released as free fatty acids. The distribution of metabolites between cells and medium shows that except for 20:5n-3 in retinal EC, each n-3 metabolite was recovered in the medium three to five times more than in the cells. Moreover, for retinal cells, there seems to be a chain-length specificity in the α-release process, the release being greater for longer-chain fatty acids (medium/cell ratio: 8.3 for 24:5n-3 > 5.0 for 24:6n-3 > 1.2 for 22:6n-3 > 0.6 for 20:5n-3). No apparent specificity was found for cerebral EC (medium/cell ratio about 4 for each metabolite). In the next experiments, we examined whether n-6 metabolites would also be released in high proportion by cerebral and retinal EC. The conversion of labeled linoleic acid ([1-^{14}C]18:2n-6) and the esterification and release of its metabolites were determined. In both cells, the major product formed by desaturation and chain elongation was 20:4n-6. Some further elongation of 20:4n-6 to 22:4n-6 was observed as well as accumulation of 20:3n-6, an anticipated intermediate between 18:2n-6 and 20:4n-6. The elongation/desaturation products, with the exception of 18:3n-6, were all preferentially retained in the cells (three to five times more than in the medium). Because the longer-chain n-6 fatty acids, such as 22:5n-6 and 24-carbon fatty acids, were not detected in our conditions, it is difficult to distinguish between fatty acid series (n-3 vs. n-6) and chain-length specificity in the release process. However, since 18:3n-6 was actually more released than the longer-chain n-6 fatty acids (medium/cell ratio: 2 vs. 0.25), and since there is no chain-length specificity in cerebral EC, these results rather suggest a preferential release of n-3 over n-6 fatty acids. This may contribute to the enrichment of 22:6n-3 in the brain and the retina. Moore (2) previously proposed a pathway in which cerebral endothelium forms and releases 20:5n-3, which is then converted by astrocytes to 22:6n-3, which is released for uptake by neurons. Since cerebral EC can also release 22:6n-3 and 24-carbon fatty acids in high proportion, we suggest that they could cooperate in supplying 22:6n-3 to neurons. Based on such a model, retinal endothelium could cooperate with retinal pigment epithelium in supplying 22:6n-3 to rod outer segments.

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Abbreviations: DHA, docosahexaenoic acid; EC, endothelial cell; HPLC, high-performance liquid chromatography.

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Effects of Dietary Oils and Cholesterol Supplement on Fluidity and Enzyme Activities of Liver Microsomes in the Rat

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Dietary fats affect the lipid composition of membranes. As a consequence of this chemical change, dynamic properties of membranes such as fluidity may be modified, affecting various biological functions, including the activity of enzymatic systems involved in the metabolism of endogenous substances and xenobiotics.

The aim of this study was to investigate a possible relationship between the ability of dietary lipids to modify liver microsomal fluidity and the activity of integral membrane enzymes participating in the metabolism of xenobiotics in rats submitted to standardized dietary conditions. We aimed to answer this question by investigating the effects of the intake of corn (C), olive (O), or fish (F) oil, and of a dietary supplement of 1% cholesterol (Ch), on microsomal fluidity, cytochrome P450 content, and the activities of phase I enzymes aniline hydroxylase (AH) and aminopyrine-*N*-demethylase (AD) and phase II enzyme UDP-glucuronyltransferase (UDP-GT).

Male Sprague-Dawley rats ($n = 6$ per group), *ca.* 100 g initial body weight, were fed semipurified diets containing 15% each oil, without or with Ch supplement.

After 20 d of feeding each of the six experimental diets, feed intake and efficiency were lower in rats fed F ($P < 0.01$) and did not differ in groups fed any of the vegetable oils, which is associated to a lower acceptability of the diet containing marine oil. Relative liver weight of rats fed with C or O oil augmented after Ch supplementation, while in F + Ch group the size of the organ was diminished compared with the nonsupplemented F group ($P < 0.001$). This is likely to be related to the accumulation of cholesterol esters in the liver in animals fed vegetable oils.

F oil intake increases n-3 polyunsaturated fatty acids in microsomal phospholipids, resulting in a higher degree of membrane unsaturation that explains the increase in fluidity. A relationship between microsomal fluidity and enzyme activity was observed: rats fed F oil exhibited the highest microsomal fluidity, cytochrome P450 content, and enzyme activities vs. groups fed C or O oils ($P < 0.001$).

A high fluidity allows the mixed function oxidases system to have an adequate structural disposition, flexible enough to facilitate the electron transport required for their optimal functioning. The intake of Ch supplement decreased membrane fluidity in all groups. The decrease in AH activity in rats fed F + Ch ($P < 0.05$) or C + Ch ($P < 0.01$) may be associated with the packing effect of this lipid. UDP-GT activity was higher in rats fed F oil vs. all vegetable oils ($P < 0.001$) and decreased after the intake of Ch ($P < 0.01$). AD was less affected by Ch supplement intake, and only F + Ch group showed lower activity ($P < 0.01$). Ch diminished the physical and functional parameters measured in animals fed F oil and, to a lesser extent, C oil. The rigidizing effect of Ch is reflected mainly on very fluid membranes, rich in long chain polyunsaturated fatty acids.

The marine oil intake exerts the most significant effects upon liver microsomal properties, which prevail over the intake of high amounts of Ch. These results also indicate that although AH and AD act in the same microsomal metabolic pathway (phase I), their localization into the membrane may be determinant of their activity and the response to dietary lipids, and also confirm that dietary lipid intake is an important factor to be taken into account when evaluating the individual response of different subjects to drug therapy and/or exposure to environmental chemicals or other xenobiotics.

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Abbreviations: AD, aminopyrine-*N*-demethylase; AH, aniline hydroxylase; C, corn oil; Ch, cholesterol; F, fish oil; O, olive oil; UDP-GT, UDP-glucuronyltransferase.

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The Effect of Palmitic Acid on Lipoprotein Cholesterol Levels and Endogenous Cholesterol Synthesis in Hyperlipidemic Subjects

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ABSTRACT: The present study assesses the effect of high vs. low palmitic acid intakes on plasma lipoprotein cholesterol levels and on rates for endogenous synthesis of cholesterol in healthy and hyperlipidemic subjects. Four diets were formulated to provide combinations of 16:0 at two levels of 18:2n-6. Subjects received each diet treatment for 21 d, followed by washout periods of 21 d. On day 21 of each diet treatment, a fasting blood sample was drawn for lipoprotein determination and to provide a measure of the background level of deuterium. A priming dose of deuterium was consumed and a second blood sample obtained 24 h after the first sample. Isotope ratio mass spectrometry was used to determine the incorporation of deuterium into the newly synthesized cholesterol molecule, and fractional synthetic rates were calculated. Serum total cholesterol and low density lipoprotein-cholesterol was not significantly affected by the high level of 16:0 when diets also contained a high level of 18:2n-6. There was no effect of dietary 16:0 on high density liproprotein-cholesterol at either the high or low levels of intake. The results indicate that 16:0 has no effect on serum lipoprotein profiles in the presence of recommended intakes for 18:2n-6.

According to the original Keys (1,2) hypothesis, palmitic acid should increase blood cholesterol levels. These commonly cited equations fail to separate the effect of three saturated fatty acids (12:0, 14:0, and 16:0), defining these as equally hypercholesterolemic. When the Keys equation is modified to treat palmitic acid as neutral (similar to stearic acid), the equation is a better predictor of changes observed in serum cholesterol levels. This observation was supported by Hayes *et al.*, who hypothesized that 16:0 is a neutral fatty acid (3). In a study which exchanged 5% of energy from 12:0 plus 14:0 for 16:0 in healthy young men consuming a low-cholesterol diet, the dietary combination of 12:0 plus 14:0 produced significantly higher serum cholesterol levels than did 16:0 (4). Other studies failed to demonstrate elevated plasma cholesterol following palmitic acid consumption. Ng *et al.* (5), compared the effects of palmitic acid and oleic acid in normocholesterolemic subjects (5).

Recently we reported a study of normal subjects in which we investigated the effect of dietary palmitic acid levels fed in high- or low-linoleic acid diets on plasma lipoprotein cholesterol content and on the rate of endogenous synthesis of cholesterol (6). Using the deuterium-uptake method, the fractional synthetic rate was assessed. When palmitic acid was exchanged for dietary 18:2n-6, plasma cholesterol levels increased. When high vs. low palmitic acid was exchanged for 18:1n-9 and was fed at both high dietary intake levels of 18:2n-6, no effect on plasma cholesterol level or the endogenous cholesterol synthesis rate was observed, thus, indicating that dietary palmitic acid has no effect on serum lipoprotein profiles in the presence of recommended dietary intakes of 18:2n-6. The present study utilized a similar dietary comparison to determine if plasma cholesterol levels can be increased in modestly hypercholesterolemic subjects by increased consumption of 16:0 when the diet contains >10% 18:2n-6.

MATERIALS AND METHODS

All procedures were approved by the Faculty of Agriculture, Forestry and Home Economics Human Ethics Review Committee. Subjects were recruited from advertisements posted at the University of Alberta campus. An in-depth questionnaire was completed by all subjects to screen for medical problems known to affect lipoprotein levels, and to characterize the subjects' activity level and sleep patterns. Subjects were nonsmokers and were not taking any medication or vitamin supplements during the study.

Subjects and diets. Moderately hyperlipidemic male and female subjects ($n = 6$) were recruited (Table 1). Caloric requirements were determined and maintained as described earlier (6). The study consisted of 21-d treatments, each consisting of a 3-d menu. All meals were prepared in the Metabolic Research kitchen and consumed at fixed times. For female subjects, diet treatments were coordinated with their menstrual cycles such that lipogenesis was measured during the luteal phase in order to be comparable to rates measured in

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Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein.

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TABLE 1
Subject Description Information

Age (yr)	45.2 ± 4.2
Body weight (kg)	74.5 ± 4.9
Height (cm)	169 ± 3.3
Body mass index	25.9 ± 1.4
Initial total cholesterol (mmol/L)	6.07 ± 0.34

male subjects (7). Diets were formulated using published nutrition composition tables (8) to contain an average of 30.6 ± 1.6% of energy from fat, 55.3 ± 1.8% of energy from carbohydrate and 15.4 ± 0.8 energy from protein. The diets were designed to provide low (2% energy) or high (8% energy) levels of palmitic acid (16:0) and approximately 10% energy as linoleic acid (18:2) (Table 2).

Experimental design. Subjects were randomly given either the low 16:0 or the high 16:0 diet and fed for 21 d. After a washout period, the next diet treatment was fed for 21 d. On day 21 of each treatment, a fasting blood sample (30 mL) was taken as described earlier (6). Subjects then consumed a priming dose of deuterium oxide (6) and a maintenance dose over the next 24 h (6). Exactly 24 h later (day 22), a second fasting blood sample (30 mL) was collected.

Serum was analyzed for lipoprotein cholesterol as described earlier (9). The remaining blood from days 21 and 22 was centrifuged at 3000 rpm for 15 min at 4°C (Jouan refrigerated centrifuge, CR 4.11) to obtain plasma. Day 21 plasma was used to determine background deuterium enrichment. Day 22 plasma was used to measure deuterium enrichment at 24 h (6) and the cholesterol fractional synthesis rate. Each diet treatment was balanced for n-3 fatty acids, cholesterol, and fiber content (6). All other analytical methods were as described earlier.

RESULTS AND DISCUSSION

Subject compliance in consuming all food provided from the metabolic research kitchen was high. Weight changes were minimal throughout the study period.

There were no significant differences between diets in regard to total fat intake. The analyzed 16:0 content of the diet varied little from the formulated values. There were no significant differences between diet treatments in regard to dietary fiber, n-3 fatty acid, and cholesterol content (Table 2). The arachidonic, eicosapentaenoic, and docosahexaenoic acids were negligible in each of the diet treatments (0.016 ± 0.006, 0.002 ± 0.012, and 0.30 ± 0.011% of fatty acids, respectively). The analyzed fat content of meals in each diet treatment was close to the formulated values and was consistent within each diet treatment.

Total cholesterol. During the entire study period, normalized total cholesterol values were between 3.17 and 4.02 mmol/L in normal subjects (Fig. 1). Feeding high 16:0 increased levels of total cholesterol from 3.30 to 3.61 mmol/L ($P < 0.001$) when the diet was low in 18:2n-6. When the diet was high in 18:2n-6, raising the level of 16:0 did not have a significant effect on total plasma cholesterol level in normal or hypercholesterolemic subjects.

Low density lipoprotein (LDL)-cholesterol. Throughout the study period, normalized LDL-cholesterol values ranged from 1.76 to 2.48 mmol/L in normal subjects, within the low range of normal (Fig. 1). Plasma LDL-cholesterol level was higher ($P < 0.0001$) when subjects consumed the higher level of 16:0 (2.18 mmol/L) at the low 18:2n-6 intake level. LDL-cholesterol levels decreased ($P < 0.01$) at the high levels of 18:2n-6 consumption (1.92 mmol/L) as compared to the lower levels of 18:2n-6 (2.12 mmol/L). Similar to the total cholesterol response, when the diet was high in 18:2n-6, raising the level of 16:0 did not have a significant effect on plasma LDL-cholesterol levels in normal or hypercholesterolemic subjects.

High density lipoprotein (HDL)-cholesterol. Throughout the study period, normalized HDL-cholesterol values varied only minimally, ranging from 1.08 to 1.24 mmol/L (Fig. 1) in normal subjects. No significant main effects of diet on HDL-cholesterol levels were observed in hypercholesterolemic subjects. At high levels of 18:2n-6, high 16:0 leads to significant reductions in HDL-cholesterol levels (1.21 vs. 1.08 mmol/L) ($P < 0.003$) in normal subjects.

Cholesterol synthesis. The diet treatments did not alter the fractional synthesis rates for cholesterol (Table 3).

In North America, palmitic acid is the predominant saturated fatty acid in the diet, contributing to approximately 7–8% of the total energy intake. Current dietary recommendations suggest that no more than 30% of the total energy be derived from fat. Of this, at least one-third, or 10% of energy, is suggested to be obtained from 18:2n-6. The diets in this study designated as high in linoleic acid contained 18:2n-6 at levels currently recommended. Clearly, the presence of adequate linoleic acid is of primary importance and cannot be overlooked. The finding that palmitic acid does not exert a cholesterol-raising effect in the presence of adequate linoleic acid has practical applications to the food industry as well as to nutritionists.

TABLE 2
Nutrient Composition of Diets Consumed by Hypercholesterolemic Subjects[a]

	Low 16:0 diet	High 16:0 diet
Energy (MJ)	12.50	12.49
Protein (energy)	16.9 ± 2.3	16.1 ± 2.1
Carbohydrate (% energy)	57.0 ± 3.3	53.5 ± 2.6
Total fat (% energy)	27.6 ± 1.1	31.1 ± 0.5
Saturated fat (% energy)	4.7 ± 0.3	10.2 ± 0.7
MUFA (% energy)	7.5 ± 0.1	8.1 ± 0.3
18:2n-6 (% energy)	12.0 ± 1.4	12.1 ± 0.6
16:0 (% energy)	2.9 ± 0.2	9.9 ± 0.2
n-3 FA (% energy)	0.5 ± 0.1	0.4 ± 0.0
Cholesterol (mg)	176.7 ± 77.5	148.8 ± 22.5
Dietary fiber (g)	32.7 ± 8.5	32.9 ± 5.3

[a]Values represent means ± SD; $n = 3$ for each diet treatment. Diet averages are based on the average calculated from each menu cycle. All values are derived from Food Processor II data, except for 16:0 and 18:2n-6 which are derived from published food composition tables. The detailed nutrient composition of diets consumed by normal subjects has been published (Ref. 6). MUFA, monounsaturated fatty acids; FA, fatty acids.

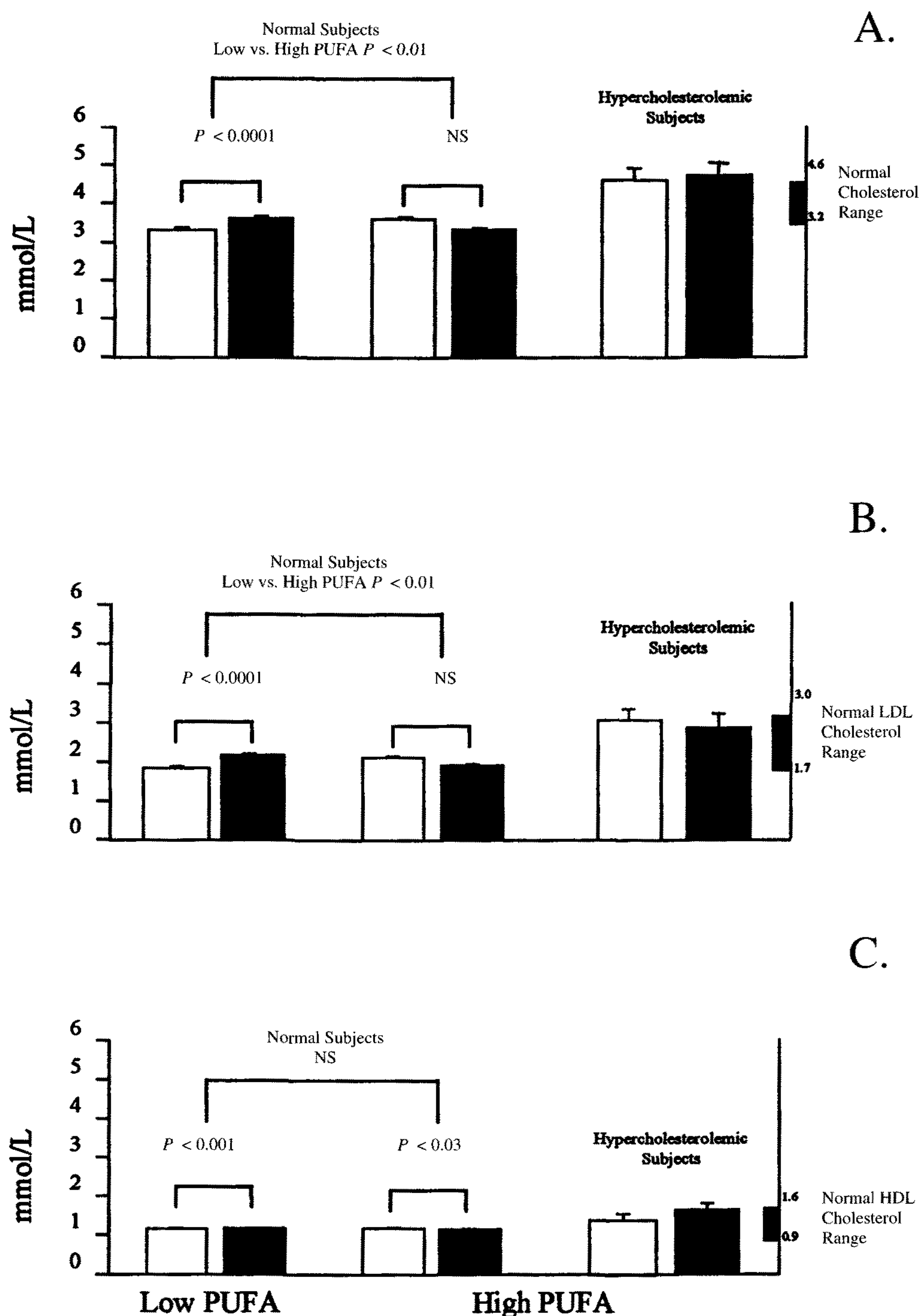

FIG. 1. Plasma lipoprotein cholesterol values. (A) Total cholesterol, (B) low density lipoprotein (LDL)-cholesterol, and (c) high-density lipoprotein (HDL)-cholesterol for subjects. Values represent means ± SEM for all subjects. NS, nonsignificant ($P > 0.05$); PUFA, polyunsaturated fatty acids. □, low 16:0; ■, high 16:0. Significant effects of diet treatments in hypercholesterolemic subjects were not found.

It is concluded that for normolipidemic and mildly hypercholesterolemic subjects who typically consume a relatively low-fat diet (30% energy from fat and containing the recommended intake of n-6 polyunsaturated fat) the consumption of palmitic acid is unlikely to have an appreciable effect on lipoprotein profiles. Furthermore, it can be inferred that an adequate amount of palmitic acid is necessary to promote a more favorable serum cholesterol response. Epidemiological data suggest that as intake of saturated fatty acids is decreased to about 10% of energy, there is a progressive fall in mortality due to cardiovascular disease. Evidence for lowering the level of saturated fat in the diet below this level is lacking, leading one to question the benefit of reductions beyond this point. Palmitoylation of protein is required for the function of receptors and in combination

TABLE 3
Fractional Synthesis Rates for Plasma Cholesterol Synthesis in Subjects Consuming Experimental Diets[a]

Number	Acid	Mean
6	Low 16:0, low 18:2	0.04 ± 0.008
6	Low 16:0, high 18:2	0.03 ± 0.008
6	High 16:0, low 18:2	0.05 ± 0.008
4	High 16:0, high 18:2	0.06 ± 0.01

[a]Diet fat effect nonsignificant at $P < 0.05$. Values are means pooled SEM. Significant effects of diet treatment were not found.

with the finding that 16:0 intake may not be cholesterolemic suggests the potential for a suboptimal intake of saturated fat if intake is lower than a level that may be needed for essential processes. This finding may be of particular importance as low and even negligible levels of dietary saturated fat are frequently recommended by health professionals to promote lowered lipid levels. Because palmitic acid is an abundant fatty acid in palm oil, meat and dairy products, and food items also high in nutritive value in other ways, reevaluation of the recommendations to limit consumption of these food items is warranted.

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Postprandial Hypertriglyceridemia Induced by Saturated vs. Monounsaturated Fatty Acids Is Related to Reduced Hepatic Lipoprotein Receptors Binding in NZW Rabbits

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Ingestion of fat is characterized by plasma accumulation of chylomicrons and very low density lipoprotein, which represent the triglyceride-rich lipoprotein (TRL). TRL accumulation depends on endovascular lipolysis by lipoprotein lipase and hepatic lipase and lipoprotein receptor-mediated uptake by the liver. The low density lipoprotein (LDL) receptor (LDL-R) and LDL R-related protein could be the major candidates implicated in this process. More recently, it was suggested that the lipolysis-stimulated receptor (LSR) could also play a role in the TRL uptake. As defect of TRL clearance generates the formation of atherogenic remnants, postprandial hypertriglyceridemia eventually leads in the long term to the development of arteriosclerosis. This study aimed to evaluate if the postprandial triglyceride response induced by saturated fatty acid or monounsaturated fatty acids is related to hepatic LDL-R and LSR activities.

MATERIALS AND METHODS

Rabbits (28 NZW) were fed during 2 or 4 wk, with either a control diet (2.7% vegetable fat) or 10%-fat diet providing either saturated fatty acid (SFA) (coconut oil) or monounsaturated fatty acid (MUFA) (olive oil).

Rabbits had a fat test-meal containing either SFA or MUFA and [^{14}C]triglycerides. Control rabbits ingested the SFA and the MUFA test-meal. Chronically coconut oil-fed rabbits for 2 or 4 wk were given a SFA-rich meal at the end of the dietary experiment. Following the same protocol, olive oil-fed rabbits were given a MUFA-rich meal. Plasma triglyceride and radiolabeled dietary triglyceride concentrations were assayed. Postheparin plasma lipoprotein lipase and hepatic lipase were checked. Lipoprotein binding to low density lipoprotein (LDL) receptor (LDL-R) and lipolysis-stimulated receptor (LSR) in control and fat-fed rabbits was measured. Hepatocyte plasmic membranes were isolated with a sucrose-gradient method. Then, ^{125}I-LDL were incubated with hepatocyte plasmic membrane in two conditions: (i) with $CaCl_2$ (binding to LDL-R calcium-dependent) and (ii) with EDTA (prevent the binding to LDL-R) and oleate (LSR activated by free fatty acids).

RESULTS

As compared to control rabbits, postprandial triglyceridemia as well as plasma dietary [^{14}C]triglyceride accumulation was slightly altered in olive oil-fed rabbits. On the contrary, postprandial plasma triglyceride and [^{14}C]triglyceride concentrations were significantly higher in rabbits fed the coconut oil-rich diet for 4 wk, as compared to control and olive oil-fed rabbits.

Chronic ingestion of the MUFA diet did not alter hepatic lipase activity, while the SFA diet decreased it after 2 wk and increased it after 4 wk. The two experimental diets did not modify lipoprotein lipase activity as compared to the control diet.

Binding of lipoprotein to the LDL-R and to the LSR is expressed as μmol ^{125}I-LDL/mg plasma membrane protein. As compared to control rabbits, the SFA diet decreased lipoprotein binding to LDL-R, while the MUFA diet significantly increased lipoprotein binding to LDL-R. As compared to control rabbits, the MUFA diet increased lipoprotein binding to LSR. On the contrary, the SFA diet decreased LSR binding activity after 2 wk.

CONCLUSIONS

As compared to a low-fat control diet, chronic ingestion of a SFA diet increased postprandial lipemia, did not noticeably modify lipoprotein lipase and hepatic lipase activities, and decreased binding activities to LDL-R as well as LSR. On the contrary, a MUFA-rich diet did not alter postprandial lipemia,

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Abbreviations: LDL-R, low density lipoprotein-receptor; LSR, lipolysis-stimulated receptor; MUFA, monunsaturated fatty acid; SFA, saturated fatty acid; TRL, triglyceride-rich lipoprotein.

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had no effect on lipase activities, but increased binding activities to LDL-R and to LSR measured on hepatocyte plasmic membrane. In both cases, postprandial triglyceridemia and accumulation of triglycerides from dietary origin were inversely correlated to LDL-R and LSR binding activities.

The reduced LDL-R as well as LSR binding activities could explain the postprandial hypertriglyceridemia observed in rabbits chronically ingesting SFA. MUFA could prevent postprandial accumulation of TRL by accelerating their hepatic uptake mediated by LDL-R and LSR.

Effects of Age and Dietary n-3 Fatty Acids on the Metabolism of [^{13}C]-α-Linolenic Acid

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Conversion of α-linolenic acid into longer-chain polyunsaturates (LCP) may be influenced by physiological and nutritional factors. For instance, desaturation may decrease with aging (1), and conversion of linoleic acid and α-linolenic acid may be inhibited by dietary linoleic acid (2). These findings, however, are mainly based on *in vitro* and rat studies using radioactive isotopes. We have therefore investigated *in vivo* the effects of age and diets rich in α-linolenic acid or eicosapentaenoic acid/docosahexaenoic acid (EPA/DHA) vs. an oleic acid-rich diet on the oxidation of uniformly labeled [^{13}C]-α-linolenic acid and its conversion into LCP in healthy human subjects.

SUBJECTS AND METHODS

Fifteen men and women participated in this study. The elderly volunteers (age > 60 yr) received a diet rich in oleic acid (n = 5), α-linolenic acid [6.8 g/d (n = 2)], or EPA/DHA [1.0 g EPA/d plus 0.6 g DHA/d (n = 3)] for 7 wk. Five younger subjects (age < 35 y) received the α-linolenic acid-rich diet. Fatty acids were provided as margarine from which pies, cakes, and chocolate paste were prepared. After 6 wk, subjects came to the department after a fasting period of 12 h. After a blood sample (t = 0), subjects were given 45 mg of uniformly labeled [^{13}C]-α-linolenic acid as methyl ester (Martek Biosciences Corporation, Columbia, MD) dissolved in 8 g of olive oil. Breath was sampled and CO_2 production was measured each hour for 12 h. Furthermore, blood samples were collected at t = 5, 11, 24, and 96 h. Subjects then returned to their home diets, and 1 wk later a final fasting blood sample was taken at t = 336 h. ^{13}C enrichments in plasma and breath samples were analyzed with a gas chromatograph-combustion-isotope ratio mass spectrometer, while fatty acid composition of plasma total lipids was measured by gas chromatography/flame-ionization detection.

RESULTS

In plasma, ^{13}C enrichments in α-linolenic acid, EPA, docosapentaenoic acid, and DHA were detected. The ^{13}C enrichments of all fatty acids were lower on the n-3-rich diets compared to the oleic acid group. Plasma concentrations of α-linolenic acid were higher in both α-linolenic acid groups than in the oleic acid group. Hardly any ^{13}C enrichment of LCP was observed in the EPA/DHA group, while plasma concentrations of EPA and DHA were highest in this group. After 336 h, plasma ^{13}C enrichments of α-linolenic acid, EPA, and docosapentaenoic acid had almost returned to baseline, while DHA was still enriched.

From the ^{13}C enrichment in breath and CO_2 production, the oxidation of ^{13}C α-linolenic acid was calculated. The mean proportion of labeled α-linolenic acid oxidized after 12 h was about 18%, and was slightly higher in the EPA/DHA group compared to the oleic acid group.

CONCLUSION

We conclude that ^{13}C α-linolenic acid is converted into its LCP. The conversion of α-linolenic acid into its LCP appears to be inhibited on diets rich in EPA/DHA, while it appears not to be influenced by age. In addition, oxidation of α-linolenic acid is not dependent on age or dietary α-linolenic acid, but may be slightly increased on diets rich in EPA/DHA.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosahexaenoic acid; LCP, longer-chain polyunsaturates.

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Carbon Recycling from Linoleate During Severe Dietary Linoleate Deficiency

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A significant portion of the ß-oxidized carbon skeleton of polyunsaturated fatty acids can be recycled into *de novo* lipogenesis (DNL), i.e., cholesterol, saturates, and monounsaturates (1–3). In the present study, we quantitated the DNL pathway in severely linoleate (18:2n-6)-deficient rats to determine whether it is a function of redundancy or is obligatory. If there is minimal recycling during severe linoleate deficiency, it will suggest that recycling from linoleate is mainly a function of linoleate intake in excess of requirement; if not, it will support the obligatory nature of this component of linoleate utilization.

Weanling male Sprague-Dawley rats consumed a semipurified diet containing linoleate at 2 en% (control) or 0.01 en% (deficient). After 13 wk, 7 μCi [1-^{14}C]-linoleate was given by gavage, and the rats were killed 48 h later. Fatty acid profiles and distribution of ^{14}C in liver sterols and individual fatty acids were determined.

In the linoleate-deficient group, fatty acid profiles reflected extreme n-6 polyunsaturated fatty acids depletion (4). In the deficient group, 69% less ^{14}C was recovered as $^{14}CO_2$ than in the control group (11 vs. 36% of the dose, $P < 0.01$; 4). ^{14}C (dpm/g) in total lipids of liver, brain, and perirenal fat of deficient rats was 2–6 times higher than in control rats (all $P < 0.01$). In livers of the linoleate-deficient group, ^{14}C (dpm/g) was 13-fold higher in linoleate, 2.7-fold higher in arachidonate (20:4n-6), and 3.5-fold higher in products of DNL (Fig. 1; all $P < 0.01$). In livers of control rats, ^{14}C distribution was: 41% arachidonate, 29% linoleate, 22% sterols, 3% oleate (18:1n-9), 3% palmitate (16:0), and 2% stearate (18:0). In liver total lipids of the controls, as much ^{14}C (dpm/g) was recovered in products of DNL as in linoleate (Fig. 1; 29–30%). In livers of the linoleate-deficient rats, ^{14}C distribution was: 63% linoleate, 19% arachidonate, 11% sterols, 4% oleate, 2% palmitate, and 1% stearate. Thus, in the deficient rats, the proportion of ^{14}C that was recovered in liver DNL was equivalent to being converted to arachidonate (Fig. 1; 18 vs. 19%).

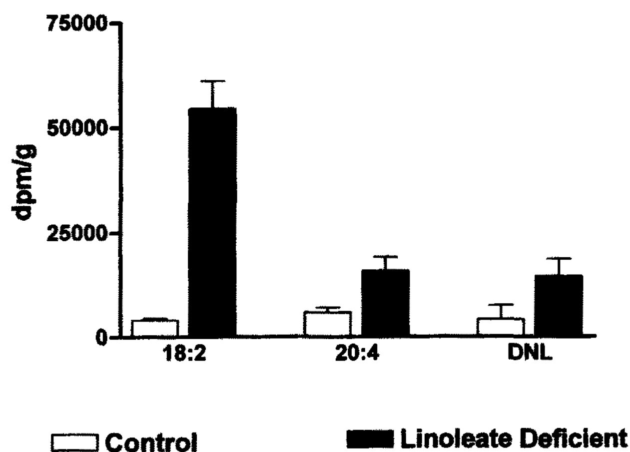

FIG. 1. Recovery of ^{14}C in liver linoleate (LA), arachidonate (AA), and the main products of *de novo* lipogenesis (DNL—cholesterol, palmitate, oleate and stearate combined) 48 h after an oral dose of [1-^{14}C]-linoleate. Values are the mean ± SD of $n = 6$/group; (□), controls; (■), linoleate-deficient; each pair of values is significantly different between the controls and deficient group.

Thus, in linoleate-deficient rats, although less ^{14}C-linoleate was expired as CO_2 (4) and more was retained in the liver pool of n-6 polyunsaturates, more ^{14}C was also recovered in DNL. Hence, linoleate deficiency shifted metabolism of ^{14}C-linoleate toward both greater conservation of n-6 polyunsaturates and toward more carbon recycling but less linoleate ß-oxidation to CO_2. We conclude that in addition to ß-oxidation to CO_2, carbon recycling into DNL accounts for a significant, obligatory component of linoleate metabolism even during severe linoleate deficiency.

ACKNOWLEDGMENT

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Abbreviations: DNL, *de novo* lipogenesis.

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A Chronic Ethanol-Feeding Study in Rhesus Monkeys

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ABSTRACT: This study describes the effect of chronic ethanol-feeding in rhesus monkeys. Animals which were maintained on a diet containing 18:2n-6 and 18:3n-3 as 1.4 and 0.08% of the calories, respectively, and consumed alcohol (mean 2.6 g kg^{-1} d^{-1}) had decreased amounts of 20:4n-6 and 22:6n-3 in their livers and plasma lipids compared with controls. Alcohol consumption did not appear to effect the absorption of $^2H_5$-18:2n-6 and 18:3n-3 esters into the blood following an oral dose. There was an increase in $^2H_5$ enrichment in plasma 20:4n-6 and 22:6n-3, indicating that alcohol may have increased production of these fatty acids. There was a greater concentration of 4-hydroxynonenal in the plasma of alcohol-exposed monkeys compared to controls.

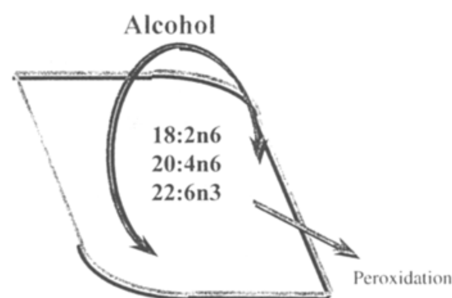

FIG. 1. Alcohol consumption causes decreases in tissue polyunsaturated fatty acids through increased catabolism.

Among its various effects, alcohol consumption alters the fatty acid (FA) composition of membrane phospholipids, causing a decrease in the concentration of tissue polyunsaturated fatty acids (PUFA) (1). The mechanism by which alcohol depletes tissues of PUFA, *in vivo*, is unknown. Evidence from *in vitro* studies suggests that alcohol may decrease Δ-6 and -5 desaturase activity (2). Other evidence suggests that ethanol does not act uniformly across desaturase systems, leaving these findings open to other interpretations. The metabolism of ethanol through P450IIE1 produces hydroxyl radicals which may react with FA-forming oxygenated compounds. The effect of chronic alcohol consumption on lipid peroxidation, the absorption of 18-carbon FA, and the *in vivo* production of 20:4n-6 and 22:6n-3 was studied in monkeys which consumed alcohol over 18 mon (Fig. 1).

METHODS

Ten adult male rhesus monkeys were maintained on a semipurified diet (3) and given access to food twice a day. Six animals were given 24 h access to an artificially-sweetened 7% ethanol solution for 18 mon. Control animals were maintained on the same diet and provided with the same amount of food as the alcohol-exposed animals (540 kcal/d). The fat content (14 en%) of the diet consisted of a 2:1 ratio of olive oil/hydrogenated coconut oil. There were 8.9 g of 18:2n-6 and 0.5 g of 18:3n-3 per 100 g of fat. Pieces of liver (20–30 mg) and plasma (1 mL) were analyzed for their FA content by gas chromatography–flame-ionization detector. The uptake and metabolism of $^2H_5$-18:2n-6 and $^2H_5$-18:3n-3 were determined in both groups by gas chromatography–mass spectrometry (4). Animals were fasted and anesthetized with ketamine-hydrochloride/acepromazine (10:1). A nasogastric tube was inserted into the stomach, and animals were given a bolus dose of 100 mg of $^2H_5$-18:2n-6 and 10 mg $^2H_5$-18:3n-3 ethyl esters. Blood (2 mL) was collected at 0, 4, 24, 130, 168, and 216 h.

RESULTS AND DISCUSSION

In the livers of animals that consumed alcohol, the concentration of nonessential FA was about 15% higher compared to controls. In particular, there were higher concentrations of 18:1n-9 (66%) and 20:3n-9 (45%) and lower concentrations of several PUFA which included 18:2n-6 (–20%), 20:4n-6 (–14%), and 22:5n-3 (–36%). There was a lower (–56%) concentration of liver 22:6n-3. In the plasma there were higher percentages of 16:1n-9 (82%), 18:1n-9 (14%), 18:1n-7 (62%), 20:3n-9 (100%), and 24:1n-9 (40%) in monkeys consuming alcohol compared with controls. There were lower percentages of 18:2n-6, 20:4n-6, 18:3n-3, and 22:6n-3 in the plasma in the alcohol group compared to the controls. The total integrated amounts of the plasma concentrations of $^2H_5$-18:2n-6 and $^2H_5$-18:3n-3 were similar in both groups over the 9 d. The total concentrations calculated as area under the curve for $^2H_5$-20:4n-6, $^2H_5$-20:5n-3, and $^2H_5$-22:6n-3 were not found to be significantly different between the two groups. However, the enrichment of deuterium into plasma 20:4n-6 and 22:6n-3 was greater in monkeys consuming alcohol compared to controls. Alcohol consumption caused a

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Abbreviations: FA, fatty acid; PUFA, polyunsaturated fatty acid.

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marked increase in the concentration of 4-hydroxynonenal in the plasma. There was a significant ($P < 0.02$) increase in 4-hydroxynonenal in the plasma of monkeys consuming alcohol (mean 1440 ± 441 pg/mL) compared to controls (68 ± 23 pg/mL). Alcohol exposure did not appear to decrease the absorption of the 18-carbon essential FA from the diet into the blood. The hypothesis that alcohol inhibits desaturases, and therefore, the *in vivo* formation of long-chain PUFA predicts that a lower concentration of $^2H_5$-20:4n-6 and 22:6n-3 should follow from oral doses of the labeled 18-carbon precursors. Finding of similar total accumulations of labeled long-chain PUFA in the plasma suggests that ethanol metabolism may not be directly linked to an inhibition of desaturase activities. However, evidence from this study does suggest that alcohol exposure had a significant impact on lipid peroxidation and suggests that alcohol may lower tissue levels of long chain essential FA primarily through increases in catabolism and not by decreases in anabolic processes.

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The Metabolism of Essential Fatty Acids in Rat Liver Is Influenced More by Dietary Fat Than Dietary Ethanol

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Ethanol ingestion has been shown to influence fatty acid composition and metabolism of cellular and subcellular membranes in various mammalian tissues (1,2). A rather consistent finding is a decrease in 20:4n-6 in rat liver after ethanol exposure, but the specific mechanism of the altered lipid composition still remains unresolved. Shorter essential fatty acids are elongated and desaturated to longer and more unsaturated fatty acids. Ethanol may influence these pathways at several levels.

MATERIALS AND METHODS

We used the Lieber DeCarli Liquid Rat diet with ethanol and the corresponding control diet. Ethanol and water were added to the diet according to the manufacturer giving an energy percentage (E%) of 36% from ethanol. This diet had a high triglyceride content, but the n-6/n-3 ratio of 132 may be capable of inducing a selective n-3 deficiency. The low-fat diet was a standard rat and mouse chow diet with an n-6/n-3 ratio of 13, and ethanol was mixed into this diet giving an E% of ethanol of 50%. The rats fed ethanol had a slower weight gain than the controls. From week 3 to week 4 after ethanol exposure (7 d), the rats fed the high-fat diet increased from 161 ± 5 g to 178 ± 6 g (mean ± SD). The control rats increased from 197 ± 4 to 251± 6 g at the same time. Parenchymal liver cells were prepared according to the method of Seglen (3). Lipids were extracted by the method of Folch *et al.* (4). Separation in lipid classes and phospholipid classes was performed by thin-layer chromatography. Separation of fatty acid was performed by reverse-phase high-performance liquid chromatography and radio gas chromatography. Separation in phospholipid molecular species was performed by reverse-phase high-performance liquid chromatography.

RESULTS

In the low-fat diet group, 32.7% of the [1-^{14}C]20:5n-3 was elongated to [3-^{14}C]22:5n-3, decreasing to 6.0% after ethanol feeding (P = 0.002) and to 16.7% after incubating control hepatocytes with ethanol *in vitro* (P = 0.048). The high-fat diet itself decreased the elongation of [1-^{14}C]20:5n-3 to 17.0%. The elongation of 20:4n-6 was 6% when fed the high-fat diet and 8% when fed the low-fat diet (not significantly different) and not affected by ethanol.

Ethanol feeding decreased the esterification of [1-^{14}C]22:6n-3 in phospholipids. After 90 min of incubation, esterification decreased from 4.6 ± 0.2 (mean ± SD) to 3.3 ± 0.1 nmoles (P = 0.001) and after 200 min incubation, from 4.9 ± 0.6 to 2.3 ± 0.1 nmoles (P = 0.001) in rats fed the low-fat diet.

Using [1-^{14}C]22:6n-3, ethanol feeding increased the ratio of esterification in phosphatidylethanolamine (PE) and phosphatidylcholine (PC). However, using [1-^{14}C]20:5n-3, the high-fat diet itself increased the PE/PC ratio compared to the low-fat diet.

Ethanol feeding decreased the relative amount of molecular species containing 18:0 compared to species containing 16:0 in PC of rats fed the low-fat diet. The 18:0/16:0 ratio was lowered by ethanol from 0.94 to 0.62 using [1-^{14}C]20:4n-6 and from 0.75 to 0.40 using [1-^{14}C]20:5n-3 after 200 min of incubation. Ethanol led to formation of species in which the labeled substrates were paired with 18:1n-9 in rats fed the low-fat diet, a species not formed in detectable amounts in these rats without ethanol in the diet.

DISCUSSION

Ethanol feeding inhibited the elongation of 20:5n-3 to 22:5n-3 in animals fed a low-fat diet. To our knowledge this observation has never been published. If this elongation was a rate-limiting step in the synthesis of 22:6n-3 in the liver, one would expect to find a decreased amount of endogenous 22:6n-3 after ethanol feeding, which we did not find. The high-fat diet itself also inhibited the elongation of 20:5n-3 compared to the low-fat diet.

A recent study concluded that the synthesis of PC was reduced by chronic ethanol treatment and that synthesis of PE and phosphatidylserine was increased (5). In agreement with this, the ratio of esterification in PE compared to PC increased when using [1-^{14}C]22:6n-3 as substrate in present study. However, the PE/PC ratio was not altered by ethanol using [1-^{14}C]20:4n-6 and [1-^{14}C]20:5n-3 as substrates. These find-

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Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine.

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ings seem interesting since in guinea pigs with fetal ethanol syndrome a reduced amount of both PE and of 22:6n-3 in liver and brain was observed (6).

In conclusion, we demonstrated that ethanol exerts selective and different effects on the metabolism of 20:4n-6, 20:5n-3, and 22:6n-3 provoked by a low-fat diet.

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Effects of n-3 and n-6 Polyunsaturated Fatty Acids on 3-Hydroxy-3-methylglutaryl-CoA Reductase in Liver and Mammary Glands of Low Density Lipoprotein-Receptor Knockout Mice

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Marine fish oils provide one of the richest sources of long chain n-3 polyunsaturated fatty acids (PUFA) in the diet. Consumption of these fatty acids is associated with a reduced risk of developing breast cancer as well as cardiovascular disease. The inhibitory effects of n-3 PUFA on breast cancer development were supported by animal studies and cell culture experiments, but the molecular mechanisms are still unclear. We recently showed that 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase is down-regulated in the mammary glands of rats fed n-3 PUFA and proposed this as a possible mechanism by which tumorigenesis is inhibited (1). HMG-CoA reductase is the rate-limiting enzyme in cholesterol biosynthesis that catalyzes the formation of mevalonate which is required for cell-cycle progression and was implicated in cancer development. Competitive inhibitors of HMG-CoA reductase (e.g., lovastatin and simvastatin) inhibit rat and mouse mammary tumorigenesis *in vivo* and the proliferation of human breast cancer cells *in vitro*. The mevalonate-derived metabolite that is required for cell-cycle progression has not yet been identified, but is known not to be cholesterol.

Cellular uptake of cholesterol-rich lipoproteins such as low density lipoprotein (LDL) is mediated through the LDL receptor (LDL-R) as well as a receptor-independent pathway. When uptake is enhanced, intra cellular cholesterol levels rise, and HMG-CoA reductase is down-regulated to prevent excessive accumulation of cholesterol. As a result, the synthesis of mevalonate that is required for cell proliferation is reduced.

In this study, we used mice with a targeted disruption of the LDL-R gene to determine whether the LDL-R mediates the down-regulation of HMG-CoA reductase by n-3 PUFA. Female mice lacking the LDL-R (–/–) and wild-type (+/+) mice were fed a 7% (wt/wt) fat diet rich in either n-3 (menhaden oil) or n-6 (safflower oil) PUFA for 1 wk. The effects of dietary n-3 PUFA were compared to those of n-6 PUFA. Total serum cholesterol was reduced by n-3 PUFA to the same extent in +/+ (43%) as in –/– (41%) mice. Serum triglyceride levels were reduced by 34% in the +/+ group and by 50% in the –/– group. n-3 PUFA also reduced serum LDL-cholesterol, but to a greater extent in the +/+ (6-fold) than in –/– mice (2-fold). HDL-cholesterol levels were reduced in +/+ mice but not in –/– mice. The specific activity of HMG-CoA reductase was measured using a radiochemical assay and expressed as pmol/mg/min. In +/+ mice, the specific activity of HMG-CoA reductase was lower in the livers (109 ± 25 vs. 336 ± 80) and mammary glands (36 ± 10 vs. 20 ± 9) of mice fed n-3 PUFA compared to those fed n-6 PUFA. In –/– mice, HMG-CoA reductase was also lower in the livers (65 ± 38 vs. 123 ± 27) of those fed n-3 PUFA, but was surprisingly greater in the mammary glands (19 ± 6 vs. 10 ± 2).

When the data were analyzed by two-way analysis of variance, there was a significant diet-genotype interaction. This indicates that not only were the effects of diet on measures of lipid metabolism dependent on LDL-R genotype, but the effects of LDL-R status were dependent on the PUFA in the diet. For example, when mice were fed n-3 PUFA, serum LDL-cholesterol was 27-fold higher in –/– than in +/+ animals, but only 10-fold greater in those fed n-6 PUFA. HDL-cholesterol levels were lower in –/– than in +/+ mice only when n-6 PUFA were fed and did not differ when n-3 PUFA were the major source of fat. With either diet, hepatic HMG-CoA reductase was lower in –/– than in +/+ mice, but the magnitude of this effect was greater in animals fed n-6 PUFA (>3-fold) than in those fed n-3 PUFA (<2-fold). +/+ Mice fed n-6 PUFA had considerably higher levels of HMG-CoA reductase in the mammary gland than –/– mice, but no differences were observed among those fed n-3 PUFA.

These results show that the LDL-R is required for n-3 PUFA to down-regulated mammary gland HMG-CoA reductase, but is not required for the reduction in serum lipids or the down-regulation of hepatic HMG-CoA reductase. The diet-genotype interactions we observed suggest that the composition of dietary fat should be considered in future studies on cholesterol metabolism using knockout or transgenic mice.

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Abbreviations: HMG-CoA, 3-hydroxy-3-methyl glutaryl; LDL, low density lipoprotein; LDL-R, low density lipoprotein receptor; PhFA, polyunsaturated fatty acid.

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Dietary Fatty Acids and the Immune System

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An effect of dietary fatty acids upon the immune system was suggested by early epidemiological studies of the incidence of multiple sclerosis and by the observations that the blood, cells, and tissues of patients with multiple sclerosis are deficient in long-chain polyunsaturated fatty acids (PUFA) (1). This suggestion was supported by observations that linoleic (18:2n-6) and arachidonic (20:4n-6) acids inhibit mitogen-stimulated proliferation of human peripheral blood lymphocytes in culture (see Ref. 2) and that subcutaneous injections of these fatty acids prolong the survival of skin allografts in mice (3). These studies focused on the n-6 family of PUFA, but more recently there has been intense interest in the effects of the n-3 PUFA. An immunomodulatory effect of these latter fatty acids is suggested by epidemiological studies which show that populations such as Greenland Eskimos, who consume large quantities of marine mammal and fish oils which are rich in eicosapentaenoic (20:5n-3) and docosahexaenoic (22:6n-3) acids, have a very low incidence of inflammatory and autoimmune disorders (4). Furthermore, a number of clinical studies reported that fish oil supplementation has some beneficial effects in rheumatoid arthritis, psoriasis, lupus, and inflammatory bowel disease and prolongs the survival of grafts (see Refs. 5,6). The potential clinical use of oils rich in n-6 or n-3 PUFA has given rise to a number of investigations of the effects of fatty acids and dietary oils upon immune cell functions (see Ref. 7).

Dietary fatty acids and immunity: An overview. A number of studies showed that feeding rats, mice, rabbits, or chickens diets rich in fish oil results in suppressed *ex vivo* lymphocyte proliferation (8–13), interleukin-2 (IL-2) production (14), natural killer (NK) cell activity (12,15–17), cytokine [(IL-1, IL-6, tumor necrosis factor (TNF)] production by inflammatory macrophages (18–20), and macrophage-mediated cytotoxicity (20–22). Recent studies indicate that relatively low levels of n-3 PUFA are required to bring about some of these suppressive effects (23,24), that dietary eicosapentaenoic and docosahexaenoic acids both inhibit lymphocyte proliferation (14,23), and that dietary eicosapentaenoic acid but not docosahexaenoic acid inhibits NK cell activity (23). Feeding laboratory animals diets containing high levels of linseed oil [rich in α-linolenic acid (18:3n-3)] also brings about suppressive effects (10,16,25,26). A recent study indicates that the precise effect of α-linolenic acid on lymphocyte functions depends on the level of linoleic acid and the total PUFA content of the diet (27). Investigations which have directly compared the effects of n-3 PUFA-rich oils indicate that fish oil is more suppressive than linseed oil. Diets rich in n-6 PUFA are less suppressive than those containing n-3 PUFA or, in some studies, without effect (see Ref. 7). Other fatty acids also appear to influence lymphocyte functions, with the levels of palmitic and oleic acids appearing to modulate the effect of linoleic acid (28,29).

Supplementation of the diet of healthy human volunteers with fish oil-derived n-3 PUFA (1.2 to 14 g/d) results in decreased lymphocyte proliferation (30–32), monocyte and neutrophil chemotaxis (33–36), and production of IL-1, IL-2, IL-6, and TNF (30–33,37). A high dose of α-linolenic acid (approx. 15 g/d) was reported to suppress human IL-1 and TNF production (37). The inverse relationship between the eicosapentaenoic acid content of mononuclear cell lipids and production of TNF and IL-1 was recently described (37).

n-PUFA decrease the expression of some adhesion molecules on the surface of cultured endothelial cells, monocytes, and lymphocytes (38–40). Addition of fish oil-derived n-3 PUFA to the diet of laboratory animals or healthy humans also appears to modulate adhesion molecule expression on the surface of monocytes and lymphocytes (12,41–43) and alters the ability of such cells to bind to ligand-bearing cells (43).

The *ex vivo* observations described above indicate that consumption of diets containing n-3 PUFA, particularly diets rich in fish oil, should result in protection against the damaging effects of inflammatory challenges, which are mediated in part by overproduction of the proinflammatory cytokines TNF, IL-1, and IL-6 (44). This would be a clinically beneficial effect of n-3 PUFA. Certainly rats or guinea pigs given large amounts of fish oil either intravenously or through the diet show markedly enhanced survival following the administration of a high dose of bacterial endotoxin (45,46) and a reduction in the anorexia which accompanies administration of IL-1 or TNF (47,48).

Fish oil feeding also ameliorates the symptoms of some animal models of autoimmune disease such as lupus-prone mice (e.g., 49), so increasing the lifespan of such animals

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Abbreviations: IL, interleukin; NK, natural killer; PUFA, polyunsaturated fatty acid; TNF, tumor necrosis factor.

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(50). These changes are associated with abolition of proinflammatory cytokine production and the induction of antiinflammatory cytokines and antioxidant enzymes (51).

Mechanisms of action. Several possible mechanisms by which n-3 PUFA influence immune cell function have been suggested (see 5,52,53). These mechanisms include modulation of eicosanoid synthesis, alteration of intracellular signaling pathways, regulation of transcription factor activity, and altered antioxidant status. These are not necessarily exclusive, and different effects of n-3 PUFA might be exerted through different mechanisms.

Some eicosanoids, such as prostaglandin E_2, are important regulators of the development and functions of T and B lymphocytes, NK cells, monocytes, and macrophages and modulate the synthesis of inflammatory and immunoregulatory cytokines (54). Other eicosanoids, such as leukotriene B_4, are potent chemoattractants and also have a role in the regulation of inflammation and cytokine synthesis. High levels of prostaglandin E_2 are present during acute and chronic inflammation and during infections, and the 4-series leukotrienes have been implicated as playing a role in a number of inflammatory diseases (55,56). Under normal conditions most eicosanoids are derived from arachidonic acid (20:4n-6). However, when high levels of n-3 PUFA are consumed in the diet, less arachidonic acid-derived eicosanoids are produced (55,57). Instead, eicosanoids are formed from eicosapentaenoic acid. This may be functionally significant, since the eicosanoids produced from eicosapentaenoic acid are usually less biologically potent than those formed from arachidonic acid (55,57). Thus, an n-3 PUFA-induced change in the amounts and types of different eicosanoids produced will have a significant influence on the functioning of immune and inflammatory cells and might partially account for the antiinflammatory and immunomodulatory actions of fish oils.

Although some of the effects of n-3 PUFA may be brought about by modulation of the amount and types of eicosanoids made, these fatty acids might elicit some of their effects by eicosanoid-independent mechanisms, including actions upon intracellular signaling pathways and transcription factor activity (see Refs. 59,60 for reviews). Recent studies showed that n-3 PUFA reduce agonist-stimulated diacylglycerol and ceramide production (14), phospholipase Cγ1 activation and subsequent inositol-1,4,5-trisphosphate production (58) and phosphatidic acid generation (59) in lymphocytes. Thus, n-3 PUFA appear to influence the generation of a range of lipid-derived second messengers in lymphocytes, and perhaps other immune and inflammatory cell types. This might account for the widespread effects of these fatty acids on the functioning of these cells.

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Dietary Fat Influences the Production of Th1- but Not Th2-Derived Cytokines

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Diets enriched in long-chain n-3 polyunsaturated fatty acids decreased the production of interleukin-2 (IL-2) by murine and human lymphocytes (1,2). IL-2 and interferon-γ (IFN-γ) are produced by the Th1 class of lymphocytes involved in cell-mediated immunity, macrophage and natural killer cell activation, transplant rejection, and inflammation. There is little information about the effects of dietary fatty acids on the production of IFN-γ or on the production of cytokines such as IL-4 which are produced by the Th2 class of lymphocytes involved in antibody-dependent responses and allergic reactions. Therefore, in the current study the effect of feeding mice diets enriched in different types of fatty acids upon IL-2, IFN-γ, and IL-4 production by spleen lymphocytes was investigated.

Male C57Bl/6 mice (n = 6 per diet) were fed for 6 wk on diets containing 200 g/kg of either coconut oil (CO), olive oil (OO), safflower oil (SO), or fish oil (FO). Spleen lymphocytes were prepared by standard procedures (3) and 2×10^6 cells were cultured for 24 h in the presence of 2.5 μg/mL concanavalin A, a T-cell mitogen; the final culture volume was 2 mL and the cultures contained 5% (vol/vol) fetal calf serum. The concentrations of the cytokines IL-2, IFN-γ, and IL-4 in the culture medium were measured by commercially available ELISA kits (Biosource International, Camarillo, CA). Lymphocyte proliferation was assessed as concanavalin A-stimulated [^{3}H]thymidine incorporation over the final 18 h of a 66-h culture period (3).

Each of the diets rich in unsaturated fatty acids (OO, SO, FO) decreased lymphocyte proliferation (i.e., thymidine incorporation) and IL-2 production compared with feeding the CO diet (Table 1). IFN-γ production was reduced by SO or FO feeding compared with feeding the CO- or OO-rich diets (Table 1). IL-4 production was not significantly affected by the type of fat in the diet, although production was lowest by cells from FO-fed mice (Table 1).

The effects of the unsaturated fatty acid-rich diets on spleen lymphocyte proliferation are in agreement with those published previously in a variety of species (3). Furthermore the reduced IL-2 production by spleen lymphocytes from FO-fed mice, at least compared with CO-fed mice, agrees with previous studies (1,2). However, this study shows that SO and FO feeding also decrease IFN-γ production. It is concluded that, compared with saturated fatty acids, polyunsaturated fatty acids decrease Th1 lymphocyte responses and that dietary fatty acids do not markedly influence Th2 lymphocyte responses. Since the Th1 response is involved in transplant rejection, autoimmunity, and inflammation, these observations might at least partly account for the beneficial effects of FO observed in these situations (see 1).

TABLE 1
Effect of Dietary Fats on Murine Spleen Lymphocyte Proliferation and Cytokine Production

Diet	Thymidine incorporation (cpm/well)	IL-2 (pg/mL)	IFN-γ (pg/mL)	IL-4 (pg/mL)
CO	34632 ± 651[a]	231 ± 44[a]	9.1 ± 1.0[a]	57 ± 10
OO	12484 ± 983[b]	127 ± 9[b]	8.5 ± 0.9[a]	70 ± 8
SO	16438 ± 6795[b]	106 ± 8[b]	3.1 ± 1.3[b]	67 ± 12
FO	10402 ± 1499[b]	137 ± 19[b]	1.3 ± 1.0[b]	48 ± 13

[a]Data are mean ± SEM for six animals fed on each diet. Values in a column indicated by different superscript letters are significantly different from one another ($P < 0.05$; one-way analysis of variance). CO, coconut oil; OO, olive oil; SO, safflower oil; FO, fish oil; IL-2, interleukin-2; IFN-γ, interferon-γ; IL-4, interleukin 4.

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Abbreviations: CO, coconut oil; FO, fish oil; IFN-γ, interferon γ; IL-2, interleukin-2; OO, olive oil; SO, safflower oil.

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Very Low Dietary Intake of n-3 Fatty Acids Affects the Immune Function of Healthy Elderly People

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The aging process, characterized by decreased antioxidant defenses, is usually accompanied by a moderate decline of immune functions. The n-3 polyunsaturated fatty acids (PUFA), abundant in fish oil, are associated with diverse human health benefits and offer some protection against cardiovascular, inflammatory, and autoimmune diseases. The efficacy of dietary n-3 PUFA in disorders involving overreactive immune responses is very likely to be due to their immunomodulatory effects (1,2). The n-3 PUFA are immunomodulatory both *in vitro*, when added to lymphocyte culture medium, and *in vivo*, after oral administration to animals and humans. In humans, most supplementation studies showing an immunomodulatory effect of n-3 PUFA used high fatty acid dosages, usually more than 2 g fatty acids per day. In these conditions of high supply, n-3 PUFA might have increased the cell susceptibility to lipid peroxidation, as both docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA), the main components of fish oil, are highly prone to peroxidation. Paradoxically, very low doses of n-3 PUFA were shown to protect cells, mainly platelets, from peroxidative stress and to exhibit some antioxidant properties (3,4). Thus, we sought to investigate the influence of a very low daily dose of n-3 PUFA on immune function in human healthy elderly people.

Healthy elderly subjects and experimental design. Twenty healthy elderly subjects (over 70 yr) were randomly assigned to a placebo group (600 mg/d sunflower oil, vitamin E 0.6 mg/g oil) or to a group consuming 600 mg/d RO-PUFA (Roche) (150 mg DHA + 30 mg EPA, Vitamin E 2 mg/g oil) for 6 wk. At the beginning of the study, all subjects had normal blood cell counts, normal cholesterolemia and triglycerides, and normal systolic and diastolic blood pressure. Blood samples were drawn by venipuncture before (on day 0) and after supplementation with either placebo or RO-PUFA (on day 42). Plasma was recovered after platelet sedimentation, and peripheral blood mononuclear cells were isolated by gradient density centrifugation. Several biochemical and immune parameters were assayed.

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Abbreviations: ConA, concanavalin A; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; OKT3, antioD3m Ab; PHA, phytohemagglutinin; PUFA, polyunsaturated fatty acid.

RESULTS AND DISCUSSION

Although no drastic modification in the fatty acid composition of plasma lipids was observed after supplementation with either the placebo or RO-PUFA, the proliferative responses of lymphocytes to several mitogens were significantly decreased in the RO-PUFA group as compared with controls (ConA: –45%, PHA: –31%, OKT3: –32%). The decreased proliferative response was accompanied by a slight lowering of their cytosolic cyclic nucleotide phosphodiesterase activity, a marked and significant increase of their particulate phosphodiesterase activity (+50%) and a slight but significant increase of cyclic nucleotide intracellular levels. In the same time, the glutathione peroxidase activity of mononuclear cells was markedly and significantly depressed in the RO-PUFA group. None of these modifications could be seen in the placebo group.

On the whole, these results demonstrate that even very low doses of n-3 fatty acids are sufficient to affect the immune responses of elderly subjects. The decreased lymphoproliferative responses observed after supplementation with RO-PUFA might be due to the modest but significant increase of cyclic nucleotide levels in mononuclear cells, cAMP being a well-recognized negative effector of the proliferative response.

On the other hand, we observed a marked decrease in the glutathione peroxidase activity of mononuclear cells, which may be interpreted as a marker of a lower peroxidative stress, as it was found, in the same study, in platelets of elderly people supplemented with RO-PUFA.

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The Effect of Dietary Fat on Cytokine Production by Murine Macrophages in Different Activation States

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Some studies report that diets rich in n-3 polyunsaturated fatty acids such as fish oil (FO) decrease pro-inflammatory cytokine production by macrophages (MØ), whereas other studies report that such diets increase production of these cytokines (1). It has been hypothesized that the use of MØ in different activation states may explain some of the contradictions in the literature (1). Thus, in this study we compared the effects of different high-fat diets on the production of pro- and anti-inflammatory cytokines by lipopolysaccharide (LPS)-stimulated resident and thioglycolate-elicited peritoneal MØ.

Male C57Bl/6 mice were fed for 8 wk on a low-fat (LF) diet (25 g/kg corn oil) or on diets containing 200 g/kg of either coconut oil (CO), safflower oil (SO), or FO. Resident MØ were collected by lavaging the peritoneal cavity with phosphate-buffered saline and were purified by adherence to tissue culture plates. Inflammatory MØ were elicited with an intraperitoneal injection of thioglycolate broth and collected from the peritoneal cavity by lavage 4 d postinjection. Purified MØ (1×10^6 cells) were cultured for 24 h in the presence of 10 μg/mL bacterial LPS; the total culture volume was 2 mL and the cultures contained 5% (vol/vol) fetal calf serum. The concentrations of the cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, and IL-10 in the culture medium were measured by commercially available ELISA kits (Biosource International, Camarillo, CA).

Inflammatory MØ from LF-fed mice produced higher concentrations of all cytokines measured than did resident MØ (Tables 1 and 2). Inflammatory MØ from FO-fed mice showed significantly decreased production of TNF-α and IL-1β compared to those from mice fed the other diets (Table 1). Resident MØ from mice fed FO had increased TNF-α production compared to those from CO-fed mice (Table 2). IL-6 production was higher from resident MØ from SO-fed mice than from those fed the LF or FO diets (Table 2). IL-10 production by both types of MØ was lowest following CO feeding (Tables 1 and 2). Diets rich in polyunsaturated fatty acids (SO, FO) significantly increased IL-10 production by resident MØ (Table 2).

TABLE 1
Effect of Dietary Fats on Cytokine Production by LPS-Stimulated Inflammatory MØ[a]

	Cytokine concentration (ng/mL)			
Diet	TNF-α	IL-1β	IL-6	IL-10
LF	3.1 ± 0.6^a	0.15 ± 0.03^a	14.9 ± 2.2	0.13 ± 0.02^a
CO	2.0 ± 0.3^a	0.13 ± 0.01^a	19.9 ± 1.5	0.08 ± 0.01^b
SO	0.7 ± 0.1^b	0.11 ± 0.02^a	16.8 ± 1.7	0.11 ± 0.01^a
FO	0.12 ± 0.02^c	0.06 ± 0.01^b	14.9 ± 1.7	0.11 ± 0.02^a

[a]Data are mean ± SEM for six mice fed on each diet. Values in a column indicated by different superscript letters are significantly different ($P < 0.05$; one-way analysis of variance). LF, low fat; CO, corn oil; SO, safflower oil; FO, fish oil; LPS, lipopolysaccharide; TNF, tumor necrosis factor-α; IL, interleukin; MØ, macrophages.

TABLE 2
Effect of Dietary Fats on Cytokine Production by LPS-Stimulated Resident MØ[a]

	Cytokine concentration (ng/mL)			
Diet	TNF-α	IL-1β	IL-6	IL-10
LF	$0.25 \pm 0.03^{a,b}$	0.05 ± 0.01	8.0 ± 1.6^b	0.09 ± 0.01^b
CO	0.14 ± 0.06^b	0.04 ± 0.01	$11.6 \pm 1.8^{a,b}$	0.07 ± 0.04^b
SO	$0.30 \pm 0.06^{a,b}$	0.03 ± 0.01	16.1 ± 3.1^a	0.19 ± 0.03^a
FO	0.35 ± 0.03^a	0.04 ± 0.01	9.9 ± 1.9^b	0.18 ± 0.03^a

[a]Data are mean ± SEM for six mice fed on each diet. Values in a column indicated by different superscript letters are significantly different ($P < 0.05$; one-way analysis of variance). See Table 1 for abbreviations.

It is concluded that dietary fat influences cytokine production by MØ but that the precise effects of dietary fat differ according to the activation state of the MØ studied. This may explain some of the contradictions in the literature (1). The significant reduction in pro-inflammatory cytokine (i.e., TNF-α, IL-1β) production by inflammatory MØ from FO-fed mice might at least partly account for the beneficial effects of FO observed in acute and chronic inflammatory conditions (1,2).

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Abbreviations: CO, coconut oil; FO, fish oil; IL, interleukin; LF, low fat; LPS, lipopolysaccharides; MØ, macrophages; SO, safflower oil; TNF, tumor necrosis factor-α.

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Phospholipid Modulation of Monocyte Oxidative Activity Measured by Luminol-Enhanced Chemiluminescence

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The lung is susceptible to inflammatory and oxidative injury leading to tissue damage and decreased function. Infiltrating neutrophils and monocytes and resident macrophages are a likely source of the damaging molecules. It is hypothesized that pulmonary surfactant rich in phospholipid may modulate the cytotoxic and inflammatory function of alveolar macrophages. Changes in membrane lipid composition may alter the immunological responses of monocytes to infection, including endotoxin (lipopolysaccharide, LPS). The present study was undertaken to determine the role of phospholipids in the modulation of monocyte respiratory burst activity.

Materials and methods. Human monocyte-derived cell line (MonoMac 6) was from the German Cell and Tissue Culture Collection, Braunsweig, Germany. Phospholipid species luminol and LPS were from Sigma Chemical Co. (Poole, United Kingdom). Phospholipids (10–500 μg/mL) were dissolved in RPMI 1640 medium and added to 1×10^6/mL MonoMac 6 cells with or without 100 ng/mL LPS for 12 h at 37°C. The cells were then washed three times, and the respiratory burst was triggered by the addition of zymosan in the presence of luminol. The production of reactive oxygen species was measured by chemiluminescence using an automated luminometer. Cells not preincubated with LPS to prime them produced little superoxide and acted as controls.

Results. The phospholipid species investigated all had effects on the priming of monocytes for superoxide production in the respiratory burst. At the concentrations used (10–500 mg/mL), they had no direct effect on the triggering of the respiratory burst with zymosan or phorbol 12,myristate 13,acetate. Therefore the phospholipids altered the monocyte responses to LPS, resulting in a primed state for enhanced superoxide generation.

Mixed lipid species of phosphatidylcholine inhibited the LPS-induced priming of the monocyte respiratory burst in a dose-dependent manner from 100–500 μg/mL. However, dipalmitoylphosphatidylcholine, the major phospholipid species in pulmonary surfactant, was stimulatory at 100–500 μg/mL but was inhibitory at concentrations below this. Mixed species of phosphatidylethanolamine had a suppressive effect on the respiratory burst in a dose-dependent manner up to 100 μg/mL, whereas at high concentrations (250–500 μg/mL) sphingomyelin had a stimulatory effect. The effects of other molecular species of the phospholipids are currently being investigated.

Conclusions. This study shows that phospholipids can modulate the respiratory burst in monocytes and that this is at the level of monocyte priming. The effect of the phospholipids was dependent both on the lipid acyl composition and on the polar headgroup composition. These results suggest that phospholipid species may regulate monocyte and macrophage oxidative responses *in vivo*, and alterations in cellular phospholipid content and composition may affect the host responses to infection. Manipulation of the extracellular lipid content may be a novel strategy in the treatment of inflammatory lung diseases.

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Abbreviations: LPS, lipopolysaccharide.

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Novel, Selective Δ6 or Δ5 Fatty Acid Desaturase Inhibitors as Antiinflammatory Agents in Mice

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Aim. The antiinflammatory properties of essential fatty acid deficiency or n-3 polyunsaturated fatty acid supplementation have been attributed to a reduced content of arachidonic acid (AA). A novel antiinflammatory approach would be to deplete AA by decreasing its endogenous synthesis *via* inhibition of the Δ5 and/or Δ6 fatty acid desaturase.

Methods. Mixed Δ6/Δ5 or selective Δ6 or Δ5 desaturase inhibitors were identified during the screening of chemical and natural product libraries. The antiinflammatory properties of CP-24879, a *p*-hydroxyaniline derivative that is a mixed Δ6/Δ5 desaturase inhibitor and SC-26196, a piperazine derivative that is a selective Δ6 desaturase inhibitor, were further evaluated *in vitro* and *in vivo*. CP-24879 was utilized for mechanism validation *in vitro* using mouse mastocytoma ABMC-7 cells. SC-26196 was nontoxic and had pharmacokinetic and pharmacodynamic profiles that allowed for the evaluation of chronic inhibition of desaturase activity *in vivo*. Effects of desaturase inhibition on eicosanoid levels were measured by enzyme-linked immunosorbent assay, while changes in lipid/fatty acid composition of liver, plasma, and peritoneal cells were quantified by gas chromatography and tandem mass spectrometry. Inflammation was quantified by measuring edema and myeloperoxidase activity in the carrageenan-induced paw edema model in the mouse.

Results. In ABMC-7 cells cultured chronically with CP-24879, there was a concentration-dependent inhibition of desaturase activity that correlated with the degree of depletion of AA and decreased production of leukotriene C_4. In mice dosed with SC-26196, there was a dose-dependent decrease in paw edema and a corresponding decrease in the level of AA in liver, plasma, and peritoneal cells. At the highest dose of SC-26196 administered (100 mpk, ip, bid, for 9 d), mitigation of edema was similar to that obtained with essential fatty acid deficiency or acute dosing with indomethacin (10 mpk, ig). In liver and plasma, AA was selectively decreased while linoleic acid was selectively increased in phosphatidylcholine, phosphatidylserine, and cholesteroyl ester lipid species. The antiinflammatory properties of SC-26196 were consistent with its proposed mechanism of action. Firstly, dose-dependent inhibition of liver Δ6 desaturase activity correlated with decreased paw edema. Secondly, the onset of edema was time-dependent, indicating that AA first had to be decreased in the target tissue. Thirdly, the AA content in liver, plasma, and peritoneal cells decreased dose-dependently. Lastly, in the presence of SC-26196, dietary AA, but not dietary oleic acid, reversed the antiinflammatory effects of desaturase inhibition; AA increased in peritoneal cells and was accompanied by dose-dependent increases in both edema and myeloperoxidase activity.

Conclusions. These results suggest that Δ5 and/or Δ6 desaturase inhibitors manifest an antiinflammatory response by decreasing the level of AA. Either desaturase may be a target for the development of antiinflammatory drugs whose mechanism of action is unique.

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Abbreviation: AA, arachidonic acid.

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In Vitro Interactions of γ-Linolenic Acid and Arachidonic Acid with Ceftazidime on Multiresistant *Pseudomonas aeruginosa*

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γ-Linolenic acid (GLA) inhibits the growth of *Escherichia coli* (1), and when applied in combination with arachidonic acid (AA) it acts bactericidally on *Pseudomonas aeruginosa* susceptible to a variety of antibiotics (2). Multiresistant *P. aeruginosa* is today a common cause of nosocomial infections accompanied by high morbidity due to the lack of active antibiotics (3). We applied the combination of GLA and AA on such strains studying also whether the presence of GLA or of AA might render them susceptible to ceftazidime since the latter is the most potent antipseudomonal agent (3).

MATERIALS AND METHODS

Nineteen isolates resistant to ceftazidime, imipenem, ciprofloxacin, and amikacin were exposed over time to GLA and/or AA at a 150 or 300 μg/mL concentration with/without ceftazidime. Acid ethyl esters (Sigma, St. Louis, MO) were dissolved in ethanol 99% (Merck, Darmstadt, Germany) (1) and added into tubes of a 10 mL final volume containing Mueller-Hinton broth (Oxoid Ltd., London, United Kingdom) and a 5×10^6 CFU/mL log-phase inoculum of each strain. Ceftazidime was applied at a concentration of 16 μg/mL which is equal to its mean serum levels. Bacterial growth was determined in each tube at 0 time, 3, 5, and 24 h of incubation. Growth in the presence of ethanol 99% and of ceftazidime served as control, and a total of 190 killing curves were performed. As for antibiotics, bactericidal effect was considered any $\geq 3 \log_{10}$ decrease of viable cells compared to controls.

RESULTS AND DISCUSSION

Mean $\log_{10}$ changes by the tested combinations are shown in Figure 1. A killing effect was found by the 300 μg/mL GLA + 300 μg/mL AA combination on 9 (47.4%) isolates. Our findings signify that GLA combined *in vitro* to AA acts bactericidally on a considerable number of multiresistant *P. aeruginosa* at a concentration of 300 μg/mL. The percentage of the achievable killing effect is significant when considering that these isolates are resistant to all antibiotics (3). It is important to state that either GLA or AA augments the activity of ceftazidime, an effect also observed in the presence of a 150 μg/mL concentration of each acid (Fig. 1).

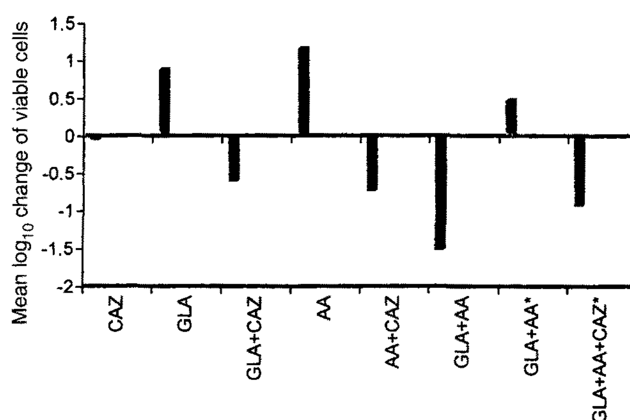

FIG. 1. Mean changes of the starting inoculum (6.69 $\log_{10}$) after 24 h of incubation. Acids were applied at a 300 μg/mL concentration. CAZ, ceftazidime; *, acids applied at a 150 μg/mL concentration; GLA, γ-linolenic acid; AA, arachidonic acid.

The clinical significance of the presented findings is supported by the fact that both the 150 μg/mL concentration of each acid and the 16 μg/mL concentration of ceftazidime are easily achievable in serum (3). The future perspectives of our study are supported by studies where the intravenous administration of GLA in patients with advanced human immunodeficiency virus (HIV) infection resulted in a moderate decrease of their HIV load and an increase of their CD4(+) count (4).

The most probable explanation of the action of GLA and AA might be lipid peroxidation, as it was found by our study group that their bactericidal activity on susceptible isolates was accompanied by the production of thiobarbiturate-reactive substances and limited in the presence of vitamin E (2). Since mul-

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Abbreviations: AA, arachidonic acid; GLA, γ-linolenic acid.

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tiresistant *P. aeruginosa* is characterized by an impermeable cell wall, limiting the entrance of antibiotics in the bacterial cell (3), these peroxides might attack the cell wall and render the resistant bacterial cell susceptible to ceftazidime (Fig. 1).

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Regulation of the Biosynthesis of 22:5n-6 and 22:6n-3: A Complex Intracellular Process

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ABSTRACT: Both 22:4n-6 and 22:5n-3 are synthesized from n-6 and n-3 fatty acid precursors in the endoplasmic reticulum. The synthesis of both 22:5n-6 and 22:6n-3 requires that 22:4n-6 and 22:5n-3 are metabolized, respectively, to 24:5n-6 and 24:6n-3 in the endoplasmic reticulum. These two 24-carbon acids must then move to peroxisomes for partial degradation followed by the movement of 22:5n-6 and 22:6n-3 back to the endoplasmic reticulum for use as substrates in membrane lipid biosynthesis. Clearly an understanding of the control of intracellular fatty acid movement as well as of the reactions carried out by microsomes, peroxisomes, and mitochondria are all required in order to understand not only what regulates the biosynthesis of 22:5n-6 and 22:6n-3 but also why most tissue lipids selectively accumulate 22:6n-3.

During the past three or four decades the results of numerous investigators led to the general concept that microsomes contain position-specific acyl-CoA-dependent 5- and 6-desaturases, but there was no evidence for the presence of an analogous 4-desaturase (1). In 1973 Ayala *et al.* (2) reported that testes and liver microsomes were unable to desaturate 7,10,13,16-22:4. We subsequently showed that neither 7,10,13,16,19-22:5 nor 7,10,13,16-22:4 was desaturated at position 4 (3,4). Instead these two 22-carbon acids were chain elongated to yield acids with their first double bond at position 9. These acids were then desaturated at position 6, but it is not known if the 6-desaturase that recognizes 24-carbon substrates is different from the enzyme that desaturates linoleate and linolenate (5). The implications of these findings were that 24-carbon acids are produced in the endoplasmic reticulum, but they must then move to another intracellular site for partial degradation. When [3-^{14}C]labeled 22- and 24-carbon unsaturated fatty acids were incubated with fibroblasts, [1-^{14}C]labeled acids were incorporated into membrane lipids. When identical experiments were carried out with fibroblasts from patients with Zellweger's syndrome, who lack peroxisomes, no chain-shortened metabolites were esterified. These studies establish that partial β-oxidation is a peroxisomal event (6,7).

The biosynthesis of 22-carbon fatty acids, with their first double bond at position 4, is thus not a process that is confined to microsomes, but rather the synthesis of these compounds requires considerable movement of fatty acids within the cell. The diagram in Figure 1 depicts an overview of some of these processes. The diagram implies that when 24-carbon fatty acids are produced in the endoplasmic reticulum they may be used for membrane lipid biosynthesis. Alternatively, they may potentially move as either acyl-CoA or free fatty acids to either mitochondria or peroxisomes. Presumably they would be completely degraded by mitochondria but only partially β-oxidized by peroxisomes. The diagram further implies that there must be structural features in the fatty acid molecule so that when 22:5n-6 or 22:6n-3 is produced, its pre-

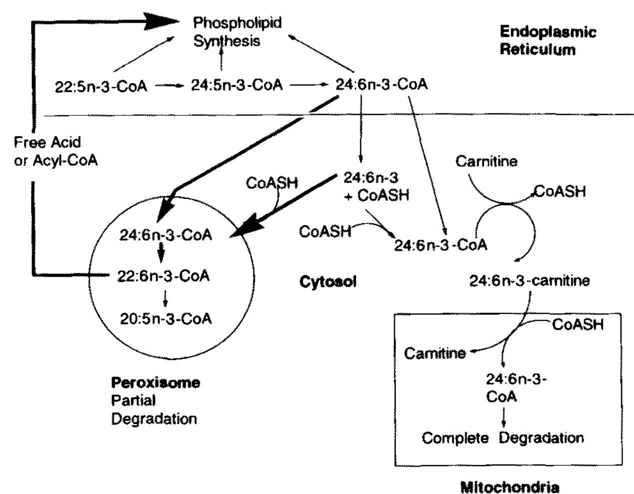

FIG. 1. The intracellular movement and metabolism of unsaturated fatty acids. The diagram implies that when 24:6n-3 is produced in the endoplasmic reticulum, it may be used as a substrate for phospholipid synthesis. Its preferred metabolic fate in liver, however, is to move to another subcellular particle. It is not established whether it moves directly as the acyl-CoA, or whether the acyl-CoA is hydrolyzed in the cytosol, followed by the reactivation of 24:6n-3 at the subcellular site, where it is to be metabolized. The diagram implies that if 24:6n-3 is degraded by mitochondria, the process would probably proceed to completion. In liver, this is a minor pathway. The dark arrows in the diagram show that the preferred metabolic fate of 24:6n-3 is to move to peroxisomes, and after one cycle of degradation, the 22:6n-3 moves back to the endoplasmic reticulum, where it is used as a substrate for phospholipid synthesis.

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ferred metabolic fate is to move out of peroxisomes rather than to serve as substrates for continued degradation. If 22:5n-6 and 22:6n-3 are to be used preferentially for membrane lipid biosynthesis, it would seem likely that they should primarily move back to the endoplasmic reticulum rather than serve as substrates for complete degradation by mitochondria. The objective of this brief review is evaluate, and indeed to speculate, as to what controls some of the processes depicted in Figure 1.

INCORPORATION OF FATTY ACIDS INTO MEMBRANE LIPIDS

If 24-carbon acids produced in the endoplasmic reticulum in liver were good substrates for membrane lipid biosynthesis, their movement to peroxisomes for partial degradation would be curtailed. When [3-^{14}C]labeled 24:5n-3 and 24:6n-3 (3) and the analogous n-6 fatty acids (4) were incubated with hepatocytes, only very small amounts of these compounds were incorporated directly into phospholipids. When these acids were converted to their acyl-CoA and incubated with microsomes and 1-acyl-*sn*-glycero-3-phosphocholine, their rates of acylation were either not detectable or very low when compared with 20- and 22-carbon acyl-CoA (8,9). These findings suggest that in liver when 24-carbon acids are produced they are neither further chain-elongated nor incorporated into phospholipids. Instead the 24-carbon acids preferentially move to another cellular compartment for subsequent metabolism. The phospholipids of many tissues contain small amounts of very long chain fatty acids with up to 40 carbon atoms and six double bonds (10,11). Clearly when 24-carbon acids are produced in the endoplasmic reticulum, in these tissues, they have other fates than to move to another cellular compartment. It is not known how many chain-elongating enzymes are found in liver or whether additional enzymes are expressed in extrahepatic tissues (12). Clearly when these long-chain compounds are produced, they are, in part, esterified, but again little is known about the numbers of enzymes that carry out these reactions or what differences exist in tissue-specific enzyme expression.

MOVEMENT OF FATTY ACIDS WITHIN THE CELL

In some cases the substrates and the products of microsomal chain elongation and desaturation are the acyl-CoA (13,14). Although it was not established, when 24-carbon acids are produced in the endoplasmic reticulum, they are likely acyl-CoAs and not free fatty acids. However, it is not known whether acyl-CoA can move directly from one cellular compartment to another or whether they are hydrolyzed by cytosolic acyl-Co hydrolyases (15). Microsomes, peroxisomes, and mitochondria all have a long-chain acyl-CoA synthetase, while a second enzyme, referred to as a very long chain activating enzyme, is not found in mitochondria, but it is present in microsomes and peroxisomes (16). It seems likely that when acyl-CoA are produced, as a product of metabolism in a cellular compartment, they are subsequently hydrolyzed in the cytosol, followed by reactivation where they are to be metabolized.

MITOCHONDRIAL FATTY ACID METABOLISM

It is generally accepted that, under normal physiological conditions, once mitochondrial fatty acid β-oxidation is initiated it proceeds to completion (17). If 24-carbon acids moved from microsomes to mitochondria and were used as substrates for total β-oxidation, they would have the potential of reducing the production of both 22:5n-6 and 22:6n-3. Rates of palmitic acid and linoleic acid activation by mitochondria were between 50–60 nmol/min/mg of mitochondrial protein. Rates of 24:4n-6 and 24:5n-6 activation were only 1–2 nmol/min/mg of mitochondrial protein. Rates of acylcarnitine synthesis, using 16:0-CoA and linoleoyl-CoA as substrates, were 10–15 nmol/min/mg of mitochondrial protein, while the rates of acylcarnitine synthesis using the 24-carbon acyl-CoA derivatives were less than 2 nmol/min/mg of mitochondrial protein. When 16:0-CoA and linoleoyl-CoA were incubated with mitochondria, 35–40 ng atoms of oxygen/min/mg of mitochondrial protein were used. When the CoA derivatives of 24:4n-6 and 24:5n-6 were used, these values were, respectively, 2 and 1.8. Collectively these studies show that 24-carbon n-6 acids were poor substrates for acyl-CoA synthesis, acylcarnitine synthesis, and mitochondrial β-oxidation (Chen, Q., and Sprecher, H., unpublished data). These results suggest that when 24-carbon n-6 and n-3 fatty acids are made in the endoplasmic reticulum, they are preferentially channeled to peroxisomes for further metabolism, rather than being degraded by mitochondria.

PEROXISOMAL FATTY ACID METABOLISM

Peroxisomes are able to activate 24-carbon acids although at a somewhat lower rate than was found for 20- and 22-carbon fatty acids. Rates of activation for the n-3 acids, 20:5, 22:5, 22:6, 24:5, and 24:6 were, respectively, 126, 72, 37, 20, and 16 nmol/min/mg of peroxisomal protein from rats whose diets contained 0.5% clofibrate (8). Similar values were obtained for the corresponding n-6 fatty acids (9). The relative contribution of the long chain and the very long chain acyl-CoA synthetase in activating these fatty acids is not known. Since 24-carbon fatty acids were very poor substrates for activation in mitochondria, the results suggest that the very long chain enzyme probably is used for activating these fatty acids in peroxisomes. All of our studies were thus carried out by generating fatty acids *in situ*, which may well be what takes place *in vivo*.

When any fatty acid is produced in the microsome, it has the potential of moving to peroxisomes for partial degradation. It is generally accepted that peroxisomes partially degrade long-chain fatty acids and that the chain-shortened products are channeled to mitochondria to complete the degradative process (18). The synthesis of 22:5n-6 and 22:6n-3, respectively, from 24:5n-6 and 24:6n-3 implies that there must be some structural feature in 22:5n-6 and 22:6n-3, so that when they are produced

they are preferentially used for membrane lipid synthesis rather than as substrates for continued degradation. Peroxisomes are unable to esterify 1-acyl-*sn*-glycero-3-phosphocholine (19). When 22:5n-6 and 22:6n-3 are produced in peroxisomes, the diagram in Figure 1 implies that they must move to microsomes where they are used as substrates for membrane lipid synthesis. We carried out a series of experiments that we termed "mixing experiments" that have as their objective to determine what structural features in a fatty acid make it a preferred substrate for movement to microsomes for acylation rather than continued peroxisomal β-oxidation. In this protocol we compare the amount of acid-soluble radioactivity produced when peroxisomes are incubated alone with labeled fatty acids vs. what is obtained when microsomes and 1-acyl-*sn*-glycero-3-phosphocholine are included in the incubation. With the later incubations, phospholipids are isolated and the amounts of metabolites esterified into the acceptor are quantified. After a 30-min incubation, using 100 nmol of [3-^{14}C]labeled 24:4n-6 or 24:5n-3, 30–40 nmol of acid-soluble radioactivity was produced. When microsomes and 1-acyl-*sn*-glycero-3-phosphocholine were added to the incubations, there was only about a 10% decrease in the production of acid-soluble radioactivity. Two cycles of fatty acid β-oxidation of [3-^{14}C]labeled 9,12,15,18-24:4 and 9,12,15,18,21-24:5 yield, respectively, arachidonate and 20:5n-3 with loss of label. These two degradative cycles require only the enzymes of saturated fatty acid degradation. When [3-^{14}C]labeled 6,9,12,15,18-24:5 and 6,9,12,15,18,21-24:6 were incubated alone with peroxisomes, only about 8–10 nmol of acid-soluble radioactivity was produced after 30 min. When microsomes and 1-acyl-*sn*-glycero-3-phosphocholine were included in the incubations, there was an approximate 50% decrease in the production of acid-soluble radioactivity. The first cycle of 24:5n-6 and 24:6n-3 degradation also only requires the enzymes of saturated fatty-acid degradation. As soon as [1-^{14}C]labeled 4,7,10,13,16-22;5 and 4,7,10,13,16,19-22:6 are produced, their continued degradation requires both NADPH-dependent 2,4-dienoyl-CoA reductase and Δ^3,Δ^2-enoyl-CoA isomerase which is a component part of the peroxisomal trifunctional enzyme (20). In all of our studies, when an acid with its first double bond at position 4 is incubated with peroxisomes, or when an acid is produced with its first double bond at position 4, metabolites with their first two double bonds at the 2-*trans*-4-*cis*-positions always accumulate (8,9,21,22). These results suggest that the reaction catalyzed by the reductase is a control step in the peroxisomal degradation of unsaturated fatty acids. As a corollary the preferred metabolic fate of a fatty acid, with its first double bond at position 4, is to move out of peroxisomes to microsomes for use in membrane lipid synthesis rather than to serve as a substrate for continued peroxisomal degradation. This general hypothesis is supported by comparing the amounts of metabolites that were esterified into the acceptor when the four 24-carbon n-3 and n-6 fatty acids were incubated with peroxisomes in the presence of microsomes and 1-acyl-*sn*-glycero-3-phosphocholine. As shown in Table 1, only small amounts of all four 24-carbon acids were esterified. Most importantly, the amounts of [1-^{14}C]labeled 22:4n-6 and 22:5n-3 that were produced, respectively, from [3-^{14}C]labeled 24:4n-6 and 24:5n-3 and esterified were much less than was found for the two 22-carbon acids with their first double bond at position 4, which are produced from [3-^{14}C]labeled 24:5n-6 and 24:6n-3.

TABLE 1
Incorporation of Radioactive Acids into Phospholipids When Peroxisomes Were Incubated with Microsomes and 1-Acyl-*sn*-glycero-3-phosphocholine[a]

	Substrate			
	24:4n-6	24:5n-6	24:5n-3	24:6n-3
	(nmols of esterified radioactive fatty acids)			
24:4n-6	3			
22:4n-6	9			
24:5n-6		2		
22:5n-6		32		
24:5n-3			3	
22:5n-3			6	
24:6n-3				6
22:6n-3				41

[a]Peroxisomes (300 μg of protein) were incubated with 100 μM each of [3-^{14}C]labeled fatty acids and 1-acyl-*sn*-glycero-3-phosphocholine along with 300 mg of microsomal protein. After 30 min the labeled acids in phospholipids were quantified.

Once 22:5n-6 and 22:6n-3 are produced in peroxisomes, they presumably must be transported back to the endoplasmic reticulum for use as substrates in membrane lipid biosynthesis. It remains to be determined in what form fatty acids are transported out of peroxisomes, i.e., acyl-CoA or free fatty acids. The production of 22:5n-6 and 22:6n-3 from dietary linoleate and linolenate is a lengthy process. It would seem likely that the primary metabolic fate of these 22-carbon acids is to serve as precursors for phospholipid synthesis. However, both of these acids must also be degraded. When we incubated [3-^{14}C]labeled 24:5n-6 and 24:6n-3 with peroxisomes in the presence of microsomes and 1-acyl-*sn*-glycero-3-phosphocholine, the production of acid-soluble radioactivity was depressed but not abolished, showing that under these conditions some 22:5n-6 and 22:6n-3 were degraded (8,9). The relative contribution of peroxisomes vs. mitochondria in degrading 22:5n-6 and 22:6n-3, however, is not established. We found that 22:5n-6 was a poor substrate for β-oxidation by liver mitochondria (Chen, Q., and Sprecher, H., unpublished data), but Nada and his colleagues (23) reported that 22:6n-3 was readily degraded by heart mitochondria. Clearly, more studies are required not only to determine the intracellular site for the degradation of long-chain polyunsaturated fatty acids but also whether there might be tissue-specific differences.

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The Growing Family of Peroxisomal Thiolases

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Thiolases catalyze the reversible thiolytic cleavage of different fatty 3-oxoacyl-CoA. Animal tissues contain two classes of thiolase: acetoacetyl-CoA specific thiolases and 3-oxoacyl-CoA thiolases that possess a broad substrate specificity and act on medium and long straight-chain 3-oxoacyl-CoA. Thiolases participate in different catabolic (fatty acid oxidation and bile acid formation) and anabolic (cholesterogenesis, ketone body synthesis, fatty acid elongation) processes. Peroxisomes play a role in most of these pathways and harbor different thiolases.

The aim of the present study was to elucidate the functional significance of multiple thiolases in mammalian peroxisomes. Methods for the purification of peroxisomal thiolases were based on the alteration in their chromatographic properties in presence or absence of low concentrations of CoA. In peroxisomes from normal rat liver, four main thiolases were detected: 3-oxoacyl-CoA thiolase A (thiolase A), sterol carrier protein 2/3-oxoacyl-CoA thiolase (SCP-2/thiolase), and two new enzymes: acetoacetyl-CoA thiolase and a long chain 3-oxoacyl-CoA thiolase (LC-thiolase).

Purified preparations of thiolase A and 3-oxoacyl-CoA thiolase B (thiolase B, the enzyme was isolated from clofibrate-treated rat liver) reacted with antibodies raised against thiolase A, but could be distinguished by their isoelectric points, their N-terminal amino acid sequences and their stability, the latter being higher for thiolase A. Thiolase A displayed a substrate specificity that is roughly similar to that of thiolase B. Both enzymes reacted with short, medium, and long straight-chain 3-oxoacyl-CoA (1).

Purified preparation of SCP-2/thiolase consisted of a 58 and a 46 kDa polypeptide. Internal peptide sequencing and immunoblot analysis revealed that the 46 kDa polypeptide is the N-terminal (thiolase) domain of SCP-2/thiolase. The enzyme exists *in vivo* as a mixture of three isoforms consisting of homo- and heterodimeric combinations of the 58 and 46 kDa subunits. SCP-2/thiolase is active with medium and long straight-chain 3-oxoacyl-CoA but also with 2-methyl-branched 3-oxoacyl-CoA and the 24-oxoacyl-CoA derivative of trihydroxycoprostanic acid (2).

Acetoacetyl-CoA thiolase is a homotetrameric protein with a subunit molecular mass of 42 kDa. The enzyme cleaves only acetoacetyl-CoA as substrate and is also active in the reverse direction (condensation of two molecules of acetyl-CoA). Kinetic analysis, internal peptide microsequencing, immunological, and some other data indicate that the peroxisomal, cytoplasmic, and mitochondrial acetoacetyl-CoA thiolases are three closely related but distinct proteins. Acetoacetyl-CoA thiolase from peroxisomes most probably catalyzes the initial step of cholesterol synthesis in these organelles.

Peroxisomal LC-thiolase has an unusual substrate specificity that distinguishes it from other known mammalian thiolases. After subfractionation of peroxisomes, the enzyme activity was recovered in the membrane fraction.

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Abbreviations: LC-thiolase, long chain 3-oxoacyl CoA specific thiolase; SCP-2/thiolase, sterol carrier protein 2/3-oxoacyl-CoA thiolase.

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α-Oxidation of 3-Methyl-branched Fatty Acids: A Revised Pathway Confined to Peroxisomes

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α-Oxidation of phytanic acid (3,7,11,15-tetramethylhexadecanoic acid), a 3-methyl-branched isoprenoid-derived fatty acid, was generally believed to consist of a hydroxylation in the 2-position, followed by an oxidative decarboxylation generating CO_2 and a fatty acid shortened by one carbon. Until recently, however, the α-oxidation process has remained a matter of debate as to its subcellular localization, the reaction intermediates, and the cofactors involved (1). The observation that formate is formed during α-oxidation of phytanic acid in human skin fibroblasts (2) has been the basis for the recent unraveling of the α-oxidation process.

For our study, we used synthetic 3-methyl-branched fatty acids that were shown to be valid substitutes for phytanic acid in the study of the α-oxidation process.

As in human fibroblasts, formate was formed during α-oxidation of 3-methyl-branched fatty acids in intact rat hepatocytes (3) and was shown to be the first product which is then converted to CO_2, presumably in an NAD^+-dependent cytosolic process (4). Consequently, α-oxidation was assessed by the sum of formate and CO_2 in all further experiments.

When studied in permeabilized rat hepatocytes and rat liver homogenates, the α-oxidation process required ATP, CoA, Mg^{2+}, Fe^{2+}, ascorbate, and 2-oxoglutarate (4). Subsequent experiments in subcellular fractions of rat liver demonstrated that this set of cofactors reflects two steps: first, activation by an acyl-CoA synthetase requiring ATP, CoA, and Mg^{2+} and then 2-hydroxylation of the resulting 3-methylacyl-CoA by a 3-methylacyl-CoA hydroxylase requiring Fe^{2+}, ascorbate, and 2-oxoglutarate (4). The whole α-oxidation pathway up to the formation of formate was clearly a peroxisomal process (4). Mihalik *et al.* (5) came to the same conclusion using a different approach. Furthermore, our results suggest that the 2-hydroxylation is a membrane-bound process (4). The 2-hydroxy-3-methylacyl-CoA is cleaved into formyl-CoA and a 2-methyl-branched fatty aldehyde by a 2-hydroxy-3-methylacyl-CoA lyase, probably located in the peroxisomal matrix and requiring no additional cofactors. In peroxisomes formyl-CoA is actively converted to formate (6), while the 2-methyl-branched fatty aldehyde is converted to a 2-methyl-branched fatty acid in the presence of NAD^+ (7). After activation to its CoA-ester, the 2-methyl-branched fatty acid can be degraded by peroxisomal β-oxidation.

Our studies show that in rat liver the whole α-oxidation process up to the formation of formate and a 2-methyl-branched fatty acid consists of five enzymatic steps, which are all confined to peroxisomes.

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Adipose Peroxisome Proliferator-Activated Receptor γ mRNA Expression in Insulin-Resistant Obese Patients: Relationship with Adipocyte Membrane Phospholipids

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Peroxisome proliferator-activated receptors (PPARs) control the expression of genes implicated in the metabolism of intra- and extracellular lipids. The subtype PPARγ is also an essential factor for adipocyte differentiation, and it has now become clear that it plays a crucial role in the action of insulin. Thiazolidinediones enhance the sensitivity of obese patients and patients with non-insulin-dependent diabetes mellitus (NIDDM) to insulin by activating PPARγ. As the amount of adipose tissue PPARγ mRNA tends to increase in response to activators and/or ligands, several attempts have been made to find disregulation of PPARγ in the tissues of obese and NIDDM patients. The results are not clear-cut, and there is no definite evidence that PPARγ expression in the skeletal muscle and adipose tissue of normal, obese, and diabetic subjects differs. Besides their role on lipid metabolism, there is also an indication of a relationship between PPAR and phospholipid metabolism. Clofibrate, a peroxisome proliferator, inhibits the synthesis of all major cellular phospholipids in cultured human fibroblasts. In a previous study, we found that changes in the phospholipid composition of the plasma membranes of adipocytes from abdominal subcutaneous fat biopsies are linked to insulin resistance in obese patients. Multivariate analysis showed that the membrane sphingomyelin (SM) content was the major independent predictor of insulin resistance markers [fasting plasma insulin and homeostasis model assessment (HOMA) values]. We have therefore examined the association between the phospholipid composition of adipocyte membranes and the expression of the PPARγ gene. This was done by assaying classes of phospholipids in adipocyte plasma membranes and by determining the PPARγ mRNA levels in a group of obese nondiabetic subjects with variable degrees of insulin resistance.

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Abbreviations: FPI, fasting plasma insulin; HOMA, homeostasis model assessment; NIDDM, non-insulin-dependent diabetes mellitus; PPAR, peroxisome proliferator-activated receptor; SM, sphingomyelin.

Abdominal subcutaneous fat tissue was obtained from 23 obese nondiabetic patients: age: 43.7 ± 10.7 yr, body mass index: 36.3 ± 10.5 kg/m^2, fasting blood glucose: 5.10 ± 1.14 mmol/L. The fasting plasma insulin concentrations (FPI: 142.0 ± 70.5 pmol/L) and the HOMA estimates: 4.6 ± 2.8 were used as markers of insulin resistance. PPARγ mRNA levels were measured by a reverse transcription-competitive polymerase chain reaction method. Eighteen of the 23 biopsies contained enough fat (>5 g) to allow isolation of adipocyte plasma membranes. The lipids were extracted, and the phospholipid composition of the membranes was analyzed by high-performance liquid chromatography.

FPI and HOMA values were positively correlated with membrane SM (FPI $r = 0.744$, $P < 0.0005$; HOMA: $r = 0.667$, $P < 005$), phosphatidylethanolamine (FPI: $r = 0.506$, $P < 0.05$; HOMA: $r = 0.531$, $P < 0.05$), and phosphatidylcholine (FPI: $r = 0.589$, $P < 0.01$; HOMA: $r = 0.480$, $P < 0.05$). The amounts of PPARγ mRNA were positively correlated with phosphatidylinositol ($r = 0.512$, $P < 0.05$) and negatively with phosphatidylcholine ($r = -0.303$, $P < 0.05$) and SM ($r = -0.551$, $P < 0.05$). Lastly, the PPARγ mRNA concentration was negatively correlated with the fasting plasma insulin ($r = -0.433$, $P < 0.05$) and HOMA values ($r = -0.496$, $P < 0.05$).

Changes in the phospholipid composition of the adipocyte plasma membranes are linked to insulin resistance in obese nondiabetic patients. The membrane SM content is strongly positively correlated with the two markers of insulin resistance studied. Although SM may accumulate in membranes for other reasons, the negative correlation between PPARγ mRNA levels and the membrane SM content supports the hypothesis that PPARγ could play a role in phospholipid metabolism. Tumor necrosis factor-α which down-regulates PPARγ and affects the SM pathway may also be important. Finally, low PPARγ mRNA levels are associated with high fasting plasma insulin and HOMA values. These results suggest that those obese women with lower insulin sensitivity are also characterized by a lower PPARγ gene expression in their subcutaneous adipose tissue.

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Mitochondrial 3-Hydroxy-3-methylglutaryl CoA Synthase and Carnitine Palmitoyltransferase II Are Potential Control Sites of Hepatic Ketogenesis Under Conditions of Peroxisome Proliferation

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3-Thia fatty acids are potent hypolipidemic fatty acid derivatives and mitochondrion and peroxisome proliferators. They increase the transport of fatty acids into the mitochondria and increase the capacity of the β-oxidation process. Administration of 3-thia fatty acids to rats was followed by a significantly increased production of acid-soluble products and ketone bodies. The levels of nonesterified fatty acids in plasma were decreased, whereas the hepatic mRNA levels of fatty acid-binding protein were increased. Increased mitochondrial carnitine palmitoyltransferase (CPT)-II and 3-hydroxy-3-methylglutaryl (HMG)-CoA synthase activities, immunodetectable proteins, and mRNA levels were also observed in the liver. In contrast, the mitochondrial CPT-I mRNA levels were unchanged, and CPT-I enzyme activity was slightly reduced. The immunodetectable CPT-I protein, however, was increased. An increase in immunoreactive protein, which is not essential for catalytic function or sensitivity to malonyl-CoA, is possible due to formation of a 3-thia fatty acid CoA-ester which *inhibits* the CPT-I activity. Acetoacetyl-CoA thiolase and HMG-CoA lyase activities involved in ketogenesis were increased. The citrate synthase activity was decreased. We conclude that under conditions of peroxisome proliferation by 3-thia fatty acids, HMG-CoA synthase and CPT-II may be regulatory sites of ketone bodies formation.

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Abbreviation: CPT, carnitine palmitoyltransferase; HMG, 3-hydroxy-3-methylglutaryl.

Suspicion of Latent Δ5-Desaturase and Peroxisomal β-Oxidation Deficiency in Elderly Women over 75 Years of Age

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The aim of the present study was to compare the plasma and erythrocyte fatty acid (FA) status in a cohort of 200 women over 75 yr of age living at home with that of a control group of 50 young female volunteers aged 20–48 yr. The data were related to the dietary habits and food intakes which were evaluated by two different methods of dietary investigation. The FA composition of total plasma lipids, plasma triglycerides, cholesterol esters (CE), and erythrocytes were determined by capillary column gas–liquid chromatography using H_2 as carrier gas.

The n-6 series precursor linoleic acid (18:2n-6) was significantly lower in elderly women (EW) than in the control group in plasma triglycerides and CE ($P = 0.029$ and $P = 0.014$, respectively), suggesting that dietary intake of this essential fatty acid was lower in this group than in the control group. This is supported by the high correlation we observed between linoleic acid in CE and vegetables, vegetal fat, polyunsaturated fatty acid and vitamin E intakes ($P < 0.001$ each). In CE, the n-3 series precursor α-linolenic acid (18:n-3) was also lower in EW ($P < 0.04$) and was highly correlated to carbohydrates, bread, fibers, and vegetal protein intakes ($P < 0.0001$ each), suggesting again a lower dietary supply. In agreement with this observation, palmitoleic acid (16:1n-7) in CE was higher in the EW group ($P < 0.0001$), an observation supported by logistic regression analysis showing that higher 16:1n-7 values were associated with the EW group in all plasma lipid fractions. These data show a tendency to EFA deficiency in the EW group.

The 20:4n-6/20:3n-6 ratio was lower in CE and phospholipids ($P < 0.02$ and $P < 0.005$, respectively) for EW, suggesting some impairment in the Δ5 desaturation, whereas 18:3n-6/18:2n-6 ratio was not reduced in EW, supporting an unaltered Δ6-desaturase activity. Contrasting with the suspected lower linoleic acid intake and Δ5-desaturase activity, no difference was observed in arachidonic acid levels. Dietary investigations suggested that high dietary protein intakes largely contributed in EW to 20:4n-6 supply by diet meat.

In PL, 22:6n-3/20:5n-3 was lower in EW ($P < 0.002$), suggesting impairment in the apparent "Δ4-desaturase" pathway. Since Δ6-desaturase capacity seemed unchanged, alteration of the peroxisomal retroconversion could be implicated. Such a peroxisomal defect has been observed in aging rodents (1,2), whereas increased Δ4-activity was observed in fibrate-treated rats (3). A fragile equilibrium largely dependent on exogenous supply of long chain FA characterizes the essential fatty acid status of the group of EW studied. It is suggested that (i) the high protein intake contributing to 20:4n-6 homeostasis in free living EW should be maintained and (ii) caution is required before dietary supplementation with large amounts of very long chain polyunsaturated fatty acids in these populations until potential peroxisomal β-oxidation deficiency can be ruled out.

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Abbreviations: CE, cholesterol ester; EW, elderly women; FA, fatty acid.

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The Mitochondrion Is the Principal Target for Nutritional and Pharmacological Control of Plasma Triglyceride

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Fish oil polyunsaturated fatty acids, fibrates, and the 3-thia fatty acid analog tetradecylthioacetic acid (TTA) are potent hypotriglyceridemic agents, which act by increasing fatty acid catabolism and decreasing triglyceride synthesis and secretion by the liver. A major unresolved issue is whether this hypotriglyceridemic effect can occur independent of induction of peroxisomal β-oxidation mediated *via* peroxisome proliferator-activated receptor (PPAR). The main n-3 fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), are still mostly referred to as fish oils without any further distinction. However, studies performed in the recent years demonstrated that they possess different metabolic properties. The present study was undertaken to determine which component of fish oil, EPA or DHA, is responsible for its hypotriglyceridemic effect. We demonstrate that EPA, not DHA, is the hypotriglyceridemic component of fish oil and that only EPA feeding induces mitochondrial proliferation in rat liver. However, our data show that DHA, not EPA, is the fatty acid responsible for the observed peroxisomal proliferating effect of fish oil. EPA feeding also led to a decrease in hepatic fat droplets, whereas DHA, which increased the peroxisomal β-oxidation, actually increased the volume fraction of hepatic fat droplets. We therefore conclude that the mitochondria, not the peroxisomes, are the principal targets for nutritional and pharmacological control of triglyceride metabolism. Interestingly, fenofibrate and TTA seem to operate by the same mechanism as EPA, i.e, by increasing the mitochondrial β-oxidation capacity. In the rat model, the mitochondrial β-oxidation increased significantly, although not to the same extent as the peroxisomal β-oxidation system. In the rabbit model, however, the increase in the mitochondrial β-oxidation was more evident than the peroxisomal. And finally, in the dog model, the mitochondrial β-oxidation was increased, whereas the peroxisomal was unaffected. This is an interesting finding, as the fibrates have a modest effect on peroxisome proliferation in humans but possess a hypotriglyceridemic effect. In contrast to fibrates and TTA, EPA did not affect hepatic apolipoprotein C-III gene expression. We conclude that increased mitochondrial β-oxidation with a concomitant decrease in triglyceride synthesis is the primary mechanism underlying the hypotriglyceridemic effect of EPA, fibrates, and TTA in rats, rabbits, dogs, and possibly also in humans. In addition, these data show that the hypotriglyceridemic action of nutritional (such as fish oil) and pharmacological agents (such as fibrates and TTA) can be dissociated from any deleterious effects from peroxisome proliferation.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; TTA, tetradecylthioacetic acid.

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Cellular Fatty Acid Transport in Heart and Skeletal Muscle as Facilitated by Proteins

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ABSTRACT: Despite the importance of long-chain fatty acids (FA) as fuels for heart and skeletal muscles, the mechanism of their cellular uptake has not yet been clarified. There is dispute as to whether FA are taken up by the muscle cells *via* passive diffusion and/or carrier-mediated transport. Kinetic studies of FA uptake by cardiac myocytes and the use of membrane protein-modifying agents have suggested the bulk of FA uptake is due to a protein component. Three membrane-associated FA-binding proteins were proposed to play a role in FA uptake, a 40-kDa plasma membrane FA-binding protein ($FABP_{pm}$), an 88-kDa FA translocase (FAT/CD36), and a 60-kDa FA transport protein (FATP). In cardiac and skeletal myocytes the intracellular carrier for FA is cytoplasmic heart-type FA-binding protein (H-FABP), which likely transports FA from the sarcolemma to their intracellular sites of metabolism. A scenario is discussed in which $FABP_{pm}$, FAT/CD36, and H-FABP, probably assisted by an albumin-binding protein, cooperate in the translocation of FA across the sarcolemma.

Long-chain fatty acids (FA) are an important source of energy for most mammalian cells. In addition, FA serve as building blocks for phospholipids, forming the structural compounds of biological membranes. Newer functions attributed to FA include altering protein function by protein acylation and serving as messenger in certain signal transduction pathways (Fig. 1).

In heart and oxidative skeletal muscle, FA are the predominant substrates. In heart, it was observed that more than 30% of FA in the vascular space was extracted upon a single pass perfusion (1), indicating that FA uptake is fast, i.e., on the metabolic time scale. Since the energy demands of both heart and muscle can vary rapidly and markedly, it is important that FA delivery to these tissues is rapid, efficient, and subject to regulation. In contrast with other substrates such as glucose and lactate, the aqueous solubility of FA is extremely low (nanomolar range), and would impose theoretical problems on the observed uptake rate by heart and skeletal muscles without the presence of specific proteins to increase their aqueous solubility and to facilitate their transport (2). In the blood and interstitial space, FA are bound to albumin, while the intracellular carriers for FA are members of a family of small cytoplasmic fatty acid-binding proteins (FABP).

Cellular uptake of FA requires passage through the sarcolemmal lipid bilayer which can be divided into three stages: (i) adsorption to the outer leaflet of the sarcolemma, (ii) translocation across the sarcolemma, and (iii) desorption from the cytoplasmic leaflet of the sarcolemma. The involvement of a protein component in either one of these steps, however, is heavily disputed. Proponents of passive diffusion as the likely mechanism base their arguments on the physicochemical properties of FA and their ability to readily dissolve in and diffuse through membranes (3). This view is strengthened by observations that flip-flop of FA across artificial bilayers, as monitored with the pH-sensitive fluorophore pyranin, was extremely fast (3,4), although these findings were disputed by others (5). On the other hand, those who are in favor of a facilitated, carrier-mediated FA transport argue that cellular FA uptake is saturable, subject to competitive inhibition, and sensitive to proteases and to heat treatment (6). As will be discussed in more detail below, our own experiments using either cardiac myocytes or sarcolemmal vesicles derived from these cells, where unidirectional influx is studied either in the presence (7) or absence (8) of ongoing metabolism, demonstrated that FA uptake could be inhibited by up to 80% in the presence of protein-modifying agents (trypsin, phloretin) or highly reactive FA derivatives such as sulfo-*N*-succinimidyl-oleate (SSO) (Fig. 2). The observation that none of these agents, either alone or in combination, can fully inhibit FA uptake (Fig. 2), suggests that for cardiac myocytes there is a noninhibitable component contributing to *ca.* 20% of cellular FA uptake and likely due to passive diffusion (7). Thus, it appears that, at least in muscle, the mechanisms of passive and facilitated diffusion of FA co-exist, whereby their relative

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Abbreviations: E-FABP, epidermal-type fatty acid-binding protein; FA, long-chain fatty acids; FABP, fatty acid-binding protein; $FABP_{pm}$, plasmalemmal fatty acid-binding protein; FAT, fatty acid translocase (CD36); FATP, fatty acid-transport protein; H-FABP, heart-type fatty acid-binding protein; mAAT, mitochondrial aspartate aminotransferase; SSO, sulfo-*N*-succinimidyloleate.

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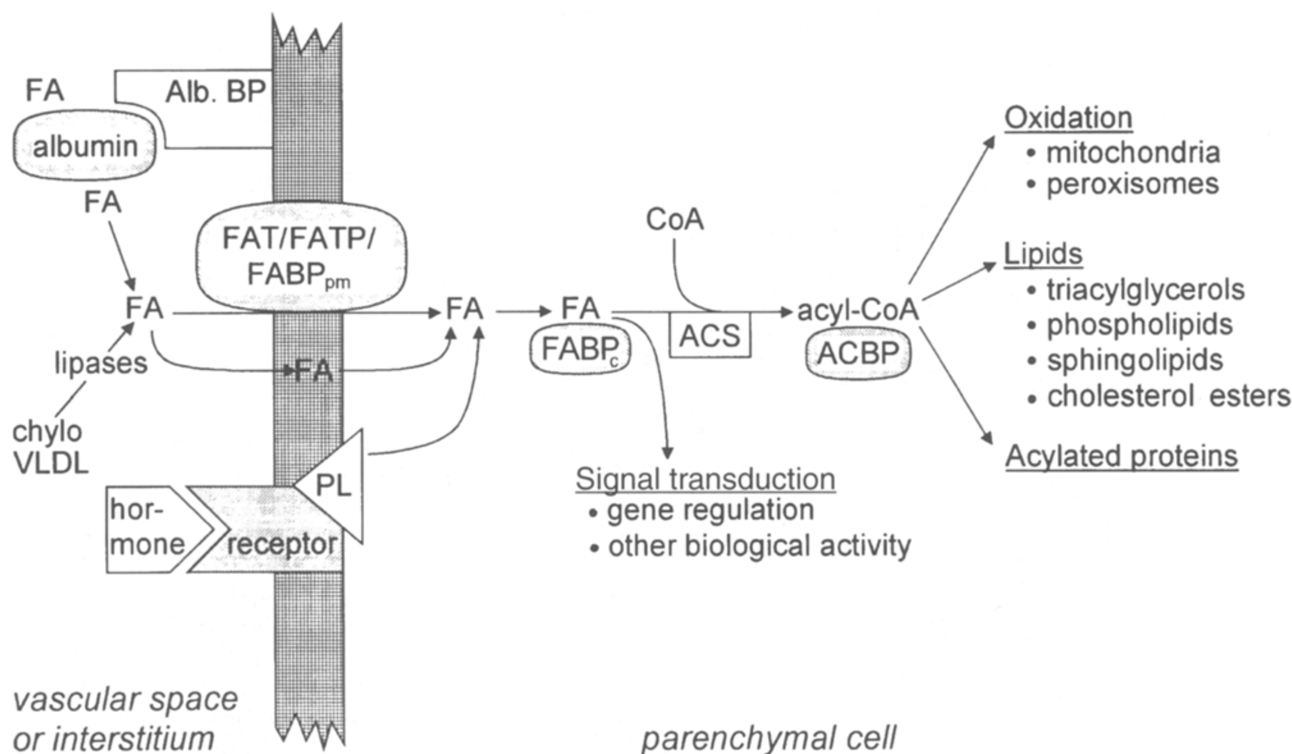

FIG. 1. Schematic presentation of the uptake and utilization of long-chain fatty acids (FA) by parenchymal cells, with emphasis on the assumed or proposed roles of various lipid-binding proteins in this process. Transmembrane translocation of FA presumably takes place by passive diffusion through the phospholipid bilayer and/or facilitated by membrane-associated proteins ($FABP_{pm}$, FAT/CD36, FATP). Intracellularly, FA are bound by cytoplasmic FABP and, after activation to fatty acyl-CoA, by ACBP. In addition, the binding of specific hormones to its cell-surface receptor may trigger the release of FA from membrane phospholipids (PL), which then act as signaling compounds (second messenger) modulating certain cellular functions. Abbreviations: chylo, chylomicrons; VLDL, very low density lipoproteins; Alb. BP, albumin-binding protein; $FABP_{pm}$, plasmalemmal fatty acid-binding protein; FAT, fatty acid translocase (CD36); FATP, fatty acid-transport protein; $FABP_c$, cytoplasmic fatty acid-binding protein; ACS, acyl-CoA synthetase; ACBP, acyl-CoA binding protein.

contribution to overall FA uptake is likely to depend on the metabolic demands of the tissue.

Driving force for FA uptake in heart and muscle. Experimental studies in heart and skeletal muscle in which the vascular and intracellular concentration of FA (sum of protein-bound and nonprotein-bound FA) was determined indicated the existence of an inwardly directed FA gradient with an extracellular FA concentration in 10–20-fold excess over the intracellular content (1,9). This gradient is regarded as the driving force for FA uptake. The intracellular (unesterified) FA concentration in cardiac myocytes is maintained at a low level of 6–8 μM by rapid metabolic conversion of the FA into fatty acyl-CoA followed by β-oxidation or esterification, which makes FA metabolism an important factor of the rate of FA uptake (7). Observations supporting this notion include the findings that (i) initial FA uptake is depressed by inhibitors of FA-metabolizing enzymes and by competitive substrates (7), (ii) in electrically stimulated cardiac myocytes, the increased metabolic demands cause an enhancement of initial uptake of FA (Luiken J.J.F.P., unpublished observations), and (iii) during the initial uptake phase (3 min) more than 50% of the sequestered FA is esterified (7).

The study of FA uptake in the absence of their subsequent metabolism has recently become possible by the introduction of giant sarcolemmal vesicles as an experimental model (8,10). These vesicles (diameter *ca.* 15 μm) are oriented fully right-side-out, and substrate metabolism is completely absent. Importantly, the vesicles contain cytoplasmic FABP that serves as an intravesicular sink for FA.

Comparison of FA uptake by cardiac myocytes and giant sarcolemmal vesicles from heart has yielded insight into the question as to what is the rate-limiting step in cellular FA uptake and metabolism. Both in myocytes and giant vesicles, i.e., in the presence and absence of FA metabolism, respectively, FA uptake showed similar saturation kinetics (7,8). Moreover, with myocytes it was observed that at high, externally added palmitate concentrations, when the initial uptake becomes saturated, there is no drastic increase in the cellular steady-state levels of (unesterified) FA, which were found to remain *ca.* 8 μM (7). Such an increase is likely to be expected when palmitate metabolism would be rate-limiting. Together these observations suggest that the translocation of FA across the sarcolemma might be a primary control point of cellular FA uptake. This notion implies that either there is an overcapacity of

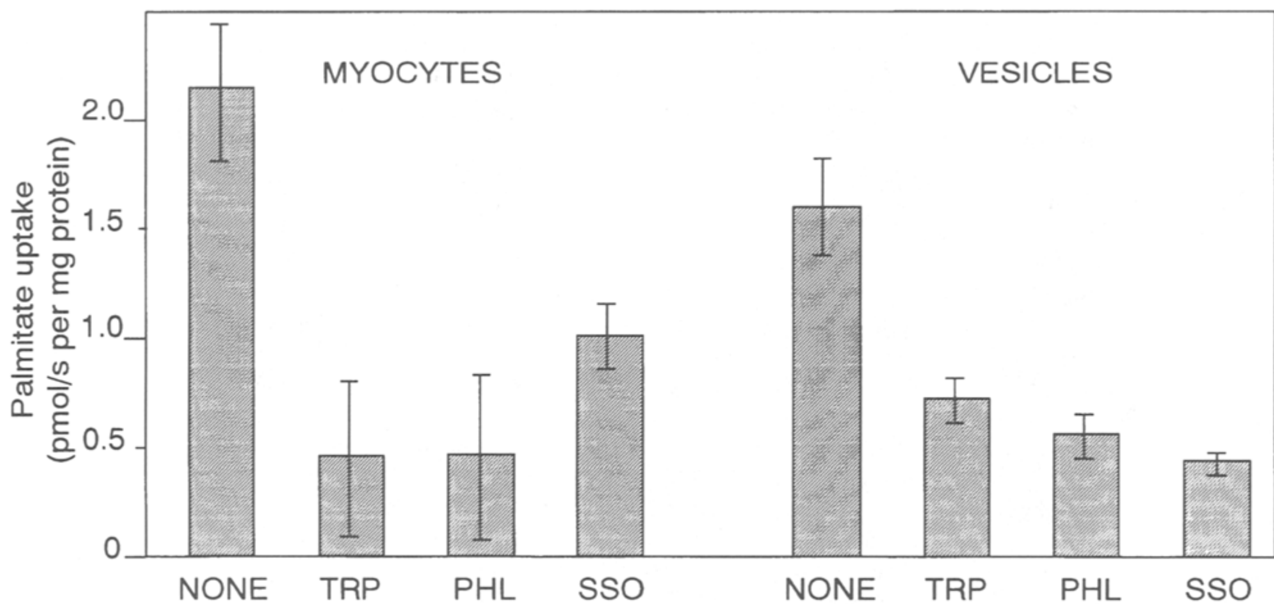

FIG. 2. Influence of plasma membrane-acting agents on palmitate uptake by isolated rat cardiac myocytes (left) or by rat heart giant sarcolemmal vesicles (right). Cells or vesicles were pretreated with trypsin (TRP) or sulfo-*N*-succinimidyloleate (SSO) or incubated in the presence of phloretin (PHL), and the uptake of ^{14}C-labeled palmitate (90 μM) complexed to albumin (300 μM) was monitored at 37°C for 3 min (myocytes) or 15 s (vesicles). Values are expressed as nmol palmitate sequestered per second and per milligram total protein and represent means ± SD for 3–5 experiments. Data obtained from References 7 and 8.

FA metabolism over the capacity of the sarcolemma to translocate FA, or that FA uptake and subsequent metabolism are finely tuned. The ability of metabolism to influence the initial uptake rate (7) strongly argues for this latter fine-tuning. It is important to maintain a low intracellular concentration of FA because these compounds are potentially detrimental for cells if allowed to accumulate (1).

The Na^+ gradient across the plasma membrane, which is the driving force for uptake of a number of nutrients, plays a role in FA uptake into neither heart nor muscle, as this process was unaffected by ouabain (11). This finding is in contrast to liver, where ouabain markedly inhibits FA uptake (12).

Membrane-associated FABP. The identification of several membrane-associated FABP during the last decade provides strong additional evidence for a facilitated transport mechanism. While the kinetic evidence in favor of facilitated transport is still explainable by passive diffusion (13,14), the evidence obtained more recently using molecular biological techniques is convincing for a role of these membrane proteins in the mechanism of cellular FA uptake. Together with the cytoplasmic FABP, these proteins, and their presence in heart and skeletal muscle, form the further focus of this review (Table 1).

Plasma membrane FABP ($FABP_{pm}$). Stremmel and coworkers (15) were the first to isolate a 43 kDa protein from hepatocyte plasma membranes that displayed a high affinity for oleate. This protein appeared to be ubiquitously present in most mammalian tissues, but most prominently in heart, skeletal muscle, brain, liver, and kidney (for review, see Ref. 16). Antibodies raised against this protein inhibited FA uptake in liver (12) and heart (17) up to 70%, providing functional evidence for an involvement in this process. Surprisingly, the amino acid sequence of this protein is homologous to mitochondrial aspartate aminotransferase (mAAT, 16). However, 3T3 fibroblasts transfected with mAAT cDNA demonstrated a plasma membrane localization of this protein rather than a mitochondrial localization. Another important finding was that overexpression of mAAT resulted in augmented FA uptake (16), again in agreement with an important role in cellular FA uptake. The finding that high salt treatment of membranes leads to the extraction of this protein (16) indicates that it is a peripheral membrane protein which therefore most likely acts in the trapping of extracellular FA.

TABLE 1
Proteins Involved in Muscular Long-Chain Fatty Acid Binding and Transport[a]

Protein	Current designation	Molecular mass (kDa)
Extracellular		
Albumin	Albumin	68
Membrane-associated		
Albumin-binding protein	ABP (Alb. BP)	18,31,60
Plasmamembrane FABP	$FABP_{pm}$	43
FA transport protein	FATP	60
FA translocase	FAT (CD36)	88
Intracellular		
Cytoplasmic FABP[b]	H-FABP	14.5
	E-FABP	15
Acyl-CoA binding protein	ACBP	10

[a]Abbreviations: FA, long-chain fatty acid; FABP, fatty acid-binding protein. [b]In muscles both heart-type FABP (H-FABP) and epidermal-type FABP (E-FABP) occur.

FA translocase (FAT)/CD36. The first step toward the identification of a putative FA transporter in adipocytes was made by labeling a highly glycosylated 88 kDa membrane protein with radiolabeled succinimidylesters of FA under conditions whereby these compounds inhibited FA uptake by adipocytes up to 70% (18). The nonglycosylated form of this protein was found to have a molecular mass of 54 kDa. This protein was designated as FAT, and its cloning (from rat) revealed a high degree of amino acid homology (85%) with human platelet glycoprotein IV, also referred to as CD36 (19), suggesting that these proteins are species homologs (20). CD36 is supposed to be involved in platelet aggregation and signal transduction (19). Besides its presence in platelets and endothelial cells (19), FAT/CD36 mRNA is expressed in tissues with a high lipid-metabolizing capacity such as heart, oxidative muscle and fat, but is absent in liver, brain, and kidney (20–22). Differentiation of preadipocytes into adipocytes showed an induction of FAT which was paralleled with an increase in FA uptake (20). In platelets, CD36 was demonstrated to be an integral membrane protein with one or two membrane-spanning regions (23,24). Extrapolation of these findings to the rat implies that FAT is also an integral membrane protein. Strong evidence for a functional role in cellular FA transport was again provided by a transfection study, whereby overexpression of rat FAT/CD36 in fibroblasts resulted in a significantly increased FA uptake rate (25). However, in another study FAT/CD36 was expressed in a heart muscle cell line (H9c2) normally lacking this protein, but this did not lead to altered FA uptake kinetics (26). This discrepancy is discussed below.

FA-transport protein (FATP). Few data are available regarding the most recently discovered candidate for facilitating the transmembrane transport of FA. This transporter, designated as FATP, was identified *via* expression cloning of an adipocyte cDNA library in COS7 cells and subsequent screening for cells exhibiting elevated uptake of a fluorescent FA analog (27). FATP has a molecular mass of 63 kDa and is an integral membrane protein with multiple transmembrane sequences, as predicted from hydropathy plots. The mRNA for FATP is present in most mammalian tissues tested with highest expression levels observed in brain, oxidative skeletal muscle, heart, and fat cells (22,27).

Mode of action of membrane-associated FABP. On the mRNA level, all three membrane-associated FABP are present in heart and muscle tissue. When comparing FA uptake in different muscle tissues using giant sarcolemmal vesicles, uptake was highest in heart, was lower in red oxidative skeletal muscle, and was lowest in white glycolytic skeletal muscle, in agreement with the oxidative capacities of these muscle tissues (8,10). The relative protein contents of both $FABP_{pm}$ and FAT/CD36 in these vesicles paralleled the observed FA uptake rates. On the other hand, the FATP content was lower in heart than in muscle. These observations, together with findings that antibodies against $FABP_{pm}$ as well as SSO, a specific inhibitor for FAT/CD36, were able to block FA uptake by heart and muscle sarcolemmal vesicles, indicate that for FA uptake into muscle both $FABP_{pm}$ and FAT/CD36 might be physiologically important (8,10). Interestingly, the inhibitory actions of SSO and anti-$FABP_{pm}$ antibodies were nonadditive (8), suggesting that $FABP_{pm}$ and FAT/CD36 may cooperate to translocate FA across the sarcolemma. This raises the attractive hypothesis that the peripheral membrane protein $FABP_{pm}$ located at the extracellular leaflet could act as a scavenger of FA in order to facilitate FA adsorption (Step I) while the integral membrane protein FAT/CD36 would function as a translocase (facilitation of Step II).

Analogous to the short-term regulation of glucose uptake into adipose tissue and muscle by insulin, which is due to translocation of GLUT4 from intracellular stores to the plasma membrane, we recently obtained evidence that FA uptake could also be regulated by a translocation event (Bonen, A., personal communication). In sarcolemmal vesicles from muscles that are electrically stimulated for 25 min, there is an increase in FA uptake concomitant with an increase in FAT/CD36, but not $FABP_{pm}$. FAT/CD36 appears directly responsible for the increased FA uptake since SSO can block this increased uptake completely. Since the duration of the period of muscle contraction is too short for *de novo* protein synthesis, this increase in sarcolemmal FAT/CD36 could only be explained by protein translocation. Interestingly, there are two independent reports claiming that rat FAT (at one extracellular site; Ref. 28) or human CD36 (at two cytoplasmic sites; Ref. 24) are palmitoylated in a reversible manner. Since acylation of proteins is often associated with a change in subcellular localization, one might speculate that muscle contractions induce signaling pathways stimulating protein-acylating enzymes to palmitoylate CD36, which in turn is translocated to the sarcolemma where it accepts FA from $FABP_{pm}$. A candidate intracellular store could be the caveolae, because in platelets this is the primary subcellular fraction where CD36 is located (29). FAT/CD36 being possibly translocated while $FABP_{pm}$ is not makes FAT/CD36 the primary site of control in the FA uptake process.

It must be stressed, however, that FATP, although probably not involved in FA uptake by muscle tissues, could be of functional importance in tissues other than heart and skeletal muscle, for instance in adipose tissue which is involved both in uptake and delivery of FA from and to the bloodstream, or otherwise in brain and liver which lack FAT/CD36.

Intracellular FABP. The intracellular FABP consist of a family of 14–15 kDa proteins with the property to bind hydrophobic ligands such as FA with high affinity. There are at least 13 members of this protein family, including four retinoic acid-binding proteins, which mutually display amino acid sequence homologies of 15–70% (for review, see Refs. 2 and 30). However, their tertiary structures share a remarkable similarity and consist of a clam shell-like structure with the lipid ligand bound in between the two halves of the clam. Each type of FABP displays a specific tissue distribution pattern, which likely relates to the specific FA metabolic needs of the respective tissues. In heart and skeletal muscle cells the most predominantly present FABP isoform is referred to as heart-type FABP (H-FABP). Myocytes also contain, in con-

siderably lesser amounts, a second FABP type which is also found in epidermal cells, and therefore designated as epidermal-type FABP (E-FABP). There is a positive correlation between the occurrence of H-FABP and the oxidative capacity of the muscle tissue (heart > red muscle > white muscle), indicating a functional involvement of these proteins in muscle lipid metabolism (2). Intracellularly also occurs an acyl-CoA binding protein, which binds the FA after activation to their acyl-CoA esters (Fig. 1 and Table 1) (2).

Functions of intracellular FABP. The proposed primary function of cytoplasmic FABP is to serve as transport vehicle for FA from the sarcolemma, through the cytoplasm to their site of metabolic conversion, i.e., acyl-CoA synthetase at the cytoplasmic leaflet of the outer mitochondrial membrane or endoplasmic reticulum. This notion is based on three independent lines of evidence. First, intracellularly sequestered fluorescent FA were found to be bound by FABP (31). Second, theoretical studies demonstrated that the aqueous solubility of FA is enhanced about 700-fold in the presence of FABP which leads to a 17-fold increased diffusional flux of FA (32). Third, studies monitoring the transfer of fluorescent FA between FABP and phospholipid bilayers indicate that FABP interacts with membranes through collisional transfer, thus without the FA being exposed to an aqueous environment (33). Furthermore, when in similar studies FABP was used as FA donor and phospholipid vesicles as acceptor, it appeared that the presence of acidic phospholipids within these vesicles enhanced the transfer rate of FA, which compounds likely interact with positive lysine residues in the portal region of the FABP molecule (34). Because the mitochondrial membrane is enriched in cardiolipin, these negatively charged phospholipids could be important in directing the intracellular transport of FA by FABP toward mitochondria.

Other functions of cytoplasmic FABP, but related to the former, may include the enhancement of cellular FA uptake by (i) extracting FA from the sarcolemma (desorption) and by (ii) trapping FA that are taken up by cells in order to maintain their intracellular nonprotein bound concentration at a low level (1,2). Transfection studies in which liver-type FABP was expressed in fibroblasts normally lacking this protein resulted in an enhanced FA uptake (35). However, expression of H-FABP in COS7 cells did not affect FA uptake rates (27). Currently, the transgenic mouse model is being used to elucidate the function of FABP. The first FABP to be knocked out was the adipocyte-type lipid-binding protein, but this resulted in a compensatory expression in adipose tissue of E-FABP (36). More recently, an H-FABP knock-out mouse was generated by Binas and coworkers (37). In contrast to the adipocyte-type lipid-binding protein knock-out mice, no compensatory upregulation of other types of cytoplasmic FABP was observed in the H-FABP knock-out model. An initial search for a phenotype revealed that these mice died with great frequency upon strenuous exercise (37). Studying FA utilization by heart homogenates showed no alteration of the tissue capacity for β-oxidation. However, in intact cardiac myocytes, both FA uptake and oxidation were depressed by about 50% when compared to cardiac myocytes isolated from hearts of control rats, strongly suggesting that H-FABP is required for an efficient intracellular utilization of FA (38).

There is also evidence that H-FABP is involved in cell growth and differentiation and in certain signal transduction pathways. For a further discussion of these putative functions, the reader is referred to recent reviews (2,30).

Molecular mechanism of cellular FA uptake. It is becoming increasingly evident that myocytes, and also other mammalian cells, govern passage of FA across the plasma membrane *via* distinct protein–protein interactions. In a first step of the putative mechanism, FA are captured from the albumin–FA complex by $FABP_{pm}$. This process could be facilitated by assistance of albumin-binding proteins which may serve as docking place for the albumin–FA complex and may accelerate the dissociation of FA (Fig. 1). The sarcolemma of cardiac myocytes is known to contain high-affinity albumin-binding sites (39). More detailed investigations led to the identification of three proteins, gp18, gp30 and gp60, showing high affinity for modified albumin species and which could function as albumin receptors (39,40). Because studies on the kinetics of palmitate uptake demonstrated a K_m for albumin of 23 μM (7), it is unlikely that *in vivo*, i.e., at physiological albumin concentrations of 300–600 μM, albumin-binding proteins could be of regulatory importance. However, they could play a permissive role by assisting the FA–albumin complex to diffuse through the unstirred fluid layer surrounding the cells. This increase in concentration of the FA–albumin complex either would lead to an increase in the concentration of unbound FA which are then extracted by $FABP_{pm}$ or would enable the direct transfer of FA from albumin to $FABP_{pm}$ without the need for these FA to enter into the aqueous phase. Subsequently, $FABP_{pm}$ might transfer the FA to FAT/CD36 which, in turn, mediates the translocation of FA across the membrane and possibly also their binding to cytoplasmic FABP. This latter concept is supported by the observation of a physical contact between FAT/CD36 and FABP (41).

A protein-mediated transport of FA across the membrane could be of importance in directly and efficiently targeting FA to their site(s) of intracellular processing, and also in protection of cells against the potentially toxic effects of (unesterified) FA. Such controlled FA uptake presumably would be more difficult to realize in case FA would partition between albumin and membrane phospholipids based on their physicochemical properties, then translocate by flip-flop to the inner leaflet of the bilayer before being bound by cytoplasmic FABP. Thus, while passive diffusion of FA into cells contributes to the mechanism of cellular FA uptake, in muscle the bulk of sarcolemmal FA translocation most likely occurs by involvement of membrane-associated FABP, possibly assisted by albumin-binding protein(s) without regulatory function.

It will be a challenge in the coming years to investigate the nature of the interactions between the various proteins involved in muscle FA uptake: albumin-binding proteins, $FABP_{pm}$, FATP, FAT/CD36, and H-FABP. Issues to be solved

include whether at some stage during the FA uptake process these proteins actually are in physical contact with each other, and whether yet other proteins are involved. Another matter worthy to be investigated in more detail is the nature of the intracellular translocation of FAT/CD36 and to what extent extracellular factors can influence this translocation.

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Expression of the Extracellular Fatty Acid-Binding Protein During Muscle Fiber Formation *in Vivo* and *in Vitro*

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Extracellular fatty acid-binding protein (Ex-FABP) is a 21-kDa protein developmentally regulated in chick embryo, first observed *in vitro* in chondrocytes at a late stage of differentiation as a protein secreted into the culture medium (1). Immunolocalization performed in cartilage tissues with polyclonal antibodies obtained against the purified protein showed that the protein is present *in vivo* in chicken embryo tibia in hypertrophic chondrocytes of the growth plate at the border between hypertrophic cartilage and newly formed bone, in the cartilage surrounding marrow cavities and blood vessels, and in the epiphysial prearticular cartilage. The complete amino acid sequence of the protein, obtained by combining protein and nucleotide sequence data, assigned the protein to the superfamily of lipocalins or lipophilic molecules carrier proteins (2). The recombinant protein was obtained in the baculovirus system, and studies were performed to identify a possible ligand. By lipidex binding assay with radioactive lipophilic compounds, a specific fatty acid binding to the protein was shown and a preferential binding of long-chain unsaturated fatty acids was observed (3). Calculated dissociation constant was 2×10^{-7} M for unsaturated fatty acids and 5×10^{-7} M for stearic acid. Short-chain fatty acids did not bind to the protein. Other hydrophobic molecules as retinoic acid, retinol, progesterone, prostaglandins, and long-chain alcohols and aldehydes did not bind to the protein. It is widely accepted that free fatty acids in blood are transported by albumin. Ex-FABP is present in chicken serum and represents the first extracellular protein able to selectively bind and transport fatty acids in extracellular fluid and serum. Ex-FABP could represent in blood the high-affinity, low-capacity, specific binding protein that transports fatty acids to target organs while albumin could represent a low-affinity, high-capacity storage protein for fatty acids. Ex-FABP is also produced by peripheric granulocytes. We performed studies aimed to elucidate a possible role of the protein during chicken embryo development, and we observed that Ex-FABP is expressed in the forming myotubes both *in vitro* and *in vivo*. The presence of the protein and of the mRNA was observed in newly formed myotubes at early stages of chick embryo development by immunohistochemistry and by *in situ* hybridization. At later stages of development, myofibers still expressed both the mRNA and the protein. Ex-FABP expression was observed also in the developing myocardium and in the muscular layer of large blood vessels. In agreement with these findings, an initial expression of the mRNA and protein secretion by cultured chicken myoblasts were observed only after the onset of myoblast fusion. Double immunofluorescence staining of these cultured cells revealed that multinucleate myotubes were stained by antibodies directed against both the Ex-FABP and the sarcomeric myosin, whereas immature myotubes and single myoblasts were not. When added to cultured myoblasts, antibodies against the Ex-FABP induced a strong enhancement of the production of the same protein. In all experiments some cell damage and a transient impairment of myotube formation were also observed. The finding that the continous removal of the Ex-FABP from the culture medium of myoblasts, due to the formation of immune complexes, resulted in an overproduction of the protein suggests a feedback (autocrine) control during myotube differentiation and maturation. We propose that the requirement for increased transport and metabolism of free fatty acid released from the membrane phospholipids and storage lipids, mediated by Ex-FABP, may be essential during differentiation of multinucleated myotubes, or that an increased local amount of fatty acids and metabolites may act as a local hormone in tissues differentiating and undergoing morphogenesis.

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Abbreviation: Ex-FABP, extracellular fatty acid-binding protein.

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Molecular Modeling and Experimental Confirmation of Selective Mobilization of Polyunsaturates from Triacylglycerols

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Research has shown that polyunsaturated fatty acids (PUFA) are selectively mobilized from adipose tissue during energy deprivation or weight cycling. In rats, weight cycles of 24 h fasting followed by 72 h refeeding resulted in stochastic loss of both linoleate and α-linolenate (1). These two fatty acids (FA) are oxidized at levels greater than intake (2). Very low calorie diets in obese female humans were found to result in selective loss of α-linolenate, despite supplementation with this FA (3,4). *In vitro* and *in vivo* studies of relative mobilization of FA from adipose tissue led Raclot and Groscolas (5,6) to formulate the following empirical observations: relative mobilization of FA from triacylglycerols (TG) increases with (i) decreasing chain length at a given degree of unsaturation; (ii) increasing unsaturation at a given chain length; and (iii) double bond(s) closer to the methyl end of the carbon chain.

Apparently more saturated, longer-chain TG structures are more likely to be stored but less likely to be mobilized, i.e., "first in, last out," but there is no rigorous physical chemical model which accounts for these observations. We present nuclear magnetic resonance (NMR) data from obese and normal mouse lipids and 3-D energy-minimized TG structures that demonstrate that storage of more saturated FA and mobilization/oxidation of PUFA are energetically favorable from a molecular standpoint.

Obese and normal mice were fed identical standard diets with 10% fat containing equal proportions of saturated fat (SFA), monounsaturated fat (MUFA), and PUFA. Extracted lipids were examined by gas chromatography and 2-D $^{13}C/^{13}C$ NOESY chemical exchange NMR spectroscopy. Normal mouse fat contained almost entirely linoleate, oleate, and palmitate in a 1:1:1 LOP (linoleate, oleate, palmitate) ratio. Obese mouse fat was enriched in oleate by a factor of two compared to normal mouse fat (i.e., 1:2:1 LOP), and obese mouse fat was more densely packed. All 27 3-D energy-minimized TG structures of palmitate, oleate, and linoleate were calculated. For *Z*α,*Z*β,*E* palmitate is strongly preferred in *E*, followed by linoleate, and then oleate. Obese mice had more oleate and less linoleate in *Z*α than normal mice. The lowest energy average structure for 1:1:1 LOP was a mixed TG with *Z*α,*Z*β,*E*, linoleate, oleate, and palmitate, respectively. At 100 ms mixing time in the 2-D NMR exchange experiment, obese mouse fat showed characteristic cross peaks. These cross peaks and the modeling both indicated that a head-foot-head-foot TG tetramer is the most likely base unit for extended adipose storage.

We constructed 3-D energy-minimized TG model structures to test the results of References 5 and 6. We considered that higher steric energies would lead to an increased tendency toward mobilization/oxidation as opposed to storage. The summed energies of three free FA (FFA) were much higher than those of the TG structures, especially for SFA, and in general FFA were not good test models. Pure SFA TG energies decreased uniformly from 8- to 20-carbon chain lengths (24.2–15.0 kcal/mol). Increasing from 2 to 6 double bonds in 22:xn-3 led to little change in energy in the FFA (19.6–21.5 kcal/mol). However, various TG structures with 18:0 and 22:6n-3 docosahexaenoic acid (DHA) increased in energy from *Z,Z,E* 18:0, 18:0, 22:6n-3 to *Z,Z,E* 22:6n-3, 22:6n-3, 18:0 (33.5–49.9 kcal/mol). DHA is a spiral molecule, and the structure/energy relationships of TG with DHA are complex and not predictable from simple progressions based on the chemistry of the FFA involved. The *Z,Z,E* 18:0, 18:0, PUFA structure is fairly realistic for adipose storage in humans consuming normal diets, and in models using α-linolenate (18:3n-3) instead of DHA, 18:0, 18:0, 18:3n-3 ac-

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Abbreviations: DHA, docosahexaenoic acid; FA, fatty acid; FFA, free fatty acid; MUFA, monounsaturated fatty acid; NMR, nuclear magnetic resonance; PUFA, polyunsaturated fatty acid; SFA, saturated fatty acid; TG, triacylgcerols.

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tually had a higher energy than 18:0, 18:0, 22:6n-3. This might account for the observed preferential loss of α-linolenate (1–4).

The double bond in 18:1 was placed in the n-3, n-6, and n-9 positions. Again, the energies for even a single FFA were nearly as high as the TG high were very dissimilar, but in TG *Z,Z,E* 18:0, 18:0, 18:1, energies increased from n-3 to n-9 (18.5 to 24.9 kcal/mol), opposite the trend from References 5 and 6. However, the structure of 18:1n-9 is unique in that the MUFA chain folds back on itself, allowing for packing nearly as efficient as a pure SFA TG in tetramer units. This may account for the prevalence of oleic acid in the obese mouse fat, as well as in storage lipids in plants.

It is concluded that the energetics of TG tetramers as a mimimum structural unit play a fundamental role in relative mobilization of FA from adipose tissue. Describing FA mobilization and kinetics of lipolysis without considering the extended 3-D structure of adipose and the packing of TG chains is likely to provide results which are too simplistic.

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Albumin Interferes with the Uptake Metabolism of Arachidonic Acid by Human Leukocytes

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Arachidonic acid in human neutrophils is metabolized primarily *via* the 5-lipoxygenase (5-LO) pathway which leads to the formation of leukotriene A_4 (LTA_4), the precursor of the potent chemotactic factor for leukocytes, leukotriene B_4 (LTB_4), formed in neutrophils. The release of arachidonic acid through the action of phospholipases within the cells is believed to be a limiting step in the production of LTB_4. Another source of arachidonic acid or LTA_4 for the biosynthesis of LTB_4 in neutrophils might be through transcellular metabolism of the precursors released from other cells.

Little is known about how arachidonic acid or other intermediates such as LTA_4 enter the cells. Albumin together with the plasma lipoproteins is the major carrier of fatty acids in the circulation, and in the present study we addressed the question of how albumin influences the conversion of arachidonic acid to LTB_4 in human neutrophils.

Polymorphonuclear leukocytes (PMNL), obtained from buffy coat, were incubated with 10 μM [1-^{14}C]arachidonic acid in phosphate buffered saline in the presence or absence of 600 μM albumin and with or without stimulation by the calcium ionophore A23187 for 1 h. In another set of experiments, the cells were prelabeled with arachidonic acid for 1 h and then incubated for another 60 min using the conditions described above. The product profile was recorded both by two-dimensional thin-layer chromatography in combination with autoradiography and by high-performance liquid chromatography with ultraviolet and radioactivity monitoring.

When the cells (1×10^7 PMNL per mL) were stimulated in the absence of albumin, both LTB_4 and ω-hydroxy-LTB_4 were formed. However, in the presence of albumin, no 5-LO metabolites were detected and around 75% of the added arachidonic acid was found to be unmetabolized compared to 3% in the absence of albumin. The incorporation of arachidonic acid into cellular phospholipids and triglycerides was not influenced by albumin.

When a concentrated suspension of neutrophils (10^9 PMNL per mL) was incubated under the same conditions, we observed a different metabolic pattern of arachidonic acid. Under all conditions, negligible amounts of free arachidonic acid were found. With unstimulated cells in the presence of albumin, the major part of the radioactivity was found in the fraction containing phospholipids and triglycerides. Some formation of 12-hydroxyeicosatetraenoic acid (12-HETE) was also observed due to contaminating platelets. When the cells were stimulated with ionophore, the major part (63%) of the arachidonic acid was converted to 12-HETE, indicating that 12-LO has a higher capacity to utilize exogenous arachidonic acid compared to the 5-LO. In this case 12-LO might act as a drainage pool for excess amount of arachidonic acid.

Since albumin seems to have a major influence on the conversion of exogenously added arachidonic acid, it was of interest to study if albumin also interfered with arachidonic acid release and metabolism if the source of arachidonic acid was cellular lipids. Leukocytes prelabeled with arachidonic acid were incubated for 1 h under the same conditions as described above. The main part of the radioactivity was associated with cellular lipids under all conditions. However, the level of free arachidonic acid was doubled when calcium ionophore and albumin were present in the incubations.

It is known that the synthesis of LT occurs in the nuclear envelope, and upon activation 5-LO translocates from the cytosol to the nuclear membrane to associate with 5-lipoxygenase-activating protein. Moreover, the cytosolic phospholipase A_2 was reported to translocate to the nuclear membrane and the endoplasmic reticulum upon activation. The source from which arachidonic acid arises during the activation was not elucidated. However, a number of authors report a rapid remodeling of arachidonic acid when the cells are stimulated. From our experiments, it is clear that exogenous arachidonic acid bound to albumin cannot serve as a precursor for LT synthesis in the leukocytes. However, it seems to be a good substrate for the 12-LO in platelets during the same conditions. Furthermore, prelabeling of the cells with arachidonic acid and stimulation of the cells without any addition of exogenous arachidonic acid did not give any detectable amounts of radioactive LTB_4 and LTB_4 metabolites. This further points out the importance of albumin as a regulator of the availability of arachidonic acid as a substrate for LT biosynthesis.

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Abbreviations: 12-HETE, 12-hydroxyeicosatetraenoic acid; LO, lipoxygenase; LTA_4, leukotriene A_4; LTB_4, leukotriene B_4; PMNL, polymorphonuclear leukocyte.

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Elongation and Trafficking of Arachidonate in Lipids of Vascular Smooth Muscle Cells

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Inflammatory cells surrounding vascular smooth muscle cells (VSMC) in atherosclerotic lesions are susceptible to release, in addition to arachidonate metabolites, high amounts of free arachidonic acid (20:4). It was then of interest to analyze the capacity of VSMC to protect themselves against excess 20:4. The A7r5 VSMC line, derived from rat embryo aorta, was cultured in Dulbecco modified Eagle medium-fetal calf serum 10% (control cells) or supplemented with 15 and 45 μM 20:4. The fatty acid composition of triacylglycerols and phospholipids was analyzed by gas chromatography–mass spectrometry after alkaline hydrolysis. The phospholipid subclasses were separated by thin-layer chromatography after benzoylation of their di-radylglycerol derivatives. Metabolic transformation of $[^3H]$20:4 into $[^3H]$22:4 and their release in chase medium were investigated using high-performance liquid chromatography on line with a radioactive counter.

The 20:4 content of phospholipids increased by 2–3 times when cells were supplemented with 15 μM 20:4 and did not change very much with higher 20:4 concentration. The 22:4, the elongated product of 20:4, incorporated in phospholipids represented around 10% of esterified 20:4. Among the phospholipids, the 22:4 fatty acid was preferentially incorporated into phosphatidylethanolamine, particularly the alkylacyl species. The 20:4 and 22:4 increases in phospholipids were at the expense of other unsaturated fatty acids, the total amount of phospholipids being not modified. The 20:4 fatty acid was not incorporated into phosphatidylcholine as di-20:4 species by *de novo* pathway, in contrast with migrating inflammatory cells, although its content in polyunsaturated fatty acids (PUFA) was fourfold increased. It then appears that, in VSMC, 20:4 and 22:4 are incorporated into phospholipids only by the remodeling pathway

The amount of triacylglycerols did not change with 15 μM 20:4 and increased by 2.5 times when cells were supplemented with 45 μM 20:4. In these newly synthesized triacylglycerols, the elongation product represented more than 30% of esterified 20:4, and PUFA content of triacylglycerols was then higher than PUFA content of phospholipids from control cells. It is likely that di- or tri-PUFA species are synthesized through *de novo* pathway involving fatty acid esterification into phosphatidic acid and diglyceride formation. The incorporation of 20:4 into triacylglycerols only in the presence of high 20:4 concentration is consistent with triacylglycerols being a storage pool protecting cells from 20:4 that is in excess. However, 20:4 can also be transferred from triacylglycerols to phospholipids and utilized as precursor of lipid mediators. Therefore, we investigated the fate of 20:4 and its elongation product, from triacylglycerols. Cells were prelabeled with 45 μM $[^3H]$20:4, then chased. The 20:4 decreased rapidly in triacylglycerols and was not transferred to phospholipids. The 20:4 is thus released directly from triacylglycerols probably by triacylglycerol lipase activity, which is specific for 20:4, as the elongation product was retained a longer time than 20:4 in triacylglycerols.

The elongation of 20:4 into 22:4 and high synthesis of triacylglycerols, together with incorporation of 20:4 into phospholipids only by the remodeling pathway, and absence of PUFA transfer from triacylglycerols to phospholipids appear to be specific ways for VSMC to protect themselves against excess 20:4.

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Abbreviations: PUFA, polyunsaturated fatty acid; VSMC, vascular smooth muscle cells.

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The Effect of n-3 Fatty Acids on Leukotriene Formation from Neutrophils in Patients on Hemodialysis

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Dietary intake of long chain n-3 polyunsaturated fatty acids, derived from fish, modulates several measures of leukocyte reactivity in healthy volunteers and in various patient groups. In the present study we evaluated the effect of dietary supplementation with n-3 fatty acids on *ex vivo* formation of leukotrienes B_4 and B_5 from stimulated neutrophils in patients with chronic renal failure. Sixteen patients treated with maintenance dialysis therapy three times a week at the Department of Nephrology, Aalborg Hospital, were included. Eight patients (five men and three women with a mean age of 53 yr) were randomized to daily supplementation with 5.2 g of n-3 fatty acids, while eight subjects (four men and four women with a mean age of 56 yr) were randomized to a similar amount of olive oil used as placebo. The oils were delivered in identical capsules, and the patients received 8 capsules/d for 12 wk in a double-blind study design. Neutrophils were isolated from whole blood before and after the supplements and were stimulated with calcium-ionophore. Leukotrienes B_4 and B_5 were measured in the supernatants by high-performance liquid chromatography. The two groups of patients were well-matched and had similar baseline levels of leukotrienes. In the placebo group there was a 2% increase in leukotriene B_4 formation, while in contrast n-3 fatty acids led to a 34% decrease in formation of leukotriene B_4. Comparisons between changes in the two groups revealed a highly significant statistical difference ($P < 0.001$, Mann-Whitney U-test). Similarly, production of leukotriene B_5 was unaltered by olive oil and increased by more than five times in the patients randomized to n-3 fatty acids ($P < 0.001$, Mann-Whitney U-test). Dietary supplementation with n-3 polyunsaturated fatty acids thus significantly reduces formation of leukotriene B_4 from activated neutrophils at the expense of leukotriene B_5. This is of potential interest because leukotriene B_4 has very powerful proinflammatory effects, with leukotriene B_5 possessing less than 10% of such effects. Introducing n-3 fatty acids into the diet may therefore lead to important antiinflammatory effects in patients treated with dialysis. Furthermore, increasing evidence exists for the role of inflammation in atherogenesis, and n-3 fatty acids may therefore reduce the risk of atherothrombotic complications in this patient group known to be at a very high risk of vascular disease. Clinically controlled trials are, however, necessary to document such a possible clinical benefit of n-3 fatty acids.

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n-3 Fatty Acids in the Prevention of Cardiac Arrhythmias

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ABSTRACT: In animals and probably in humans n-3 polyunsaturated fatty acids (PUFA) are antiarrhythmic. A report follows on the recent studies of the antiarrhythmic actions of PUFA. The PUFA stabilize the electrical activity of isolated cardiac myocytes by inhibiting sarcolemmal ion channels, so that a stronger electrical stimulus is required to elicit an action potential and the relative refractory period is markedly prolonged. This appears at present to be the probable major antiarrhythmic mechanism of PUFA.

McLennan *et al.* reported (1,2) that feeding rats a diet in which the fat content was largely saturated or monounsaturated fats resulted in a high incidence of irreversible ventricular fibrillation (VF) when their coronary arteries were subsequently experimentally ligated. When vegetable oils were the major source of the dietary fat, there was a reduction in arrhythmic mortality by some 70%. With tuna fish oil, however, they reported irreversible ventricular arrhythmias to be completely prevented with or without reflow to the ischemic myocardium. They confirmed their findings in marmosets (3). These striking observations led us to pursue the possible mechanism(s) for such an antiarrhythmic action of the fish oil.

To see first if we could confirm their findings, we studied a canine model of sudden cardiac death. A surgically induced myocardial infarction was produced by ligating the left main coronary artery, and an inflatable cuff was placed around the left circumflex artery. The dogs were allowed about a month to recover from the surgery and their myocardial infarction during which they were trained to run on a treadmill. The animals were then screened for susceptibility to a fatal ventricular arrhythmia when the left circumflex artery was occluded while they were running on a treadmill. About 60% of animals were found susceptible and these were the dogs studied. Once an animal is "susceptible," it remains susceptible on further exercise-ischemia trials. In 10 of the 13 such dogs, infusion of an emulsion of a concentrate of the fish oil free fatty acids, polyunsaturated fatty acids (PUFA) infused intravenously just prior to the exercise-ischemia test prevented the fatal VF ($P < 0.005$) (4). In the control exercise-ischemia tests 1 wk prior to the test with the infusion of the PUFA, and 1 wk following that test all animals developed ischemia-induced VF, requiring prompt defibrillation. In additional studies, we found that pure eicosapentaenoic acid (20:5n-3) or docosahexaenoic acid (22:6n-3), or α-linolenic acid (18:3n-3) are apparently equally antiarrhythmic in this dog preparation. We purposely infused the n-3 fatty acids rather than fed the dogs fish oil to be certain exactly what ingredient of the fish oil prevented the fatal VF. In dietary studies invariably several things must change. When the free fatty acids were infused intravenously just prior to producing the ischemia and preventing the fatal VF, then that effect convincingly results from what has been just infused.

Having confirmed directly the findings of the earlier workers, we wanted to determine the mechanism of this striking antiarrhythmic effect of n-3 PUFA. To have a simple, available model to study in which we could visualize the production of arrhythmias and possible prevention of the arrhythmias by the PUFA, we studied cultured neonatal rat cardiac myocytes (5). Hearts are quickly removed from 1- to 2-d-old decapitated rat pups. The cardiac cells are separated with trypsin digestion, and the cells are plated on microscope cover slips. By the second day of culture, one sees clumps of growing myocytes of a few to several hundred cells. Each group of cells is contracting spontaneously, rhythmically, and synchronously. With a microscope, a video camera, and an edge monitor we can focus on a single myocyte in a clump of cells and see and record the rate and amplitude of contractions. With this *in vitro* model we produced arrhythmias with a number of chemicals known to produce fatal VF in humans: elevated extracellular [Ca^{2+}] toxic levels of the cardiac glycoside ouabain (5), excessive β-adrenergic agonist isoproterenol (6), lysophosphatidylcholine, acylcarnitine, and even the calcium inophore A23187 (7). With each agent a tachyrhythmia was induced. If the PUFA were added to the fluid perfusing the isolated myocytes before the arrhythmogenic toxins were administered, they would in every instance prevent the expected arrhythmia. If the arrhythmia was first induced by the toxin and the PUFA added to the superfusate in the continued presence of the toxin, within a short time the arrhythmia would be terminated and the cells would commence beating again regularly. Then in the continued presence of the toxin, the PUFA can be extracted from the cells with delipidated bovine serum albumin and the

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Abbreviations: PUFA, polyunsaturated fatty acid; VF, ventricular fibrillation.

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arrhythmia promptly resumes. These results indicated that it was only the free PUFA partitioning into the membrane phospholipids that prevented the arrhythmias. If the fatty acid had been covalently bound to any constituent in the membrane, we would not have been able to extract them from the membrane. When the ethyl ester or the triglyceride of the PUFA was tested, they were not antiarrhythmic in this model; the free carboxylic acid group is essential for this antiarrhythmic action.

We then tested which PUFA were antiarrhythmic (5). Both the n-3 and n-6 classes of PUFA are antiarrhythmic, but arachidonic acid (20:n-6) was anomalous. Cyclooxygenase metabolites of arachidonic acid cause arrhythmias (8). For this reason we have recommended that only the n-3 PUFA should be tested in clinical trials as antiarrhythmic agents.

The structural requirements for an antiarrhythmic compound that acts in the manner of these PUFA are a long acyl or hydrocarbon chain with two or more C=C unsaturated bonds and a free carboxyl group at one end. With this guideline we found all-*trans*-retinoic acid also to be specifically antiarrhythmic (9).

The antiarrhythmic action of the PUFA results from their effects on the electrophysiology of cardiac myocytes (10). They cause slight hyperpolarization of the resting or diastolic membrane potential and an increase in the threshold voltage for the opening of the Na^+ channel. This results in an increased depolarizing stimulus of about 50% required to induce an action potential. In addition, the relative refractory period, phase 4 of the cardiac cycle, is prolonged by some threefold. These two effects on every myocyte in the heart would account for the increased electrical stability and resistance of the heart to lethal arrhythmias.

These effects in turn result from an action of the PUFA to modulate the conductance of ion channels in the plasma membranes of the heart cells. By whole-cell voltage clamp measurements, we found that the PUFA inhibit the voltage-dependent Na^+ (I_{Na}), (11), K^+ (the transient outward current, I_{to}, and the delayed rectifier current, I_K, but not the inward rectifying current, I_{K1}) (Xiao *et al.*, unpublished results), as well as the L-type Ca^{2+} current, $I_{Ca,L}$, (12). In the case of the Na^+ channels we showed that only the antiarrhythmic PUFA displace ^{3}H-batrachotoxinin-20-α-benzoate bound to the sodium channel pore protein noncompetitively (13). This is similar to our finding that PUFA noncompetitively displaced ^{3}H-nitrendipine (a specific L-type calcium channel antagonist) from its binding site at the external pore of the calcium channel protein (14). Because the displacement in each case, though specific, was noncompetitive, we cannot resolve the urgent question of whether the PUFA bind specifically and primarily to ion channel proteins directly or interact primarily with the phospholipid of the cell membranes to allosterically change the conformation of transmembrane protein channels.

In the cultured neonatal rat heart cells, the PUFA shifted the steady-state potential for inactivation of the Na^+ channels to more hyperpolarized potentials (11). A recent study of the effects of the PUFA on the Na^+ currents in α-subunits of the human myocardial Na^+ channel expressed in human embryonic kidney cells (15) showed no effect on the activated opening of the Na^+ channel. The only effect seen was a large shift in the hyperpolarization of the membrane potential necessary to close the Na^+ channel. Attaining a closed resting, but activatable, state is required before the channel is again susceptible to initiation of a new action potential. This makes the I_{Na} voltage-dependent in the presence of the PUFA, as we found (11,15). Myocytes apparently must maintain their normal resting membrane potentials in order to avoid the inhibitory actions of the PUFA on I_{Na}.

These effects primarily on inhibition of the Na^+ and Ca^{2+} currents, we now think, may account for the potent antiarrhythmic effects of these PUFA. In ischemic myocardium, myocytes become slightly depolarized due to functional reduction of the Na,K-ATPase pump. These cells become "hyperexcitable" and subject to induction of premature action potentials and arrhythmias. Their resting membrane potential is more positive and closer to the threshold for the gating of the I_{Na} so that any small depolarizing current may elicit a premature action potential and initiate an arrhythmia. Because they have a reduced resting membrane potential, it is just these ischemic "hyperexcitable" myocytes that are eliminated quickly from further mischief by PUFA. After their first activation, the necessity for a more negative resting potential in order to revert the channel to an activatable resting state makes the channel unresponsive and eliminates it as an arrhythmogenic risk.

The potent inhibitory action of the PUFA on L-type Ca^{2+} currents, $I_{Ca,L}$ (12), complements this action on I_{Na}. Significant inhibition of $I_{Ca,L}$ is observed at 10 nM Ca^{2+} in the medium, bathing the human cardiac Na^+ channel (15). This effect prevents triggered arrhythmias induced by overload of cytosolic Ca^{2+} and increased cytosolic Ca^{2+} fluctuations. Most arrhythmogenic cardiotoxins may induce fatal ventricular tachycardia (VT) or VF by triggered after-potentials from excessive cytosolic Ca^{2+} which are prevented by the PUFA., e.g., cardiac glycosides, lysophosphatidylcholine, excessive catecholamines, thromboxane A_2, etc.

Evidence that PUFA will prevent lethal arrhythmias in patients is slim, but two secondary prevention trials, which unexpectedly showed prevention of ischemia-induced sudden cardiac death, are encouraging. One dietary study (16) was a prospective, randomized, single-blinded, secondary prevention trial which compared the effect of a Mediterranean α-linolenic acid-rich diet to the usual "prudent" diet. The subjects on the more fat-restricted experimental diet receiving the α-linolenic acid (18n-3) showed a remarkable reduction in mortality and morbidity of some 70%, including prevention of sudden death. The other study (17) was also a randomized, prospective, secondary prevention trial in which advice to eat oily fish two or three times weekly was compared with no such advice. This study did not record arrhythmic deaths. It, however, found no reduction in new events but a 29% reduction in mortality, suggesting a reduction in sudden deaths which constitute 50 to 60% of the acute mortality from heart attacks (18). In both studies the survival curves showed a very

early beneficial separation of the experimental vs. control groups, quite unlike the 2 yr required in the cholesterol-lowering trial (19) before the lower mortality was significant. A case-control study (20) reported an inverse relationship between fish consumption and sudden cardiac death, suggesting an antiarrhythmic effect from ingestion of fish. The Indian infarct survival study reported a significant reduction in arrhythmic and total cardiac deaths in a randomized, blinded, controlled study in patients fed fish oil 1.08 g/d for 1 yr (21). Reanalysis of the Physicians Health Study (22) reported a 52% reduction of risk of sudden cardiac death in subjects eating fish at least once a week.

In conclusion, it is apparent that there exists a basic control of cardiac function by common dietary fatty acids which has been largely overlooked. The n-3 PUFA have been part of the human diet for some 2–4 million yr (23), while our genes were adapting to our diet as hunter-gatherers. With some 250,000 sudden cardiac deaths annually largely due to VF in the US alone (18) there may be a potential, large public health benefit from the practical application of this recent understanding. Carefully planned and executed clinical trials are now needed to determine the antiarrhythmic effectiveness of these fatty acids in humans at high risk of fatal arrhythmias.

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The Inhibition of Endothelial Activation by Unsaturated Fatty Acids

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ABSTRACT: Dietary long-chain fatty acids (FA) may influence pathological processes involving endothelial activation and leukocyte-endothelial interactions, such as inflammation and atherosclerosis. We previously showed that the n-3 FA docosahexaenoate (22:6n-3, DHA) inhibits cytokine-stimulated expression of endothelial-leukocyte adhesion molecules and soluble cytokines in the range of nutritionally achievable plasma concentrations. More recently we assessed structural determinants of VCAM-1 inhibition by FA. Cultured endothelial cells were incubated first with various saturated, monounsaturated, n-6 or n-3 polyunsaturated FA alone and then together with interleukin-1 or tumor necrosis factor. Saturated FA did not inhibit cytokine-induced endothelial activation, while a progressive increase in inhibitory activity was observed, for the same chain length, with the increase in double bonds accompanying the transition from monounsaturates to n-6 and, further, to n-3 FA. Comparison of various FA indicated no role of the double-bond position or configuration; the greater number of double bonds could explain the greater inhibitory activity of n-3 vs. n-6 FA. In order to ascertain mechanisms for these effects, we demonstrated inhibition of nuclear factor-κB (NF-κB) activation by DHA in parallel with a reduction in hydrogen peroxide (a critical mediator of NF-κB activation) released by endothelial cells either extracellularly or intracellularly. This suggests that a property related to fatty acid peroxidability (the presence of multiple double bonds) is related to inhibitory properties of hydrogen peroxide release and, consequently, of endothelial activation.

Highly unsaturated fatty acids (FA) and, particularly, n-3 FA, are receiving increasing attention as potential anti-atherogenic and antiinflammatory agents. This paper will summarize our recent research line in attempting to provide some mechanistic explanations for their preventive or therapeutic use to this regard.

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Abbreviations: DHA, docosahexaenoate acid; FA, fatty acid; IL, interleukin; LPS, lipopolysaccharide; NF-κB, nuclear factor-κB.

n-3 FA AND ATHEROGENESIS

The epidemiological association between dietary n-3 polyunsaturated fatty acids (n-3 FA) and protection from cardiovascular disease (1–8) results at least in part from a decreased incidence of atherosclerosis. Apart from numerous animal studies showing decreased atherosclerosis in animals treated with n-3 FA (reviewed in Ref. 9), recent evidence has been obtained about such effects in humans, through autopsy studies in Alaskan natives (consuming high amounts of fish-derived products) and nonnatives, mostly consuming Western-type diets. In the study by Newman and coworkers, reporting decreased percentage of intima covering with fatty streaks and raised lesion in Alaskan natives [with a high n-3 FA dietary intake (6)], vs. nonnatives, the magnitude of difference in fatty streak development appears larger in younger age groups (10), suggesting an effect of diet mainly in the early events leading to fully developed atherosclerotic lesions. A recent study of n-3 FA supplementation after coronary bypass surgery indicates that such a treatment significantly reduces vein graft stenosis (11), a process which may be regarded as an accelerated form of atherosclerosis.

We therefore hypothesized that n-3 FA may modulate atherogenesis by affecting processes of early atherosclerotic development leading to the formation of fatty streak. Such processes are mostly comprised under the name of "endothelial activation."

ENDOTHELIAL ACTIVATION AS AN EARLY CONTROLLING STEP IN ATHEROGENESIS

Atherosclerosis and inflammation share similar basic mechanisms involving the adhesion of leukocytes to vascular endothelium in their early phases. Multiple protein families, each with a distinct function, provide "traffic signals" for leukocytes. These include (i) the "selectin" family of adhesion molecules; (ii) chemoattractants, some of which ("classical" chemoattractants), such as *N*-formyl peptides, complement components, leukotriene B_4 and platelet-activating factor, act broadly, on neutrophils, eosinophils, basophils and monocytes, while more recently described "chemokines," such as monocyte chemoattractant protein-1 and interleukin (IL)-8, have selectivity for

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leukocyte subsets; (iii) the immunoglobulin superfamily members on the endothelium (ICAM-1, ICAM-2,ICAM-3, and VCAM-1), recognizing "integrin" ligands on the leukocyte surface. For neutrophil and, probably, lymphocyte adhesion, selectins mediate initial tethering of the circulating leukocyte over the endothelium, allowing it to roll over the endothelium, considerably slowing down its speed, and allowing leukocytes to "sense" the presence of chemotactic gradients. Final firm attachment of leukocytes to endothelium requires the interaction of integrin ligands on the leukocyte surface with immunoglobulin superfamily members expressed on the endothelium, such as ICAM-1, ICAM-2, and VCAM-1. The multiple molecular choices available for each of these ligand–ligand interactions provide great combinatorial diversity in signals, allowing the selective responses of different leukocyte classes to inflammatory agents, the preferential recirculation patterns of lymphocyte subpopulations, or the selective binding of monocytes to arterial endothelium during early phases of atherogenesis.

Since monocyte recruitment into the intima of large arteries is specific for atherosclerosis as compared to other forms of leukocyte-endothelial interactions, it was hypothesized that these localized monocyte–endothelium interactions reflect specific molecular changes in the adhesive properties of the endothelial surface, leading to endothelial surface expression of "athero-ELAMs," i.e., endothelium-leukocyte adhesion molecules expressed in the early phases of atherosclerosis. The first such protein, originally identified in the rabbit hypercholesterolemic model, is VCAM-1, a member of the immunoglobulin superfamily, expressed on human vascular endothelium at least in two molecular forms. Both forms are able to bind a heterodimeric integrin receptor, VLA4, whose leukocyte selectivity of expression, on monocytes and lymphocytes, but not on neutrophils, can explain the selectivity of monocyte recruitment in early atherogenesis (12). Endothelial cells express VCAM-1 early during cholesterol feeding in the rabbit, before the appearance of macrophages/ foam cells in the intima of developing fatty streak, in a temporal pattern consistent with its pathogenetic role in lesion develoment. Pathophysiologically relevant stimuli for VCAM-1 expression in atherogenesis could include minimally oxidized low density lipoprotein or β-very low density lipoprotein, the advanced glycation end products associated with diabetes, lipoprotein (a), or perhaps homocysteine, elevated in homocysteinuria and in subtler forms of congenital or acquired enzyme defects in its biosynthetic pathway. In addition to these humoral stimuli, VCAM-1 endothelial gene expression also responds to hemodynamic forces, thus potentially explaining the localization of atherosclerosis in particular points of the arterial vasculature. For a general review on these issues, see Reference 12.

EVIDENCE THAT n-3 FA MAY CONTROL ENDOTHELIAL ACTIVATION

We used human adult saphenous vein endothelial cells activated by cytokines, in an *in vitro* model of these early steps in atherogenesis, first assessing the effects of various fatty acids on the surface expression of endothelial leukocyte adhesion molecules, and subsequently characterizing mechanisms and functional relevance of such effects. One n-3 fatty acid, docosahexaenoic acid (DHA), when added to cultured endothelial cells hours to days before the stimulation with cytokines, early enough to allow a significant incorporation of this fatty acid in cell membrane phospholipids, significantly inhibited events connected with endothelial activation, including the expression of adhesion molecules such as VCAM-1, E-selectin and, to a lesser extent, ICAM-1 after stimulation with virtually any stimulus able to elicit the coordinated expression of such genes (13,14). Thus, this inhibition could be demonstrated with IL-1α and β, tumor necrosis factor α (TNFα), IL-4, and lipopolysaccharide. Inhibition of adhesion molecule expression occurred in a range of DHA concentrations compatible with nutritional supplementation of this fatty acid to normal Western diet, occurred at any time point after the appearance of cytokine effect, modifying the specific kinetics of surface expression of adhesion molecules, and was strictly related in its magnitude to the extent of incorporation into total cell lipids. Indeed, the extent of VCAM-1 inhibitory effect paralleled the incorporation of DHA and the overall increase in incorporation of n-3 FA and was inversely related to the content of n-6 FA. Experiments following the fate of ^{14}C-labeled DHA into cell phospholipids showed a significant incorporation of DHA into the phosphatidylethanolamine pool, i.e., in a specific and not the most abundant phospholipid pool, likely in the inner plasma membrane, and therefore in a possibly strategic position to alter intracellular signal transduction pathways. This effect was not limited to the expression of transmembrane molecules involved in leukocyte recruitment but appeared to be true for other cytokine-activated products, such as the soluble proteins IL-6 and IL-8 involved either in the amplification of the inflammatory response (IL-6) or in specific chemoattraction for granulocytes (IL-8), and was accompanied by a functional counterpart, i.e., reduced monocyte or monocytoid cell adhesion to cytokine-activated endothelium. Compared to DHA, eicosopentaenoic acid was a weaker inhibitor of the expression of these molecules and of monocyte adhesion, although still more potent than other fatty acids. We also showed that DHA's effects on VCAM-1 expression are accompanied by parallel reductions in VCAM-1 mRNA steady-state levels, as assessed by Northern analysis (see Refs. 13,14). Similar results, in experiments with remarkably similar design, were later reported by Weber *et al.* (15). These authors also carried these investigations one step further, demonstrating, by electrophoretic mobility shift assay, an inhibition by DHA of the activation of the nuclear factor-κB (NF-κB) system of transcription factors (15), which controls the coordinated expression of adhesion molecules and of leukocyte-specific chemoattractants upon cytokine stimulation (16,17).

THE DOUBLE BOND AS THE MINIMAL NECESSARY AND SUFFICIENT REQUIREMENT FOR THE INHIBITION OF ENDOTHELIAL ACTIVATION BY FA

We further analyzed endothelial effects of various FA differing in chain length, number, position (n-3 vs. n-6 vs. n-9) and

cis/trans configuration of the double bonds. From a large number of such experiments, using VCAM-1 surface expression (by enzyme immunoassay or flow cytometry) as a readout, we concluded that (i) saturated FA are inactive; (ii) potency of polyunsaturated FA increases with the number of unsaturation; (iii) potency does not depend on chain length; (iv) the single double bond present in the monounsaturated fatty acid oleic acid is indeed sufficient to produce all the effects obtainable with higher unsaturated FA, albeit at higher concentrations; (v) for such an effect to occur even the configuration (*cis* vs. *trans*) of the double bond does not really matter, since oleic acid (19:1n-9 *cis*) and its *trans* stereoisomer elaidic acid are of equal potency (18). Indeed, inhibition of NF-κB activation could also be reproduced upon incubation of endothelial cells with oleic acid (19).

POSSIBLE MECHANISMS BY WHICH UNSATURATED FA MAY INHIBIT ENDOTHELIAL ACTIVATION

In order to ascertain mechanisms for these effects, we demonstrated inhibition of NF-κB activation by DHA (the most potent FA inhibitor of endothelial activation) in parallel with measurements of production of hydrogen peroxide by cultured endothelial cells. This reactive oxygen species (or one of more of its downstream unstable products) appears to be a critical mediator of NF-κB activation, because of our previous demonstration that treatment of endothelial cells with polyethylene glycol-complexed superoxide dismutase (a cell membrane-permeable form of this enzyme catalyzing the conversion of superoxide anion to hydrogen peroxide) does not much affect VCAM-1 mRNA production, contrary to a treatment with polyethylene glycol-catalase, which acts by accelerating the degradation of hydrogen peroxide, and which clearly diminished VCAM-1 mRNA levels (20). These results suggested that some specific reactive oxygen species (hydrogen peroxide or some downstream products) are involved more directly than others (e.g., superoxide anion) in the activation of NF-κB. We assessed the production of extracellular hydrogen peroxide by endothelial cells stimulated with the cytokine-transforming growth factor-β and, more pertinent to results described above, the production of intracellular hydrogen peroxide (and/or its downstream products) by measuring the intracellular fluorescence after endothelial cell loading with dichloro-fluoresceine before or after stimulation with IL-1 or tumor necrosis factor. In both these experimental systems we could document (preliminary results) a decrease in baseline production of hydrogen peroxide (or some of its downstream products) after cell membrane enrichment with DHA, and an even more pronounced dampening of the increase produced by stimulation with cytokines. Saturated FA served as a negative control in these experiments. This suggests that a property related to FA peroxidability (the presence of multiple double bonds), and usually regarded as a detrimental consequence of polyunsaturated FA enrichment of cell membranes, is indeed also directly related to inhibitory properties in the release of some reactive oxygen species crucial for cell responsiveness to cytokines.

CONCLUSIONS AND OPEN QUESTIONS

These results have led to a reappraisal of how FA may act on endothelial cells in modulating general phenomena such as atherogenesis (mostly investigated by our experimental systems), but also, potentially, inflammation or some immune responses. Since all these effects could be confirmed to occur even in the presence of inhibitors of metabolic conversion of FA to eicosanoids, they provide a novel explanation for the modulating effect of n-3 FA in atherogenesis, distinct from the "classical" hypothesis of substrate substitution (21). The results with oleic acid might also be an explanation for at least some of the beneficial effects of olive oil-rich ("Mediterranean") diets on atherogenesis (19). It is noteworthy, to this regard, that oleic acid mostly appeared to incorporate to the expense of saturated FA, thus disclosing the possibility of additive effects with n-3 FA, which mostly substitute less unsaturated FA in the membrane phospholipid pools. If extended to cell types different from endothelial cells, such as the monocyte-macrophage, also undergoing "activation" phenomena upon cytokine or LPS stimulation, they may provide a coherent explanation for a number of previous observations such as the inhibition of cytokine formation from lipopolysaccharide-activated macrophages (22). As to the mechanism(s) involved, these effects might be intrinsically linked to polyunsaturated FA peroxidability, usually regarded as a detrimental effects of higher unsaturated FA, but which could simply be the other side of the same coin. Future research will have to further elucidate molecular aspects of these phenomena as well as the greater scope of this research line to help explain other many biological effects of unsaturated FA as modulators of biological responses to cytokines.

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Dietary Fish Oil Promotes Positive Inotropy and Efficiency of Digitalis

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Digitalis is clinically effective in heart failure, but it has an extremely narrow therapeutic index. Inclusion of fish oil (FO) in the diet may have cardioprotective effects by different mechanisms (1,2): decreased heart rate and occurrence of arrhythmias, increased mechanical activity, and ejection fraction. The aim of this study was to investigate if dietary FO with incorporation of n-3 polyunsaturated fatty acids in membranes could promote positive inotropy of ouabain.

Methods. Twenty-five male Wistar rats were fed for 60 d: (i) supplemented with FO concentrate [180 mg eicosapentaenoic acid (EPA) + 120 mg docosahexaenoic acid (DHA)/g oil] at a dose of 0.5 g/kg/d (group FO), (ii) without supplementation (group C). Inotropic and toxic responses to cumulative doses of ouabain (from 100 nM to 0.3 mM) were evaluated in the isolated perfused rat heart and the relative variations in cardiac energetic metabolism, and pH were obtained using ^{31}P magnetic resonance spectroscopy. Activity, contribution, affinity, and expression of Na,K-ATPase isoforms were determined on purified membrane-bound Na,K-ATPase. Fatty acid composition of cardiac membrane fractions was analyzed by gas chromatography.

Results. The rat is the only mammalian species with a well-characterized biphasic inotropic response to ouabain which is coupled to consecutive inhibition of the $\alpha 2$- and $\alpha 1$-isoforms of high and low affinity for ouabain, respectively. Results show that (i) there is a twofold increase in the low-affinity response to ouabain (0.1 mM) (% inotropy = 40 ± 3 in group C vs. 79 ± 7 in group FO, $P < 0.001$), (ii) there is a lower inhibition of $\alpha 1$ Na,K-ATPase activity ($IC_{50} = 7.2 \pm 2.0 \times 10^{-5}$ M in group C and $3.4 \pm 0.8 \times 10^{-4}$ M in group FO, $P < 0.05$), (iii) the higher inotropic response to ouabain is not associated with significant changes in energetics, (iv) alteration of function and energetics in response to the toxic dose of 0.3 mM is delayed in the FO group, (v) there are no differences in overall Na,K-ATPase activity or in the proportion of its isoforms, and (vi) fatty acid composition of rat hearts submitted to FO diet shows that the proportion of EPA and DHA had significantly risen.

In conclusion, improving ouabain efficacy may represent an additional mechanism whereby FO exert their cardioprotective action in rats and possibly in humans.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FO, fish oil.

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n-3 Fatty Acids and the Risk of Sudden Cardiac Death Assessed by 24-Hour Heart Rate Variability

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Dietary n-3 polyunsaturated fatty acids (PUFA) may reduce the incidence of sudden cardiac death (SCD) in humans possibly due to an antiarrhythmic effect. However, only sparse information exists on such an effect in humans. Therefore, we performed three double-blinded intervention trials with n-3 PUFA in three different groups: in patients with a previous myocardial infarction (MI) (1,2), in patients with chronic renal failure and at high risk of SCD (3), and in healthy volunteers (Christensen, J.H., Christensen, M.S., Dyerberg, J., and Schmidt, E.B., unpublished data). The end point in the trials was 24-h heart rate variability (HRV), and the clinically important HRV parameter SDNN [standard deviation of all normal RR intervals during a 24-h electrocardiogram (ECG) recording] was used. HRV is a powerful predictor of mortality and of arrhythmic events in humans.

The relationship between fish consumption, the content of n-3 PUFA in platelets, and 24-h HRV was examined in 55 post-MI patients (mean age 63 ± 7 yr) with left ventricular dysfunction (mean ejection fraction 0.33 ± 0.05). The patients were divided into three groups: (i) Those who never ate fish, (ii) those who ate fish once a week, and (iii) those eating fish at least twice a week. A close positive correlation was observed between fish intake and the cellular content of n-3 PUFA. HRV tended to be higher among those who consumed one fish meal per week compared to no fish (122 vs. 103 ms, $P = 0.07$). Furthermore, a significant positive correlation was found between the cellular content of n-3 PUFA and HRV ($r = 0.30$, $P < 0.05$). These post-MI patients were now randomly allocated to receive either 5.2 g of n-3 PUFA daily or placebo oil for 12 wk. After dietary supplementation, HRV increased from 115 to 124 ms (significant compared to baseline and to placebo, $P = 0.01$).

The relationship between the cellular content of n-3 PUFA and HRV was also examined in patients with chronic renal failure. Twenty-nine patients (mean age 52 ± 15 yr) received 5.2 g of PUFA or olive oil daily for 12 wk. After dietary supplementation, a close positive correlation was found between the cellular content of n-3 PUFA and HRV ($r = 0.71$, $P < 0.01$).

Finally, 60 healthy subjects (25 women, mean age 38 yr; 35 men, mean age 38 yr) were randomized to either (i) 2.0 g of n-3 PUFA daily, or (ii) 6.6 g of n-3 PUFA daily, or (iii) placebo for 12 wk. At baseline, a close positive correlation was found between n-3 PUFA in granulocytes and HRV. Furthermore, the subjects with a low HRV at baseline had a dose-dependent increase in HRV after n-3 PUFA supplementation.

In conclusion, the results showed a beneficial effect of n-3 PUFA on HRV in three different populations, strongly indicating an antiarrhythmic effect of n-3 PUFA. Our data may explain the findings from other studies, namely that a low occurrence of SCD is observed in subjects with and without coronary heart disease who regularly eat fish.

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Abbreviations: HRV, heart rate variability; MI, myocardial infarction; PUFA, polyunsaturated fatty acid; SCD, sudden cardiac death.

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Stearic Acid-Rich Diets Do Not Increase Thrombotic Risk Factors in Healthy Males

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Stearic acid is a saturated fatty acid found in meat fat and chocolate. Studies from more than 30 yr ago indicated that stearic acid might promote blood clotting through increased platelet aggregation (1). More recent studies have indicated that stearic acid is unique as a saturated fatty acid in that it does not cause an increase in blood cholesterol levels (2). Several recent studies indicate that stearic acid might not be a thrombotic-promoting fatty acid; however, the data have not been convincing to all observers. Five intervention studies carried out (between 21–40 d for each dietary intervention) with a range of 10–18 subjects (mean age 23–37 yr) gave varying results regarding the cholesterolemic and thrombogenic influences of stearic acid. In one of the studies (3), the stearic acid-rich diet increased the thrombosis potential (increased factor VIIc); however, since the study did not compare stearic acid with another saturated fatty acid, this reduced the importance of this result. A second study found a nonsignificant rise in this parameter (4), while a third study showed there was a decreased factor VIIc activity (5). The study by Schoene *et al.* (6) showed that stearic acid-rich diets decreased mean platelet volume, which indicates a reduced platelet activation (less thrombotic) and Tholstrup *et al.* (4) showed that platelet aggregation induced by low levels of collagen and ADP was lowered 24 h after consumption of stearic acid.

The aim of this study was to compare the thrombotic risk factors of two diets rich in saturated fatty acids, palmitic acid, and stearic acid. Palmitic is a very common saturated fatty acid in foods including meat fats. In this study, 13 male subjects consumed each test fat for 4 wk with a 7-wk wash-out between test diets in a random cross-over design. The test fats were supplied to the subjects in the form of baking and spreading margarines, cakes, and biscuits. The mean intake of stearic acid was 19.4 g/d in the high stearic acid period compared with 7.3 g/d in the baseline period. For palmitic acid, the baseline level was 13.6 g/d compared with 22.5 g/d in the test period. The diets led to significant ($P < 0.05$) increases in palmitic (8%) or stearic acids (17%) in the platelets during the test diet periods. Despite this, there were no significant differences in platelet aggregation, fibrinogen levels, or Factor VIIc activity between the two test diets. The main difference was in mean platelet volume (MPV) which was significantly reduced ($P < 0.05$) on the stearic acid diet (–6%, $-0.57 \pm 0.09\ 10^{-15}$ L) compared with the palmitic acid diet which increased MPV (+2%). Reduced MPV values are an index of reduced platelet activity, and this supports the data of Schoene *et al.* (6) who also reported that stearic acid-rich diets reduce MPV. These data indicate that an additional 12 g/d of stearic acid is not pro-thrombotic over a 4-wk period in men. Additional studies should be conducted using more sophisticated markers of platelet reactivity as well as consideration to use stearic acid in the food industry to provide the appropriate physical properties for various food stuffs. There is reported interest in increasing the stearic acid content of soybeans through the use of plant breeding techniques, as indicated by several papers presented at the 1998 annual meeting of the American Oil Chemists' Society.

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The 3-Thia Fatty Acid, a Novel Bioactive Compound, Which Changes the Plasma Profile from Atherogenic to Cardioprotective

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The hypolipidemic effect of sulfur-substituted long chain fatty acids (tetradecylthioacetic acid, TTA), where sulfur substitution prevents the β-oxidation of TTA, is well-documented in rats, rabbits, dogs, and possibly also in humans. The hypotriglyceridemic properties of TTA which activates peroxisome proliferator-activated receptor α are probably due to increased mitochondrial β-oxidation with a concomitant decreased triglyceride synthesis. Therefore, the mitochondria and not the peroxisomes are the principal targets for this effect. The lowering of low density lipoprotein (LDL)-cholesterol is possibly due to inhibition of 3-hydroxy-3-methylglutaryl-CoA reductase and acyl-CoA cholesterol acyltransferase. TTA possesses a sulfur atom, and it is therefore expected to have reducing properties. TTA was a potent compound in inhibiting the human LDL oxidative modification induced either by Cu^{2+} ions or azo-compounds. The electrophoretic mobility of LDL returned to normal values in the presence of TTA. This fatty acid analog was effectively able to reduce, in a dose-dependent manner, the formation of 8-hydroxydeoxy guanosine from 2-deoxy guanosine. TTA also changed the antioxidant defense system in plasma in a beneficial way, i.e., reduced glutathione levels increased. The total antioxidant status elevated, and thiobarbituric acid-reactive substances were decreased. An additional "olive oil" effect of TTA was seen as LDL purified from TTA-treated rats and dogs were protected from oxidation. The LDL from TTA-treated animals were enriched with 18:1n-9 and a Δ^9-desaturated metabolite of TTA, probably due to upregulated Δ^9-desaturase gene expression. Antioxidants were suggested to reduce restenosis. In a preliminary study, TTA reduced proliferation of smooth muscle cells. Data from a study of oral administration with TTA suggest that it is effective reducing the development of restenosis on rabbit artery after balloon injury (overstretching). The exact mechanism is not known, but it seems possible to modulate the injury response pharmacologically. The pharmacological properties of the 3-thia fatty acids suggest that TTA may be of potential interest for a new therapeutic approach to atherosclerosis.

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Abbreviations: LDL, low density lipoprotein; TTA, tetradecylthioacetic acid.

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Dietary n-3 Polyunsaturated Fatty Acids and Oxidants Increase Rat Mammary Tumor Sensitivity to Epirubicin Without Change in Cardiac Toxicity

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We previously reported that polyunsaturated fatty acids (PUFA) and oxidants (OX) increased *in vitro* efficacy of anticancer drugs that generate reactive oxygen species, through an increase in lipid peroxides formation (1). The aim of the study was to examine whether n-3 PUFA and OX modulate *in vivo* the antitumor activity and cardiotoxicity of epirubicin, an anthracyclin that stimulates oxidative stress.

Thirty-six rats with *N*-methyl nitrosourea-induced mammary tumors were fed a basal diet supplemented with n-3 PUFA and separated in three groups receiving n-3 PUFA alone (15% sardine oil, control group) or with lipid peroxidation inhibitor (vitamin E, 100 IU/kg, vit E group) or with lipid peroxidation inducers (dehydroascorbate + menadione, respectively, 20 and 0.2 mg/d, OX group). When the target tumor reached 1 cm in diameter, epirubicin (3 mg/kg; i.p.) was administered weekly for 3 wk. Tumor response was assessed in two ways: the change in tumor area and the score of new tumors occurring after chemotherapy. Four weeks after the end of treatment, cardiac functioning was evaluated by measuring the left ventricular end-diastolic pressure at basal conditions or during volume expansion.

During a 6-wk period, the growth of the target tumor was totally inhibited in the OX group by the epirubicin treatment, while it kept going in the control and in the vit E groups. Similarly, the number of newly appeared tumors was reduced three times in the OX group in comparison to both the other groups. No difference in the cardiac pressures measured at rest or during volume expansion was observed between the dietary groups. However, almost 10% of rats in all dietary groups were found to present an elevated left ventricular end-diastolic pressure, which is characteristic of the cardiotoxicity induced by anthracyclin.

It is concluded that dietary n-3 PUFA in the presence of OX increased the antitumor activity of epirubicin *in vivo*, without affecting cardiac toxicity. This observation suggests that supplementing the diet with n-3 PUFA plus OX increases the cytotoxicity of epirubicin toward tumor cells rather than toward normal cardiac cells. A phase I-II is now contemplated in metastatic breast cancer patients in Tours.

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Abbreviations: OX, oxidant; PUFA, polyunsaturated fatty acid.

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Roles of Lipid-Activated Receptors in the Adipogenic Action of Fatty Acids

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Fatty acids are precursors of triglycerides, phospholipids, and eicosanoids. In addition to their role as substrates, fatty acids affect many cell functions acting either indirectly, *via* arachidonic acid metabolites, or directly by modulating various cellular signaling pathways (1–3). During the past years, it has been demonstrated that fatty acids play a central role in the control of gene expression in many tissues and participate in the control of adipose tissue development. *In vivo* studies showed that high-fat diets lead to hypertrophic and hyperplastic development of adipose tissue in adult rodents (4,5). These nutritional obesities occur within weeks and are preceded by a proliferation of preadipose cells taking place a few days after the shift to high-fat diet. The finding that diets containing saturated fat are more active than polyunsaturated fat-enriched diets (6) suggested a direct action of fatty acids on adipose tissue rather than involvement of fatty acid metabolites such as eicosanoids. During the last few years, the use of preadipose cell lines has greatly improved our knowledge on the cellular and molecular mechanisms which are behind the adipogenic effects of fatty acids. Particularly, these studies led to the identification of nuclear receptors activated by fatty acids and related molecules and to the description of their roles in adipogenesis. These findings were an important step in the understanding of the control of adipose differentiation and have provided a molecular explanation of the relationships between the amount of fat in diet and adipose tissue development.

ADIPOGENIC ACTION OF FATTY ACIDS

The action of fatty acids has been extensively investigated in our laboratory using the Ob1771 preadipose cell line. These cells, established from genetically obese mouse, undergo a typical adipose conversion process when maintained in appropriate conditions. Adipose differentiation is a complex process that corresponds to transcriptional activation of numerous genes and occurs in a sequential manner (6,7). The first step occurs just at growth arrest and is characterized by transcriptional activation of "early genes," such as pOb24, CAAT enhancer-binding protein (CEBP) β and δ, and lipoprotein lipase (8). These committed preadipose cells undergo several rounds of mitosis which are critical for terminal differentiation. This last step is characterized by expression of a large set of genes, including those involved in triglyceride synthesis such as glycerophosphate dehydrogenase (GPDH), adipocyte lipid-binding protein (ALBP), fatty acid transporter (FAT), or acylCoA synthetase (ACS). Notably, in standard culture conditions, terminal differentiation occurs only in a limited proportion of the cells leading to the formation of colonies of adipose cells separated from each other by cells resting in the preadipose state, i.e., cells expressing only the early markers of adipose differentiation. Adipose tissue from adult animals contains such dormant preadipose cells capable of proliferation and terminal differentiation throughout the life. Adipose differentiation is under multihormonal control involving circulating hormones and factors such as insulin, growth hormone, triiodothyronine, retinoids and prostacyclin, which activate adipose marker expression and lipid deposition.

Exposure of Ob1771 cells to fatty acid-enriched medium during the course of differentiation leads at the end of the process to a tremendous increase of adipose cell number due to an enhancement of the percentage of differentiated cells and an amplification of the proliferation of preadipose cells that occurs after confluence. This morphological adipogenic action of fatty acid supplementation is characterized at the biochemical level by an increase of triglyceride amount per cell (fivefold increase when compared to cells maintained in standard differentiation medium) and is not a passive filling-up of the cells as accompanied by increase of specific activities of enzymes of lipid metabolism such as GPDH and ACS and enhancement of adipose differentiation program expression. Interestingly, saturated and unsaturated long-chain fatty acids (LCFA) were found to be equally potent, whereas middle- and short-chain fatty acids were ineffective on adipose differentiation of Ob1771 cells. Furthermore, 2-bromopalmitate, as compared to palmitate, exerted its adipogenic action at lower concentrations (half-maximal effect observed at 10 μM vs. 75 μM)(9). As 2-bromopalmitate was shown to be less metabolized than palmitate in Ob1771 cells (10), this last observation suggested that adipogenic effect of LCFA is likely related to a direct effect of the molecule rather than to an increase in substrate availability for triacylglycerol synthesis.

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Abbreviations: ACS, acylCoA synthetase; ALBP, adipocyte lipid-binding protein; CEBP, CAAT enhancer-binding protein; cPGI2, carbaprostacyclin; FAAR, fatty acid-activated receptor; FAT, fatty acid transporter; GPDH, glycerophosphate dehydrogenase; LCFA, long-chain fatty acid; PGJ_2, prostaglandin J_2; PPAR, peroxisome proliferator-activated receptor.

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In order to further characterize this adipogenic action of LCFA, some efforts were devoted to determine the differentiation step affected by 2-bromopalmitate. These experiments showed that chronic exposure to the compound was not required for maximal effect as treatment from confluence to day 5 exerted similar effects on adipose differentiation. By contrast, treatments delayed to day 5 were found to be ineffective (9). These findings led to the conclusion that an increase in intracellular concentration of unprocessed LCFA occurring in preadipose cells, i.e., not terminally differentiated cells, acts as a trigger for terminal differentiation events.

TRANSCRIPTIONAL EFFECTS OF LCFA IN PREADIPOSE CELLS

The effects of LCFA on the pattern of gene expression were investigated in preadipose Ob1771 cells just after confluence (11–13). At this stage, genes related to terminal differentiation, including those involved in fatty acid metabolism, are completely unexpressed, and the cells are not able to synthesize fatty acids and triglycerides. Treatment of such cells by LCFA led to potent transcriptional activation of genes encoding proteins directly involved in fatty acid metabolism, such as ALBP, FAT, ACS and lipoprotein lipase, whereas the other adipose-related genes remained transcriptionally inactive. In such transcriptional effect, saturated and unsaturated LCFA as well as nonmetabolized LCFA derivatives were found to be active, while middle- and short-chain fatty acids were less effective. Time-course experiments revealed that LCFA effects occurred within hours and were rapidly and completely reversible upon their withdrawal from culture medium (11,12). The use of 2-bromopalmitate and serum-free culture medium allowed the conclusion that this LCFA derivative exerted its transcriptional effect at intracellular concentrations ranging from 10 to 100 nanomolar.

Taken together, these findings indicated that accumulation of very low amounts of unprocessed LCFA led to transcriptional activation of genes directly involved in fatty acid uptake and activation and that these short-term effects of LCFA resulted, after several days, in an amplification of the terminal differentiation process. Direct extrapolation of these results to *in vivo* situation remains hazardous, but the demonstration that LCFA act as transducing molecules in preadipose cells favors the hypothesis of a direct link between the LCFA amount of the diet and adipose tissue development and could explain, at least partially, the hypertrophic and hyperplasic action of high-fat diets.

ROLE OF NUCLEAR RECEPTORS IN FATTY ACID GENE REGULATION

It is now fully established that fatty acids and fatty acid metabolites exert some of their transcriptional effects through activation of nuclear receptors, namely PPAR (peroxisome proliferator-activated receptors). So far, three different PPAR isoforms have been identified in mammals. Various authors have showed that PPAR exerted their transcriptional effects by heterodimerization with retinoid × receptors and binding to a responsive element, called peroxisome proliferator-responsive element. These regulatory sequences, first identified in the acyl-CoA oxidase promoter (14), are imperfect 6-base pair direct repeats of the sequence RGGTCA spaced by a single variable nucleotide. Additional peroxisome proliferator-responsive elements have been identified in regulatory sequences of various genes, and especially those encoding proteins involved in lipid metabolism (15).

The differential expression of PPAR isoforms suggested specific regulatory functions. PPARα (16), mainly expressed in liver and brown adipocytes, could play a central role in fatty acid catabolism as suggested by the finding that targeted disruption of this isoform in mice resulted in abolishment of liver peroxisomal proliferation and fatty acid oxidation (17). PPARγ is predominantly expressed in adipose cells and plays, in combination with other transcription factors, a crucial role in activation of genes of adipose differentiation process and adipogenesis (18). PPARδ, also called FAAR (fatty acid-activated receptor), shows high level of expression in various lipid-metabolizing tissues such as adipose tissue, small intestine, and muscles and could play a role in the process of fatty acid uptake and activation as suggested by transfection experiments, demonstrating that its ectopic expression in fibroblasts confers fatty acid responsiveness to FAT, ALBP, and ACS genes (19).

Despite intense efforts, the identification of physiological ligands and activators of the various PPAR isoforms still remains an open question. By using transactivation assays, it has been shown that PPAR are activated by a large variety of molecules including fatty acids, arachidonate metabolites, and pharmaceutical compounds. These studies carried out with different recipient cells and with different reporters genes led to contradictory results. However, from the most recent data, it can be proposed that PPARα is activated by unsaturated LCFA and fibrates; that PPARγ is activated by 15-deoxy-Δ12-prostaglandin J_2 (PGJ_2), thiazolidinediones or hydroxy-LCFA and insensitive to native LCFA. LCFA, carbaprostacyclin ($cPGI_2$), or 2-bromopalmitate activates FAAR.

From the numerous studies performed in recent years, it can be concluded that PPAR play a central role in the control of expression of genes involved in lipid metabolism of various tissues. These lipid-activated nuclear receptors are probably very important actors in the processes of adaptation of the organism to high-fat feeding by increasing expression of enzymes of fatty acid catabolism in certain tissues such as liver and brown adipose tissue, and increasing the capacity of storage of fatty acids in white adipose tissue.

FATTY ACIDS, PPAR, AND ADIPOGENESIS

PPARγ and FAAR are induced during adipose differentiation. FAAR is a very early marker of differentiation occurring during the preadipose state whereas PPARγ is induced later during terminal differentiation (19,20). A regulatory role for PPARγ in adipogenesis has been established by ectopic ex-

pression experiments. Expression of physiological level of this nuclear factor by retroviral vector promotes activation of an adipose differentiation program and adipogenesis in fibroblasts upon treatment by PPARγ activators (21). A cooperative process between PPARγ and other transcription factors of the C/EBP family in induction of adipogenesis has also been described (22). However, the recent demonstration that fatty acids do not activate PPARγ (23) argues against its direct implication in development of obesity induced by high-fat feeding. Very recent data from our laboratory suggested that activation of FAAR by fatty acids could be the first event involved in adipogenic action of LCFA. Stable 3T3-C2 cell lines expressing the FAAR protein (3T3-C2/FAAR) and control cell lines (3T3-C2/Biz) have been isolated by retroviral infection and their response to various cocktails of fatty acids and other adipogenic agents has been investigated. Exposure of 3T3-C2/FAAR cells to FAAR activators such as LCFA, 2-bromopalmitate, or $cPGI_2$ led within hours to a strong activation of FAT and ALBP genes and, after a few days, to a delayed accumulation of PPARγ mRNA. At that stage, addition of PPARγ activators, such as thiazolidinediones or 15-deoxy-$\Delta 12$–PGJ_2 resulted in lipid accumulation and expression of the overall adipose differentiation program. By contrast, treatment by PPARγ activators of cells not previously exposed to FAAR activators did not promote adipogenesis. Furthermore, the finding that control 3T3-C2/Biz cells remained unresponsive to the various treatments confirmed that this new phenotype is related to FAAR expression. These data strongly suggest that a direct FAAR activation by LCFA leads to PPARγ gene induction and that, in turn, PPARγ activation by its specific activators promotes adipogenesis.

How fatty acids promote obesity *in vivo* remains an open question. However, keeping in mind that it has been clearly established that adipose tissue from adult animals contains some preadipose "dormant" cells expressing the early markers of adipose differentiation including FAAR, it is tempting to speculate that under conditions leading to an increase of fatty acid supply, such as high-fat feeding, FAAR activation could promote PPARγ gene expression and subsequent adipose conversion. Further studies are clearly required to validate such a model. In that respect, PPAR gene disruption experiments could provide very interesting animal models for these studies.

In conclusion, fatty acids act as transducing molecules and behave as adipogenic hormones. These effects are, at least in part, mediated by activation of nuclear receptors of the PPAR subfamily. The characterization of the mode of action of these lipid-activated nuclear receptors could be useful to define new pharmaceutical strategies in the treatment of nutritional obesities.

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Dietary Polyunsaturated Fatty Acids and Hepatic Gene Expression

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ABSTRACT: Dietary polyunsaturated fatty acids (PUFA) have profound effects on hepatic gene transcription leading to significant changes in lipid metabolism. PUFA rapidly suppress transcription of genes encoding specific lipogenic and glycolytic enzymes and induce genes encoding specific peroxisomal and cytochrome P450 (CYP) enzymes. Using the peroxisome proliferator-activated receptor α (PPARα)-null mouse, we showed that dietary PUFA induction of acyl CoA oxidase (AOX) and CYP4A2 require PPARα. However, PPARα is not required for the PUFA-mediated suppression of fatty acid synthase (FAS), S14, or L-pyruvate kinase (L-PK). Studies in primary rat hepatocytes and cultured 3T3-L1 adipocytes showed that metabolites of 20:4n-6, like prostaglandin E_2 (PGE_2), suppress mRNA encoding FAS, S14, and L-PK through a G_i/G_o-coupled signal transduction cascade. In contrast to adipocytes, 20:4n-6-mediated suppression of lipogenic gene expression in hepatic parenchymal cells does not require cyclooxygenase. Transfection analysis of S14CAT fusion genes in primary hepatocytes shows that peroxisome proliferator-activated PPARα acts on the thyroid hormone response elements (−2.8/−2.5 kb). In contrast, both PGE_2 and 20:4n-6 regulate factors that act on the proximal promoter (−150/−80 bp) region, respectively. In conclusion, PUFA affects hepatic gene transcription through at least three distinct mechanisms: (i) a PPAR-dependent pathway, (ii) a prostanoid pathway, and (iii) a PPAR and prostanoid-independent pathway. PUFA regulation of hepatic lipid metabolism involves an integration of these multiple pathways.

Understanding the molecular basis for dietary fatty acid regulation of cell function and how dietary fat contributes to human health is a central issue in modern nutrition research (1–4). First, it is important to understand how dietary fat affects specific biochemical events under normal physiological conditions and then to determine whether these pathways contribute to the onset and/or progression of specific chronic diseases. Our studies have focused on defining how dietary fat affects gene expression under normal physiological conditions, with an emphasis on understanding the role dietary fat plays in the control of hepatic lipid synthesis and metabolism (5–10).

The liver plays a central role in whole-body lipid metabolism. Fatty acids originating from the diet (as triglycerides) or extrahepatic tissues enter hepatocytes and are rapidly converted to fatty acid CoA esters (FA-CoA). These FA are processed to complex lipids, such as triacylglycerols and phospholipids or are diverted to oxidation in mitochondria (β-oxidation), peroxisomes (β-oxidation), or microsomes (cytochrome P450, CYP). Complex lipids are assembled into lipoprotein complexes and secreted in the form of very low density lipoproteins (VLDL) for export to extra-hepatic tissues. The liver also carries out *de novo* FA synthesis (lipogenesis), which involves the conversion of carbohydrate to palmitate. In rodent liver (and probably humans), lipogenesis is induced by high-carbohydrate fat-free diets, triiodothyronine (T_3), and insulin and is inhibited by starvation, streptozotocin-induced diabetes, or treatment with hormones that elevate hepatocellular cAMP levels (4).

In addition to these endocrine regulatory mechanisms, hepatic lipogenesis and triacylglycerol synthesis are suppressed in rats fed diets containing polyunsaturated fatty acids (PUFA), particularly highly unsaturated n-3 PUFA found in fish oil (see Refs. 3,4). PUFA suppression of lipogenesis may be linked to fuel partitioning. Suppressing lipogenesis keeps malonyl CoA low. Malonyl CoA is an inhibitor of carnitine palmitoyl transferase I (11). Thus, by suppressing lipogenesis, mitochondrial FA oxidation is favored. Another explanation for this effect is that newly synthesized FA might facilitate triglyceride assembly into VLDL. Certainly, feeding rats fish oil has profound inhibitory effects on lipogenesis and suppresses hepatic VLDL secretion leading to diminished circulating VLDL-triglyceride (12–15).

Studies described here focus on the effects of dietary fat on the transcription of the hepatic gene encoding the S14 protein. The S14 protein is thought to play a role in orchestrating the glucose/insulin regulation of hepatic FA synthase (FAS), malic enzyme, and ATP-citrate lyase (16). These studies will show that FA activate multiple mechanisms to influence hepatic gene transcription.

PUFA regulation of S14 gene transcription is directed at the S14 proximal promoter region. n-3 and n-6 PUFA suppression of mRNA encoding hepatic lipogenic [fatty acid synthase (FAS), S14, SCD1] and glycolytic (L-PK) enzymes is due to

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Abbreviations: AOX, acyl CoA oxidase; CYP, cytochrome P450; Fa-CoA, fatty acid CoA; FAS, fatty acid synthase; L-PK, L-pyruvate kinase; PPAR, peroxisome proliferator activated receptor; PUFA, polyunsaturated fatty acids; RR, regulatory region; S14, S14 protein; T_3, triiodothyronine; VLDL, very low density lipoprotein.

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rapid inhibition of transcription of these genes (5–9). Moreover, these same effects on gene expression are seen in primary rat hepatic parenchymal cells treated with specific n-3 and n-6 PUFA, indicating that PUFA action does not require extrahepatic factors. Using a transfection approach, the proximal promoter region of the S14 gene was found to contain a *cis*-regulatory region (PUFA-RR) that was targeted by PUFA. By targeting this region, PUFA can selectively control gene expression without having broad effects on either thyroid, insulin, or glucose-regulated gene transcription. Here, we describe our current understanding of the mechanism of PUFA regulation of S14 that is directed at the PUFA-RR (−220/−80 bp) (Fig. 1).

Transfection studies showed that the S14 PUFA-response region (PUFA-RR) contains a functional Y-box at −104/−99 bp upstream from the 5′ transcription start site (17). A Y-box is an inverted CCAAT-box that can potentially bind several CCAAT-box binding proteins like c/EBP-related factors, NF1, NFY, dbpA, and dbpB (YB-1). Deletion or mutation of the S14 Y-box essentially abrogates S14 gene transcription. Gel shift analyses show that c/EBP-α or β, NF1, and NFY bind this element. Transfection analyses showed that NFY, but not c/EBP, is required for the operation of the two upstream enhancers (thyroid and glucose/insulin). Such studies suggest that PUFA might regulate the composition of the Y-box, which leads to changes in S14 gene transcription. However, direct test of this hypothesis showed that genes containing a Y-box binding only NFY are marginally sensitive to PUFA control. The 100 bp region (−220/−120 bp) immediately upstream from the Y-box also did not confer PUFA control to a *cis*-linked gene when tested without the functional Y-box. However, when both the Y-box and the C-region were together, PUFA control to the S14 gene was restored. Thus, PUFA control of S14 gene transcription requires a functional interaction between NFY and factors binding the C-region.

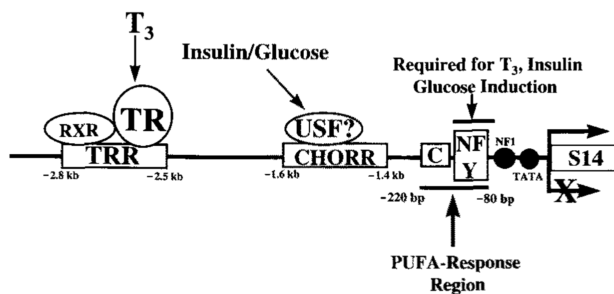

FIG. 1. Functional elements involved in the hormonal/nutrient control of the rat liver S14 protein (S14) gene. The S14 gene proximal promoter region extends to −220 bp and contains a functional TATA-box, NF1, and NFY elements. The Y-box and the C-region, (C), functionally interact to form a polyunsaturated fatty acid (PUFA)-response region (PUFA-RR) within the S14 proximal promoter. Two enhancers are found upstream. The carbohydrate-response region (CHORR) is located between −1.4 and −1.6 kb. This region contains an E-box that may bind USF-related factors. The thyroid hormone response region (TRR) is located between −2.5 and −2.8 kb and contains three thyroid hormone response elements (TRE). Each TRE binds thyroid hormone receptors (TR) and the retinoid x receptor (RXR) as heterodimers. T_3, triiodothyronine.

The identity of the factors binding the C-region is currently under investigation.

Peroxisome-proliferator-activated receptors (PPAR) regulation of hepatic gene transcription. A second mechanism for FA control of hepatic gene transcription involves PPAR (18,19). Peroxisome proliferators encompass a wide variety of compounds, including hypolipidemic drugs (WY14,643, gemfibrozil, clofibrate, etc.), plasticizers (DEHP) steroids (DHEA, DHEA-S), and dietary FA. PPAR are members of the steroid/thyroid superfamily of nuclear receptors. PPAR-mediated effects on gene transcription are achieved through the formation of PPAR-RXR heterodimers which bind DNA motifs called peroxisome proliferator response elements located in promoters of target genes.

PPAR also have inhibitory effects on gene transcription. For example, apolipoprotein CIII and transferrin gene expression are inhibited by PPAR-RXR competition for an HNF-4 binding site within the promoters of these genes (20). PPARα was also shown to interfere with thyroid hormone action by sequestering RXRα, a factor required for thyroid hormone receptor binding to DNA (19,21).

Because PPAR were reported to bind FA (22), they may potentially serve as monitors of intracellular FA levels and target the genome to control specific metabolic pathways. The fact that PPAR are activated by FA in conjunction with the known effects of PUFA (n-3) on peroxisomal and microsomal FA oxidation led us to question whether PUFA regulated peroxisomal, microsomal, glycolytic, and lipogenic pathways through a common transcriptional mediator, i.e., PPAR. While several PPAR subtypes [α, β (also known as δ , Nuc1, FAAR), γ1 and γ2] were identified in rodents, PPARα is the predominant form in rodent liver. Recent gene targeting studies clearly demonstrate that PPARα is required for the pleiotropic response to peroxisome proliferators, including an increase in hepatic mRNA encoding peroxisomal and microsomal enzymes (23).

To examine the role of PPARα in PUFA regulation of hepatic mRNA, we used the PPARα-deficient mouse. These studies included an analysis of mRNA encoding hepatic lipogenic [FAS and the S14 protein (S14)], microsomal [cytochrome P450 4A2 (CYP4A2)] and peroxisomal [acyl CoA oxidase (AOX)] enzymes (18). PUFA ingestion induced $mRNA_{AOX}$ (2.3-fold) and $mRNA_{CYP4A2}$ (8-fold) and suppressed $mRNA_{FAS}$ and $mRNA_{S14}$ and $mRNA_{L\text{-}PK}$ by >50–80% in wild-type mice. In PPARα-deficient mice, PUFA did not induce $mRNA_{AOX}$ or $mRNA_{CYP4A2}$, indicating a requirement for PPARα in the PUFA-mediated induction of these enzymes. However, PUFA still suppressed $mRNA_{FAS}$ and $mRNA_{S14}$ in the PPARα-deficient mice. These results indicate that while PPARα is required for the FA induction of AOX and CYP4A2, it is not required for PUFA suppression of L-PK, FAS, or S14.

Prostanoid regulation of hepatic gene expression. Arachidonic acid (20:4n-6) is a substrate for the synthesis of prostaglandins, leukotrienes, and related compounds (24). In cultured adipocytes, 20:4n-6 is converted to prostanoids and suppresses $mRNA_{S14}$ and $mRNA_{FAS}$ through a pertussis toxin-sensitive G_i/G_o-linked pathway (10). In liver, both 20:4n-6 and

20:5n-3 are equipotent inhibitors of glycolytic and lipogenic gene transcription. While 20:4n-6 is a precursor to prostanoids, 20:5n-3 is a poor substrate for prostanoid conversion. This finding suggested that PUFA might not go through a prostanoid pathway to affect hepatic gene expression. Thus, the mechanism of 20:4n-6 control of lipogenic gene expression in the liver and white adipose tissue is different.

To examine this issue further, primary rat hepatocytes were treated with inhibitors of 20:4n-6 conversion to prostanoids (flurbiprofen, a cyclooxygenase inhibitor) or leukotrienes [nordihydroguaiaretic acid (NGDA) a lipoxygenase inhibitor] (10). Both inhibitors failed to block the 20:4n-6-mediated inhibition of S14CAT expression. Interestingly, treatment of primary hepatocytes with prostaglandin E_2 (PGE_2) (10 μM) suppressed the mRNA encoding S14 and FAS as well as S14 CAT activity. Like PUFA regulation of S14, one of the *cis*-regulatory targets of PGE_2 action was the S14 Y-box. In adipocytes, PGE_2 suppressed S14 and FAS gene expression through a G_i/G_0 system, involving changes in intracellular free calcium (10). Whether a similar mechanism is operative in hepatic parenchymal cells awaits further investigation.

PUFA regulates hepatic gene expression through multiple mechanisms. These studies indicate that the pleiotropic effects of PUFA on hepatic gene transcription can not be attributed to a single pathway or mediator. In fact, at least three distinct pathways are operative in the liver (Fig. 2). They are: (i) PPARα-dependent; (ii) prostanoid-dependent; and (iii) PPARα and prostanoid-independent. In rat hepatic parenchymal cells, the PPARα pathway is activated by 20:5n-3, but not by 18:2n-6, 18:3n-3, 18:3n-6, or 20:4n-6. When compared to WY14,643, 20:5n-5 is a weak activator of PPARα (18). Despite this weak activation, 20:5n-3 requires PPARα to induce enzymes involved in FA oxidation, *e.g.*, AOX and CYP4A2. In contrast to FA oxidation, PPARα is not required for the PUFA-mediated suppression of lipogenic or glycolytic gene expression. An exception to this is seen with the potent peroxisome proliferator, WY14,643 (19). WY14,643-activated PPARα interferes with T_3 regulation of gene expression by sequestering RXR. RXR is a co-receptor required for heterodimer formation with thyroid hormone receptors and DNA binding. Based on these studies, we speculate that under normal dietary conditions, dietary PUFA may have little impact on PPARα-regulated gene transcription.

A second mechanism of PUFA control involves a paracrine regulatory mechanism. n-6 PUFA are not converted to prostanoids within hepatic parenchymal cells due to the lack of cyclooxygenase. However, these FA can be converted to prostanoids in nonparenchymal hepatic cells, like Kupffer or endothelial cells. Upon appropriate activation, released prostanoids, like PGE_2, can activate G-protein-coupled prostanoid receptors (*e.g.*, EP3) on hepatic parenchymal cells, initiating a signal transduction cascade that leads to a suppression of S14 and FAS mRNA. One *cis*-regulatory target for PGE_2 action of the S14 promoter is the Y-box. How this mechanism participates in the regulation of hepatic lipogenic gene expression in healthy subjects ingesting normal quantities of dietary PUFA is not clear. However, this mechanism may have a more significant impact in septic animals with hepatic inflammation.

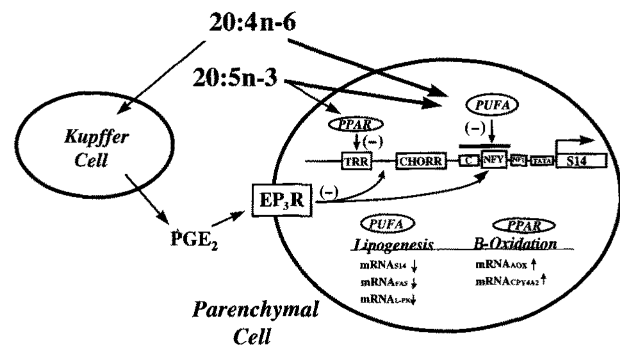

FIG. 2. Direct and paracrine regulation of S14 gene transcription by PUFA and PUFA metabolites. Hepatic parenchymal cell S14 gene transcription is suppressed by PUFA, peroxisome proliferater-activated receptor (PPARα), prostanoids (PGE_2, $PGF_{2\alpha}$);. 20:5n-3 is a weak activator of PPARα in rat liver. Activation of PPARα leads to the induction of mRNA encoding acyl CoA oxidase (AOX) and cytochrome P450 (CYP)4A2. Strong activation of PPARα by WY14,643 targets the S14 TRR leading to interference of T_3 regulation of this gene. PGE_2 is not generated in rat hepatic parenchymal cells. However, parenchymal cells respond to prostanoids through an EP3-like receptor. Thus, 20:4n-6 can be converted to a prostanoid in a nonparenchymal cell and through a paracrine mechanism activate an EP3 receptor. This leads to a suppression of mRNA encoding S14, FAS, and L-PK; 20:4n-6 and 20:5n-3 act directly on rat hepatic parenchymal cells and target the proximal promoter region of S14. Targeting this region leads to an interference with the enhancer-mediated transactivation of S14 gene transcription. FAS, fatty acid synthase; L-PK, L-pyruvate kinase; CYP, cytochrome P450. See Figure 1 for other abbreviations.

A third pathway for PUFA regulation of hepatic gene expression is independent of PPARα and prostanoids and involves the control of glycolytic and lipogenic gene transcription. In contrast to the two preceding pathways, a wide spectrum of FA activates this pathway, including 18:3n-6, 18:3n-3, 20:4n-6, and 20:5n-3. This pathway targets a *cis*-regulatory region within the S14 proximal promoter that includes the Y box, binding NFY and the C-region binding factors that remain to be identified. Based on our previous studies, this pathway is probably the operative mechanism for the control of lipogenesis under the following physiological conditions: (i) during the suckling period prior to weaning, (ii) starvation; (iii) uncompensated diabetes, and (iv) ingestion of PUFA-containing diets.

The finding that PUFA regulation of hepatic gene transcription involves multiple pathways suggests that lipogenesis, per se, may play an important regulatory role in hepatic carbohydrate/lipid metabolism. In addition to its role in the palmitate synthesis, this pathway may also function in nutrient partitioning (FA oxidation vs. storage), triglyceride assembly into VLDL, or myristate formation (25). Numerous physiological states are known to affect this pathway, like suckling/weaning, starvation/re-feeding, diabetes, high-carbohydrate fat-free diets, insulin resistance, obesity, and sepsis. With our new understanding of the pleiotropic effects of

dietary fat on gene expression, we can begin to understand how effects of dietary fat on the genome contribute to alterations in hepatic lipid/carbohydrate metabolism associated with chronic diseases like obesity and insulin resistance.

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The Double Bond in Unsaturated Fatty Acids Is the Necessary and Sufficient Requirement for the Inhibition of Expression of Endothelial Leukocyte Adhesion Molecules Through Interference with Nuclear Factor-κB Activation

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We previously showed that n-3 polyunsaturated fatty acids (FA) inhibit endothelial activation, i.e., early events involved in early atherogenesis and inflammation. These include the expression of endothelial leukocyte adhesion molecules, such as VCAM-1, ICAM-1, E-selectin, and endothelial chemoattractants (1). We further analyzed endothelial effects of various FA differing in chain length, number, position, and *cis/trans* configuration of the double bonds, concluding that saturated FA are inactive, while potency of polyunsaturates increases with the number of unsaturation (2). We now analyze the extent and mechanisms of modulation of endothelial activation by the monounsaturated FA oleic acid (18:1n-9), as the basis for such effects of the single double bond, and a possible contribution to healthy effects of olive oil in the Mediterranean diet.

Methods. Sodium oleate, in medium with 15% serum, was incubated with human umbilical vein endothelial cells for 24–72 h followed by coincubation of oleate with interleukin-1, *Escherichia coli* lipopolysaccharide (LPS), or phorbol myristate acetate (PMA) for a further 1–16 h. U937 monocytoid cell adhesion was measured by adhesion assays, the endothelial expression of VCAM-1, E-selectin, and ICAM-1 by cell surface enzyme immunoassays, steady-state levels of VCAM-1 mRNA by Northern analysis, and nuclear factor (NF)-κB activation by electrophoretic mobility shift assays (EMSA). Fatty acid composition of total cell lipid extracts was monitored with gas–liquid chromatography.

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Abbreviations: EMSA, electrophoretic mobility shift assays; FA, fatty acid; LPS, lipopolysaccharide; NF-κB, nuclear factor; PMA, phorbol myristate acetate.

TABLE 1
Results for VCAM-1 Expression, After Lipopolysaccharide (for 16 h)

Sodium oleate (μM)	0	1	25	50
VCAM-1 expression	100	99 ± 3	60 ± 10*	44 ± 9*

*$P < 0.05$ vs. control (no fatty acid).

Results. At 25–100 μM, concomitant with a selective displacement of saturated FA from cell lipids, oleate inhibited U937 cell adhesion and the expression of all adhesion molecules induced by any of the stimuli used (Table 1).

The effect of oleate required a preincubation of hours before the addition of cytokines or LPS or PMA, being absent upon coincubation, and increasing up to 48–72 h. This was associated with a reduction of VCAM-1 mRNA levels at Northern analysis, and inhibition of NF-κB activation at EMSA.

Conclusions. These results indicate that the single double bond present in oleic acid is a necessary and sufficient requirement for FA inhibition of endothelial activation. Since this occurred independent of the stimuli used and was present also with PMA, bypassing membrane receptors, these results point to an interference with a common signal transduction pathway for cytokine signaling. These results are accounted for by an inhibition of NF-κB activation, here demonstrated at the same concentrations required for the inhibition of surface protein. Relevance of these observations to explaining the alleged beneficial antiatherogenic effects of the Mediterranean diet, in which oleic acid in olive oil is a major nutritional component, could lie in the relatively selective displacement of saturated FA by oleate addition to endothelial cells which was also shown to occur in our system. Thus, an increase in the unsaturation index in cell membranes by oleic acid-rich diets is theoretically additive to the effects of polyunsaturated FA. By these mechanisms, monocyte recruitment in response to atherogenic stimuli might be decreased (3).

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Reduction of Scavenger Receptor Expression and Function by Dietary Fish Oil Is Accompanied by a Reduction in Scavenger Receptor mRNA

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During atherosclerosis, macrophages (MØ) within plaques take up oxidized low density lipoprotein (LDL) *via* the scavenger receptor (SR) to become lipid-laden foam cells. Dietary fish oil (FO) provides some protection against the development of atherosclerosis in a variety of species including mice, pigs, nonhuman primates, and man (1,2). We hypothesized that at least part of this beneficial effect might be due to a reduction in SR expression on MØ. Thus, in this study the effect of feeding mice high-fat diets with different fatty acid compositions upon class A SR (SRA) expression (mRNA and protein) and function [uptake of acetylated-LDL (Ac-LDL)] was investigated.

Male C57Bl/6 mice were fed for 12 wk on a low-fat (LF) diet (25 g corn oil/kg) or on diets containing 200 g/kg of either coconut oil (CO), olive oil (OO), safflower oil (SO), or fish oil (FO). Four days prior to sacrifice, the mice were injected intraperitoneally with thioglycolate broth to elicit MØ migration to the peritoneal cavity. At sacrifice the MØ were collected by lavaging the peritoneal cavity. Flow cytometry was used to assess cell surface SRA expression (staining with a fluorescently-labeled monoclonal antibody [clone 2F8; Serotec, Kidlington, United Kingdom]) and function (uptake of fluorescently-labeled Ac-LDL [Biogenesis, Poole, United Kingdom] over 1 h); flow cytometry data are expressed as % of positive cells and median fluorescence intensity (MFI). The 2F8 monoclonal antibody recognises both the type I and type II forms of the SRA (3) and both forms are involved in Ac-LDL uptake by MØ. RNA was extracted, and SRA type I and type II mRNA levels were quantified using reverse transcriptase-polymerase chain reaction (RT-PCR); mRNA levels are expressed relative to cyclophilin mRNA levels also determined by RT-PCR.

FO feeding significantly reduced the percentage of SRA-positive MØ compared with the other high-fat diets (Table 1). This was mirrored by a trend toward a reduced number of MØ able to take up Ac-LDL following FO feeding. The level of

TABLE 1
Effect of Dietary Fats on Murine Scavenger Receptor Expression and Function[a]

	Scavenger receptor expression		Ac-LDL uptake		Scavenger receptor mRNA levels	
Diet	% Positive	MFI	% Positive	MFI	Type I	Type II
LF	68.2 ± 6.1^{a,b}	74.1 ± 12.2	54.4 ± 4.2	2029 ± 266	0.38 ± 0.06^{a}	0.57 ± 0.07^{a}
CO	79.3 ± 2.8^{a}	74.1 ± 8.5	58.4 ± 5.4	2389 ± 492	0.37 ± 0.04^{a}	0.49 ± 0.06^{a,b}
OO	73.1 ± 3.5^{a}	83.3 ± 17.3	59.4 ± 4.3	2563 ± 612	0.20 ± 0.04^{b}	0.34 ± 0.07^{b}
SO	73.6 ± 2.9^{a}	76.4 ± 9.9	59.4 ± 4.8	2373 ± 408	0.27 ± 0.06^{a,b}	0.52 ± 0.06^{a,b}
FO	59.0 ± 4.6^{b}	54.0 ± 1.9	51.5 ± 4.8	1884 ± 404	0.28 ± 0.07^{s,b}	0.36 ± 0.08^{b}

[a]Data are mean ± SEM for 8 to 10 mice fed on each diet. Values in a column not sharing a common subscript letter are significantly different ($P < 0.05$; one-way analysis of variance). LDL, low density lipoprotein; LF, low fat; CO, corn oil, OO, olive oil; SO, safflower oil; FO, fish oil; MFI, median fluorescence intensity.

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Abbreviations: AC-LDL, acetylated low density lipoprotein; CO, corn oil; FO, fish oil; LDL, low-density lipoprotein; LF, low-fat; MFI, median fluoresence intensity; MØ, macrophages; OO, olive oil, SO, safflower oil; SR, scavenger receptor.

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expression of the SRA on positive MØ, as measured by MFI, was also reduced by FO feeding, but this did not reach statistical significance. Once again, this was mirrored by a reduced level of Ac-LDL uptake by those cells which were active. MØ from OO-fed mice contained significantly lower levels of SRA type I mRNA than those from mice fed the LF and CO diets (Table 1). MØ from OO- and FO-fed mice contained significantly lower levels of SRA type II mRNA than those from LF-fed mice (Table 1).

We conclude that FO feeding reduces macrophage SRA expression by acting at the level of gene transcription and that this might contribute to the protective effect of dietary FO toward atherosclerosis.

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The Effect of Eicosapentanoic Acid on Matrix Metalloproteinase Gene Expression

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Matrix metalloproteinases (MMP) are a family of serine proteases of which 21 members are known to date. The members of this gene family share a number of biochemical properties which include possession of a zinc ion in their active site, secretion in a latent form requiring proteolytic activation in the cytoplasm, and the capability of degrading at least one component of the extracellular matrix; also all members of the MMP family are inactivated by their natural inhibitors, the tissue inhibitors of matrix metalloproteinases (TIMP). Matrix metalloproteinases are involved in remodeling the extracellular matrix, which is vital to numerous cell processes including proliferation and differentiation. However, extensive remodeling of the cell matrix by MMP has been observed in a number of human disease conditions such as arthritis (1), coronary heart disease (2), and cancer (3).

MMP play a pivotal role in tumor metastasis in which excessive extracellular matrix degradation facilitates cancer cell migration to secondary sites. During the process of tumor invasion increased MMP production has been linked to an increased invasive phenotype in a number of human cancers. Dietary fats such as those comprising the polyunsaturated fatty acids, which include the n-3 and n-6 subclasses, have been reported to effect both growth and metastasis of a number of human tumors both *in vitro* and *in vivo* (4). Investigators generally report protective effects by n-3 fatty acids such as eicosapentanoic acid (EPA) and docosahexanoic acid on growth and metastasis whereas n-6 fatty acids are reported to enhance the growth and invasion of cancer cells. In this present study we investigated the effects of EPA on the gene expression of one of the most abundant metalloproteinases, MMP-2, and one of its inhibitors, TIMP-2, in a renal cell carcinoma by reverse transcription polymerase chain reaction.

Materials and Methods. Cell culture. The renal cell carcinoma, caki-1, was routinely maintained in McCoy's 5A supplemented with 10% fetal bovine serum with 5 mg/mL penicillin/streptomycin. Caki-1 were grown in a humidified atmosphere at 37°C and 5% CO_2.

Fatty acids. EPA and arachidonic acid (AA) were purchased from Sigma (United Kingdom) in 99% pure free acid form and were diluted in 100% ethanol.

Growth assay. The effect of EPA and AA on the proliferation of caki-1 cells was assessed using the MTT [3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide] assay (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's protocol.

RNA isolation. Total RNA was isolated from 1×10^6 caki-1 cells harvested by trypsinizing following culturing with fatty acids. RNA was extracted using a commercial total RNA isolation kit (Glassmax RNA isolation kit, Gibco BRL, United Kingdom). RNA was isolated according to manufacturer's protocol. Extracted RNA was measured at 260 nm, aliquoted and stored at −20°C.

Reverse transcription polymerase chain reaction (RT-PCR). RT-PCR was performed using a single tube method which utilized an enzyme mix containing both a reverse transcriptase and a polymerase enzyme (One-step RT-PCR kit, Gibco BRL). Total RNA (500 ng) was used in each RT-PCR reaction according to manufacturer's instructions. The RT-PCR conditions were as follows: 50°C for 30 min, 94°C for 3 min followed by 30 cycles of 94°C for 50 s, annealing temperature for 60 s, and 72°C for 40 s. This was followed by a final extension step of 72°C for 10 min and storage at −20°C. The annealing temperatures used for MMP-2 and TIMP-2 were 57 and 60°C, respectively. Glyceraldehyde phosphate dehydrogenase (GAPDH) was used as an internal control for the RT-PCR reactions; our GAPDH primers had an annealing temperature of 56°C. Following the RT-PCR reactions the products were visualized by agarose gel electrophoresis and analysed by one-dimensional analysis (Phoretix software package).

Results and Discussion. From data obtained from the MTT proliferation assay we were able to determine that concentrations of EPA greater than 30 μM caused a decrease in the proliferation of caki-1 cells, and concentrations of AA above 10 μM showed a similar decrease in proliferation. For this reason all subsequent experiments used concentrations of fatty acids below these levels.

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Abbreviations: AA, arachidonic acid; EPA, eicosapentanoic acid; GAPDH, glyceraldehyde phosphate dehydrogenase; MMP, matrix metalloproteinases; MTT, 3-(4,5-dimethylthiazo-2-yl)-2,5-diphenyltetrazolium bromide; RT-PCR, reverse transcription polymerase chain reaction; TIMP, tissue inhibitors of metalloproteinases.

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FIG. 1. Reverse transcription polymerase chain reaction (RT-PCR) of matrix metalloproteinase (MMP)-2 gene. Lanes 1–5, respectively: 100 bp molecular weight marker, no fatty acid, 10 μM eicosapentaenoic acid (EPA), 20 μM EPA, 30 μM EPA.

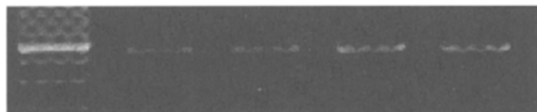

FIG. 2. RT-PCR of tissue inhibitor of metalloproteinase (TIMP)-2 gene. Lanes 1–5; respectively: 100 bp molecular weight marker, no fatty acid, 10 μM EPA, 20 μM EPA, 30 μM EPA. For other abbreviations see Figure 1.

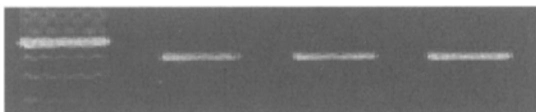

FIG. 3. RT-PCR of MMP-2 gene. Lanes 1–4, respectively, 100 bp molecular weight marker, no fatty acid, 5 μM arachidonic acid (AA), 10 μM AA. For other abbreviations see Figure 1.

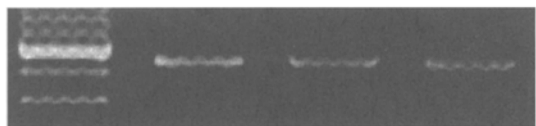

FIG. 4. RT-PCR of TIMP-2 gene. Lanes 1–4, respectively: 100 bp molecular weight marker, no fatty acid, 5 μM AA, 10 μM AA. For abbreviations see Figures 1–3.

Figure 1 shows the effect of increasing EPA concentration on the gene expression of MMP-2. Figure 1 illustrates a decrease in the MMP-2 RT-PCR bands as EPA increases to 30 μM. Figure 2 also shows a dose response effect of increasing EPA in the expression of TIMP-2, which increases with increasing EPA concentration. Caki-1 cells cultured in AA showed an increase in MMP-2 and a decrease in TIMP-2 as illustrated in Figures 3 and 4. The GAPDH gene expression did not change with treatment (data not shown). The results demonstrate alterations in the gene expression in MMP-2 and TIMP-2 which may reduce the metastatic potential of caki-1 when supplemented with n-3 fatty acids and an alteration which may enhance the metastatic potential by increasing MMP-2 and decreasing TIMP-2 when supplemented with n-6 fatty acids. The results suggest a possible role for dietary supplementation with fish oils high in n-3 polyunsaturated fatty acids for the treatment of renal cancer.

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Opposite Regulation of Prostaglandin H Synthase Isoforms by Eicosapentaenoic and Docosahexaenoic Acids

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Long-chain n-3 fatty acids such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids have been described as inhibitors of platelet response by reducing prostanoid synthesis from arachidonic acid (AA), both in decreasing its availability from membrane phospholipids and its conversion by prostaglandin H (PGH) synthase (1), and at least for DHA, in reducing thromboxane receptor sensitivity (2). The effect of EPA and DHA on the other potent prostanoid, prostacyclin (PGI_2), has been however controversial as *ex vivo* experiments have suggested that PGI_2 production is preserved after fish oil intake (3), whereas its production by cultured vascular endothelial cells was inhibited (4). The latter has been reevaluated by investigating the expression of enzymes responsible for the conversion of AA into PGI_2.

Primary cultures of bovine aortic endothelial cells were incubated with 25 μM albumin-bound EPA, DHA, or oleic acid (molecular ratio fatty acid/albumin of 1.3:1) for 22 h and compared with those incubated with albumin alone (control). Under these conditions, the majority of each fatty acid tested was esterified in cell phospholipids, mainly at the expense of endogenous oleic, linoleic and arachidonic acids, except for the oleic acid-enriched cells which only lost linoleic and arachidonic acids. On the other hand, PGI_2 production from exogenous as well as endogenous AA was decreased by 50% in EPA- or DHA-rich cells but remained unchanged in oleic acid-rich ones. This indicated an inhibition of PGI_2 production downstream from phospholipase(s), i.e., at the level of PGH synthase and/or PGI_2 synthase specific activities and/or quantities. Western blotting analyses of the constitutive isoform of PGH synthase (PGHS)-1, showed a significant decreased immunoreactive amount in EPA- and DHA-rich cells but not in oleic acid-rich ones, while no alterations of PGI_2 synthase could be detected. Further investigations by Northern blotting revealed that the PGHS-1 mRNA was similarly and significantly decreased by cell preenrichment with EPA and DHA but not with oleic acid. Finally, studies on the stability of PGHS-1 mRNA in the presence of the transcription inhibitor actinomycin D failed to show any difference in the time-dependent decay in control and DHA-rich cells, strongly suggesting that the inhibition of PGHS-1 expression observed in EPA- or DHA-rich cells likely occurred at the transcriptional level (5).

Whereas the inducible isoform of PGHS, PGHS-2, could not be detected by Western blotting in any of the above-mentioned conditions, it could be measurable after 2 to 4 h incubation with phorbolmyristate acetate (PMA) used at concentrations as low as 10 nM. After 4 h incubation, the expression of PGHS-2 was already strong enough to mask any effect of cell preenrichment with DHA. However, in the weaker stimulation condition of 2-h incubation with PMA, the cell preenrichment with DHA markedly and significantly increased the expression of PGHS-2, showing a potentiation of such an expression. This indicates that PGHS-2 may be oppositely regulated by long chain n-3 fatty acids when compared to PGHS-1.

We conclude that, in addition to the well-known metabolic effect on n-3 fatty acids on prostanoid generation, a regulation of the PGHS protein expression could occur and contribute to the overall effect of those fatty acids on prostanoid homeostasis.

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PGH, prostaglandin H; PGHS, PGH synthase; PGI_2, prostacyclin; PMA, pherbol myristate acetate.

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Effects of Fish Oil and n-3 Fatty Acids on the Regulation of Δ^9-Fatty Acid Desaturase mRNA and -Activity in Rat Liver

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This study was designed to study the effects of treatment with dietary fish oil, or with long-chain n-3 polyunsaturated fatty acids, on Δ^9-desaturase in rat liver. The desaturase activity was measured in isolated microsomes in parallel with measurements of liver content of Δ^9-desaturase mRNA. Fish-oil diet [20% (w/w) of dietary fat] led to 85% decrease in Δ^9-desaturase activity, while the decrease in Δ^9-desaturase mRNA was about 90%. In comparison, soybean oil-diet rich in linoleic and α-linolenic acids [20% (w/w)] decreased Δ^9-desaturase activity by 50%, and the hepatic mRNA content by about 60% (Fig. 1). Treatment with docosahexaenoic acid (DHA) *per os* decreased the Δ^9-desaturase mRNA by 70%, while treatment with eicosapentaenoic acid (EPA) decreased the mRNA level only slightly less than with palmitic acid (PMA) (Fig. 2). DHA and EPA were kindly supplied by Pronova Biocare A/S (Lysaker, Norway). Clofibrate treatment, in contrast, caused about a sixfold stimulation of Δ^9-desaturase activity and about twofold increase in mRNA concentration. At the same time the amounts of acylCoA oxidase- and acylCoA dehydrogenase mRNA did not vary significantly with high-fat diets or long-chain n-3 fatty acids, but increased 2- and 2.5-fold, respectively, with clofibrate. The experiments show that fish oil has a strong decreasing effect on the amount of Δ^9-desaturase mRNA and that docosahexaenoic acid in the oil may be a potent component in this respect.

The effect of fish oil on the transcription of Δ6- and Δ5-desaturase has not been studied because the enzymes have

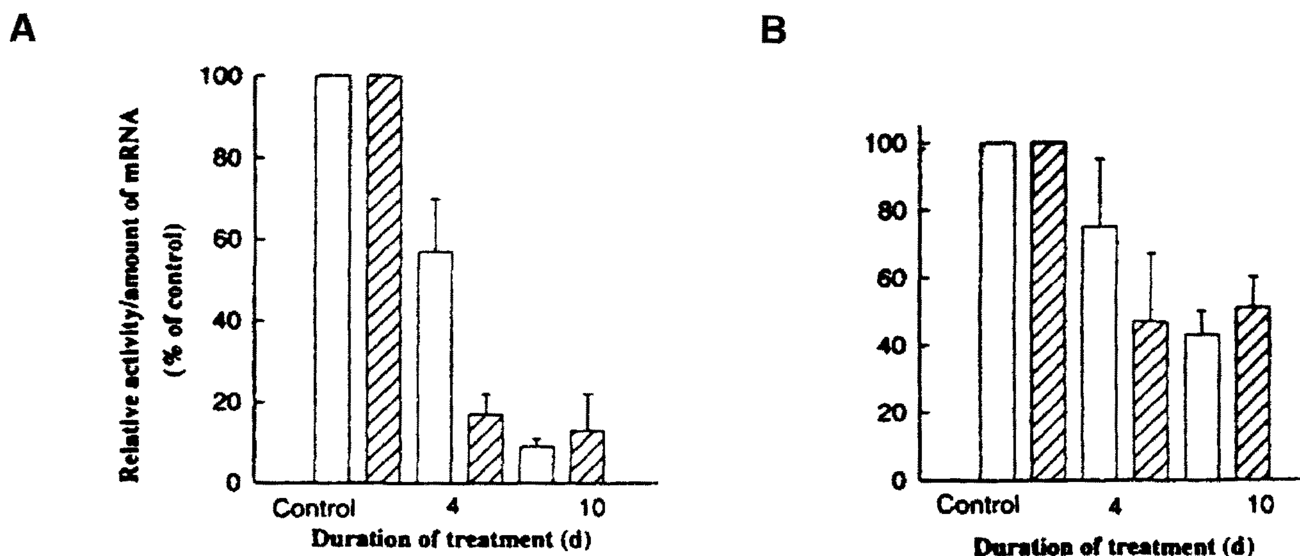

FIG. 1. Effects of fish- and soybean oil-diets on hepatic Δ^9-desaturase activity and amount of mRNA in percentage of control values. Rats were fed on high-fat diets containing 20% (w/w) fish oil (A), or soybean oil (B) for 10 d. The amounts of Δ^9-desaturase mRNA (in %) were measured, and activities of Δ^9-desaturase were assayed as described in Reference 1. Control value (pellet-fed) of Δ^9-desaturase activity was 0.15 ± 0.02 nmol/mg/min (100% activity). The values represent duplicate measurements each from four rats. Cross-hatched box; Δ^9-desaturase activity, open box; Δ^9-desaturase mRNA.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PMA, palmitic acid.

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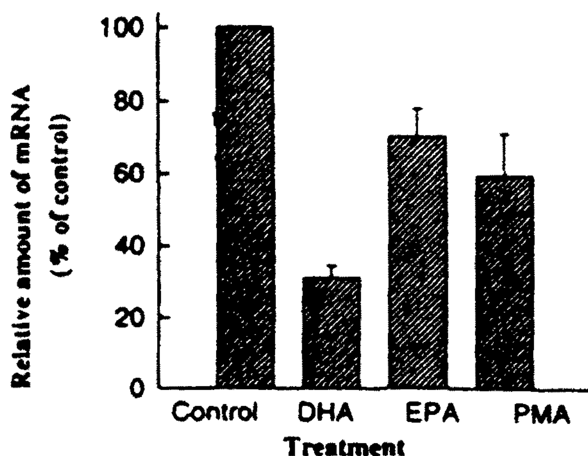

FIG. 2. Effects of ethyl-docosahexaenoate (DHA), ethyl-eicosapentaenoate (EPA), and palmitic acid (PMA) on amount of hepatic Δ^9-desaturase mRNA in percentage of control values. Rats were given the fatty acid by gastric tube delivery for 10 d as described in Reference 1. Controls were pellet-fed. The values represent duplicate measurements each from 4–8 rats.

still not been purified to satisfactory levels. However, Christiansen *et al.* (2) studied their enzymatic activities; Δ6 desaturase activity decreased 50% compared to a pellet diet and 80% compared to a sunflowerseed diet (rich in linoleic acid), while Δ5-desaturase activity did not change compared to a pellet diet and decreased 60% compared to a sunflowerseed diet. These results with fish oil compared to a sunflowerseed oil diet on Δ6- and Δ5-desaturase activities are in the same range as the presented results were when we compared fish oil with a soybeen oil diet for Δ9-desaturase activity. A clue to a possible gene-regulation of Δ6- and Δ5-desaturases was given by Kawashima *et al.* (3) who studied their regulation by clofibric acid. They found a strong stimulation on both desaturases, and inhibition by cycloheximide which is a potent inhibitor of protein synthesis.

In conclusion fish oil was found to strongly decrease the amount of Δ9-desaturase-mRNA in parallel to Δ9-desaturase activity in rat liver. This suggests that the regulation of activity is exerted either on the level of transcription or at the level of control of mRNA stability. An active component in the fish oil in this respect seems to be DHA. There are indirect indications for gene regulation of Δ6- and Δ5-desaturase activities as well; however, the direct experiment has to our knowledge still not been performed.

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Fatty Acid Synthase Gene Expression in Rat Mammary Carcinoma

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Fatty acid synthase (FAS) [EC 2.3.1.85] is a lipogenic enzyme catalyzing *de novo* fatty acid biosynthesis. FAS is present in various mammalian tissues and at a high level in a few specific tissues, such as liver. It is well-established in these tissues that FAS activity and gene expression depend on nutritional, hormonal, and physiological parameters. We were interested in FAS because recent data showed that FAS is overexpressed in various types of human cancers, such as breast cancer, prostate cancer, and others. This abnormal expression has been associated with a poor prognosis. Yet, in contrast to normal tissues, very little study was carried out on FAS regulation mechanisms in tumoral tissues.

The objective of the present study was to investigate *in vivo* a potential dietary regulation of FAS in a rat model of breast cancer. To do so, we checked if FAS was expressed in a model of chemically induced mammary tumors. Then, we investigated if FAS gene expression was regulated by dietary factors, particularly by quantity or quality of dietary fat.

Mammary tumors were obtained by chemical induction with a direct carcinogen: *N*-methyl nitrosourea in female Sprague-Dawley rats receiving different dietary fat. To check whether FAS was expressed in this experimental model, we analyzed FAS mRNA in rat mammary tumors as compared to liver specimens from the same rat fed on a high-fat diet (15% wt). Next, to check whether FAS expression varied according to the quantity of fat in the diet, we analyzed FAS mRNA from rat mammary tumors fed either a low-fat (6% wt) or a high-fat (15% wt) diet. And then, to see if the quality of fat [degree of unsaturation, type of poyunsaturated fatty acid (PUFA)] influenced FAS expression, we tested a high level of either saturated or unsaturated fat (15% wt) and a high level of n-6 PUFA or n-3 PUFA (15% wt) on FAS mRNA level.

First, Northern blot analysis revealed that FAS gene was abundantly expressed in rat mammary tumors, but it was not detectable in the liver under these dietary conditions (high fat). Secondly, Northern blot analysis of FAS mRNA from mammary tumors of rats fed a low- or a high-fat diet has shown no difference in FAS mRNA level under the two dietary conditions. Moreover, FAS mRNA level was similar in mammary tumors whatever the degree of unsaturation of fat or the type of PUFA present in the diet.

So, the main finding of the study with the rat NMU model is that rat tumors expressed a high level of FAS, similar to previous reports on breast cancer and breast cancer cell lines. The elevated FAS level suggests that chemically induced mammary tumors are able to synthesize *de novo* fatty acid. Moreover, the level of FAS mRNA in rat mammary tumors is not regulated by dietary fat.

We conclude that this model can serve as an additional tool in evaluating *in vivo* the role of FAS in cancer development and its specific role in tumoral tissue. It can, as well, serve as a model for studying FAS regulation mechanisms in tumoral tissue.

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Abbreviations: FAS, fatty acid synthase; NMU, *N*-methyl-nitrosourea; PUFA, polyunsaturated fatty acid.

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Effects of Maternal Docosahexaenoic Acid Supplementation on Visual Function and Growth of Breast-Fed Term Infants

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The low docosahexaenoic acid (DHA) content of the milk of women in the United States has led some to suggest DHA supplementation of breast-feeding women. However, data concerning the efficacy of supplementation are lacking. To provide such data, we determined the effects of DHA supplementation of lactating women on the visual function and growth of the recipient infants. Mothers were assigned randomly and blindly to receive ~200–250 mg of DHA daily as either algal DHA (Group 1; $n = 42$), refined high-DHA fish oil (Group 2; $n = 42$), or placebo (Group 3; $n = 42$) for 120 d after delivery. The fatty acid pattern of maternal milk and infant plasma phospholipid (PL) was determined 120 and 240 d postdelivery. Visual function of infants was assessed at the same ages by transient visual-evoked potential (VEP) latency and amplitude; visual acuity was measured at the same times by sweep VEP and the Teller Acuity Card Procedure. Growth was monitored throughout the study. At 120 d, milk total lipid DHA content of Group 1 and Group 2 was approximately twice that of Group 3 (0.37 and 0.39 vs. 0.17 mol%, respectively, of total fatty acids, $P < 0.001$); infant plasma PL DHA content was 45–48% higher ($P < 0.001$) and arachidonic acid, 22:4n-6, and 22:5n-6 contents were 11–13, 31–34, and 38–63% lower ($P = 0.04$, <0.001, and <0.001). There were no statistically significant differences among groups in VEP latency (125 ± 11 vs. 127 ± 11 vs. 122 ± 11 and 116 ± 8 vs. 117 ± 9 vs. 115 ± 8 ms in Group 1 vs. Group 2 vs. Group 3 at 120 and 240 d, respectively), sweep VEP acuity (9 ± 1.4 vs. 9 ± 1.4 vs. 9 ± 1.3 and 10.4 ± 1.7 vs. 9.6 ± 1.3 vs. 10.2 ± 1.5 cyc/deg) or Teller Card acuity (5.2 ± 2.7 vs. 5.1 ± 2.0 vs. 5.6 ± 2.6 and 11.8 ± 4.8 vs. 10.7 ± 3.9 vs. 12.9 ± 7.1 cyc/deg) at either age. Transient VEP amplitude was lower in infants whose mothers received the algal DHA supplement (Group 1) than infants in Group 2 and Group 3 at 120 d of age (22.8 ± 7.7 vs. 33.6 ± 13.5 vs. 33.1 ± 12.4 μvolts), but not 240 d of age (23.6 ± 6.6 vs. 25.0 ± 12.7 vs. 28.7 ± 11 μvolts). No measure of visual function correlated significantly with either milk DHA or infant plasma PL DHA content at 120 d of age. There were no statistically significant differences in weight, length, head circumference, or triceps skinfold thicknesses among groups at either 120 or 240 d of age. These data show that maternal DHA supplementation increases the DHA content of maternal milk as well as the plasma PL DHA content of the recipient infant but confers no detectable benefits with respect to visual function.

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Abbreviations: DHA, docosahexaenoic acid; PL, phospholipid; VEP, visual-evoked potential.

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The Postpartum Docosahexaenoic Acid Status of Lactating and Nonlactating Mothers

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Results of a cross-sectional study conducted by Al *et al.* (1) indicate that the maternal docosahexaenoic acid (DHA) levels in plasma phospholipids are lower with each following pregnancy. This suggests that mothers invest DHA in the development of their infants, possibly by mobilization of their own DHA stores. This view is supported by another observation of Al *et al.* (2) that the amount of DHA in the maternal plasma phospholipids increases by more than 30% during pregnancy.

Since breast milk contains DHA, breast-feeding, in contrast to bottle-feeding, may continue to impose on the maternal DHA after delivery. Therefore, we compared the recovery of the maternal DHA postpartum between lactating and nonlactating mothers.

Methods. Healthy pregnant women in their last trimester were recruited through midwives in the area of Southern Limburg. A written inform consent was obtained from each participant before the start of the study, which was approved by the Medical Ethics Committee of the University Hospital Maastricht. Subjects were excluded if they received a blood transfusion at delivery.

Blood samples were collected into EDTA-containing tubes at week 36 of pregnancy, on the 2nd and 5th day after delivery, and at 1, 2, 4, 8, 16, 32, and 64 wk postpartum. Fatty acids were analyzed in plasma phospholipids as described previously (2). The DHA levels of the two groups were compared using multiple regression analysis, with correction for parity.

Results and conclusion. The preliminary results until 16 wk postpartum of 17 lactating mothers aged 33.1 ± 0.6 yr and 10 nonlactating mothers aged 30.0 ± 1.1 yr are reported. Both the lactating and the nonlactating mothers had uncomplicated pregnancies and delivered full-term singleton newborns (39.8 ± 0.2 vs. 39.9 ± 0.4 wk, respectively). At week 16, the mothers in the lactating group were still breast-feeding their infants.

There were no differences in the DHA levels of the two groups shortly after delivery (on the 2nd and the 5th day postpartum). The DHA levels started to decrease in both groups from week 1 postpartum. This decline became more pronounced in the lactating group, and at weeks 8 and 16 the DHA levels of the lactating mothers were significantly lower than those of the nonlactating mothers.

A comparison of the DHA levels at weeks 8 and 16 with data of 80 nonpregnant women showed that the levels of the lactating mothers were significantly lower than those of the nonpregnant women, whereas the levels of the nonlactating mothers were quite comparable with the nonpregnant group.

It can be concluded that maternal plasma DHA declines after delivery. This reduction is more pronounced in lactating as compared to nonlactating mothers. The DHA levels of lactating mothers reach values lower than in nonpregnant women. This observation suggests that lactating mothers may become DHA-depleted unless they increase their DHA intake during lactation.

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Abbreviation: DHA, docosahexaenoic acid.

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The Female Docosahexaenoic Acid Status Related to the Number of Completed Pregnancies

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Essential fatty acids (EFA) are important constituents of all cell membranes. High concentrations of the long chain polyenes (LCP) docosahexaenoic acid (DHA, 22:6n-3) and arachidonic acid (20:4n-6) are present in the structural components of the brain and the retina. EFA and their LCP are therefore particularly important during fetal development, when processes like brain development and organogenesis take place. Because the fetus cannot synthesize EFA, the fetus depends on the transport of EFA from the maternal to the fetal circulation to acquire EFA. It is important that the maternal EFA status during pregnancy is adequate to cover the fetal requirement. The maternal DHA status decreases during pregnancy and seems to decrease further with each following pregnancy (1). This could imply that the maternal DHA status does not fully normalize after a mature pregnancy. To further test the suggestion that pregnancy is associated with a maternal DHA-depletion phenomenon, the existence of a relationship between the DHA status of nonpregnant women and the number of completed pregnancies has been investigated.

Materials and methods. For this purpose, the fatty acids in plasma and red blood cell (RBC) phospholipids obtained from women who completed zero ($n = 41$), one ($n = 21$), two ($n = 34$), three ($n = 26$), or four ($n = 7$) mature pregnancies have been analyzed (cross-sectional design). The average time lapse between blood sampling and last delivery was 3.7 yr (range 0.8 to 12.3 yr). Fatty acids were analyzed as described before by Al *et al.* (2). The relationships between the maternal DHA status and the number of completed mature pregnancies was estimated by means of multiple regression analysis. The influence of the time period between the different pregnancies on the maternal DHA status was also investigated. Because the total amount of fatty acids (mg/L) in the plasma and RBC phospholipids in the nulli-, primi-, and multipara was not significantly different, only the relative amounts (% w/w) of fatty acids have been considered and statistically analyzed.

Results. The amounts of plasma DHA (% w/w) in nullipara and mothers were not significantly different; a significant correlation between parity and plasma DHA did not exist either. The RBC DHA in mothers was significantly lower than that of nullipara ($P = 0.013$), but no significant correlation between RBC DHA and parity was observed. The time-interval between the different pregnancies did not influence the maternal DHA status. Because the average time lapse between blood sampling and last delivery was (rather long) 3.7 yr, we recalculated the results for a subsample of 27 mothers whose last delivery was at least 1 yr ago, but no longer than 2 yr ago. We got the same results: no differences at all.

Discussion. No relationship was found between the DHA status in blood and the number of pregnancies in nonpregnant women, by whom the last partus at the moment of research occurred at least 1 yr ago. The maternal DHA status probably normalizes within 1 yr after the last partus. Therefore, a maternal depletion phenomenon cannot be observed. The question remains why multiparous women have lower plasma DHA levels than primiparous women during pregnancy. We only can hypothesize about this. The study by Al *et al.* (1) was cross-sectional; maybe a longitudinal study will give different results. In addition, it could be that the plasma DHA levels have normalized after 1 yr, whereas tissue values (stores) have not and therefore during pregnancy, when there is a high demand for DHA, plasma levels do not rise as much as during the first pregnancy. More studies are needed to solve these questions.

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Abbreviations: DHA, docosahexaenoic acid; EFA, essential fatty acid; LPC, long chain polyene; RBC, red blood cell.

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Effects of Third Trimester Consumption of Eggs High in Docosahexaenoic Acid on Docosahexaenoic Acid Status and Pregnancy

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Worldwide, maternal intakes of docosahexaenoic acid (DHA) are highly variable; however, few studies have looked at the effect of variable DHA intake on DHA in maternal and infant blood. We postulated that eggs from chickens fed DHA-rich microalgae (DHA Gold™, OmegaTech, Inc., Boulder, CO) would be accepted as a food source of DHA and increase levels of this fatty acid in blood lipids of mothers and infants. The primary aim of this study was to compare maternal third trimester DHA status and infant DHA status among women who: (i) were randomly assigned to consume up to one dozen regular eggs, each containing about 28 mg DHA, per week (regular egg); (ii) were randomly assigned to consume up to one dozen eggs from chickens fed a diet containing the dried microalgae, each containing approximately 135 mg DHA, per week (high-DHA egg); or (iii) routinely consumed few if any eggs (low egg). Secondary aims of the study were: (i) to determine the usual DHA intakes from eggs by women who delivered in our hospital, and (ii) to obtain data about pregnancy outcomes that might be relevant to safety and efficacy of the high-DHA eggs.

Methods. Pregnant women who were 24–28 wk postmenstrual age without diabetes, organ disease, or pre-eclampsia were asked to consent to the study. Blood samples (pre-study) were obtained, and the women were asked about frequency of food intake. Women who ate eggs were randomly assigned to regular or high-DHA eggs; women who ate few eggs were grouped without being randomized. The two egg groups were given one dozen eggs per week and instructed to eat as many of the eggs as they could. Egg intakes were recorded from written records and double-checked biweekly by telephone interviews.

Poststudy maternal bloods were taken at delivery. After delivery, cord bloods were obtained, the placentas and infants weighed, and infants' lengths and head circumferences measured. Plasma and red blood cells (RBC) were separated by centrifugation, and total lipids from each were extracted and separated into lipid classes by thin-layer chromatography. Plasma and RBC total phospholipids and plasma triglycerides were recovered from the plates, transmethylated with boron trifluoride–methanol, and the resulting fatty acid methyl esters analyzed by capillary gas–liquid chromatography (SP-2560, 100 m × 0.25 mm).

Results. Fifty-three women completed the study (16 low egg, 19 regular egg, 18 high-DHA egg). The respective mean pre-study DHA intakes from eggs for these groups were 5.4, 16.0, and 19.6 mg/day. The mean study DHA intakes from eggs for the same groups were 16.2, 51.2, and 205.2 mg/d based on weekly egg intakes of 2.7, 8.8, and 10.1 for 12.9, 12.6, and 12.8 wk of study, respectively.

In the control groups (low egg and regular egg), DHA decreased in each lipid class during the study while it increased in comparable lipids of the high-DHA egg group. The difference between groups did not reach statistical significance because of variable egg intakes within groups. However, regression analysis showed a significant positive relationship between study DHA intake and DHA in plasma and RBC lipids of women and infants at delivery.

The study did not have adequate power to detect an effect of high-DHA eggs on pregnancy outcome; however, several favorable outcomes were suggested. Despite the variability in number of eggs consumed, the high-DHA egg group had fewer low-birth-weight (<2500 g) (0% vs. 13 and 26%) and preterm (<37 gestation) (6% vs. 25 and 26%) infants than women from the low-egg and regular-egg groups, respectively. They also had larger placentas (760 g vs. 658 and 663 g), even after preterm infants were excluded (762 g vs. 665 and 710 g). A higher proportion of women in the control and comparison groups compared to the high-DHA group also had surgical delivery and received antibiotics in hospital. Three women in the regular-egg group but none in the high-DHA group or low-egg group developed gestational diabetes after enrollment.

Summary. High-DHA eggs appear to be a practical way to increase maternal DHA status. The data suggest that high-DHA eggs could decrease the incidence of preterm and low-birth-weight births, and this could be tested in a study with more statistical power. The neurodevelopment of infants from women fed low- and high-DHA eggs could also provide helpful information.

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Abbreviations: DHA, docosahexaenoic acid; RBC, red blood cell.

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Essential Fatty Acid Status in Malnourished Children

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There are several hundred millions of malnourished children in the developing world. The high morbidity and mortality as well as many specific deficiencies have been extensively documented in this sad situation. On the contrary, essential fatty acid deficiency (EFAD) has only been dealt with scarcely and mainly in the extreme stages of kwashiorkor and marasmus. We report here preliminary observations in 32 cases from the hospital Instituto Clínico-Nutricional "Menca de Leoni" of Caracas (10 mild, 6 moderate, and 16 severe) and 22 controls. The staging of weight for age proposed by the World Health Organization was used (mild: <percentil 3 to −3 SD, moderate: −3 SD to −4 SD and severe: <−4 SD). None of our cases was complicated by infections. Only one reached the staging of kwashiorkor by Kanawati/McLaren index. Controls belong to the range of 90 to 10 percentile.

Biochemical methods. Three mL of blood in EDTA were obtained. Plasma phospholipids were extracted in chloroform/methanol, and then phospholipids separated by thin-layer chromatography in silica gel. Fatty acids (FA) were measured by gas–liquid chromatography in an open column of polyethylene-glycol-adipate 5% of 1.8 m at isothermal of 180°C. FA are expressed as percentage of total area of FA.

Results. Plasma phospholipid FA of the control children can be observed in Table 2. There is no major difference with what we observed in numerous analyses in the adult normal population. On the contrary, most of our malnourished children showed EFA deficiency (EFAD). In general both series, n-3 and n-6, were deficient. The extent of this problem could be very conveniently shown by calculating the index Σ non-EFA/Σ EFA in plasma phospholipids. The resulting indexes are shown in Table 3.

TABLE 1
Distribution of Malnourished Children

	N	Age (yr)	Boys	Girls
Mild	10	1–5	4	6
Moderate	6	1–2	2	4
Severe	16	1–3	8	8

TABLE 2
Fatty Acids of Plasma Phospholipids in Control Children

	16:0	16:1	18:0	18:1	18:2n-6	20:3n-6	20:4n-6	20:5n-3	20:6n-3
Mean	26.4	1.6	14	14	26.2	4.4	9.2	.8	.4
SD	3	.8	1.3	2.3	3	.8	1.8	.7	1.3

TABLE 3
Σ Non-EFA/Σ EFA in Plasma Phospholipids[a]

	Mean	SD	N	*P* (Mann-Whitney)
Controls	1.53	.2	22	
Mild	1.85	.3	10	.002
Moderate	2.47	.6	6	<.001
Severe	2.22	.7	16	<.001

[a]EFA, essential fatty acid.

TABLE 4
Mead Acid Frequency

	Yes	No	*P* Fisher's exact test
Controls	2	20	
Total malnourished	27	5	<.001
Mild	9	1	<.001
Moderate	4	2	<.001

Discussion. Reported evidence of EFAD in malnourished children deals with primary severe malnutrition or with secondary deficiency related to malabsorption syndromes. Two general patterns have previously been observed. (i) The protein malnutrition prevails, so that the pool of C_{18} parent EFA cannot be elongated and unsaturated. In this case, no Mead acid is generally observed. (ii) Enzymes are preserved, but there is a very low C_{18} EFA pool. In this case Mead acid will be present and arachidonic acid will be low. Our cases belong to this second category. If the situation observed in our children is prevalent in other underdeveloped areas of the world, we can conclude that EFAD may be a very common nutritional deficiency. In general the treatment of this specific deficiency is not adequately standardized to replenish rapidly the membranes of most cells. Further research is needed in this interesting field.

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Abbreviations: EFA, essential fatty acid; EFAD, essential fatty acid deficiency; FA, fatty acid.

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A Hypothesis to Explain the Reduced Blood Levels of Docosahexaenoic Acid in Inherited Retinal Degenerations Caused by Mutations in Genes Encoding Retina-Specific Proteins

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ABSTRACT: Some humans and animals with inherited retinal degenerations (RD) have lower blood levels of docosahexaenoic acid (22:6n-3) than controls. As a result of recent studies, clearly the low blood 22:6n-3 phenotype is found in multiple RD phenotypes and no mutation thus far identified in humans or animals is involved in lipid metabolism. Therefore, it seems reasonable to suggest that the primary defect is not in 22:6n-3 metabolism, but rather in some common convergent pathway that ultimately leads to the reduction of blood and tissue 22:6n-3 levels. One possibility is that the different mutations produce a metabolic stress that provokes structural and biochemical adaptive changes in photoreceptor cells and their rod outer segments. If the stress is oxidant, the retina could downregulate 22:6n-3 and upregulate antioxidant defenses. How such a stress could lead to changes in blood levels of 22:6n-3 is not obvious. However, the consistent finding of the 22:6n-3 phenotype in many different retinal degeneration genotypes suggests that some form of communication exists between the retina and other tissues that serves to reduce blood levels of 22:6n-3.

Retinitis pigmentosa (RP) is the name given to a group of retinal degenerations that may be inherited in an autosomal dominant, recessive, X-linked, or sporadic manner. The first symptom is usually night blindness, which is caused by the loss of rod photoreceptor cells in the peripheral retina. With time, the loss of vision slowly progresses until only "tunnel vision" remains, indicating that only cone photoreceptors in the central retina are still viable. In most cases of RP, cone photoreceptors eventually die, leading to total blindness.

Over the last 15 yr, numerous publications have appeared describing reduced levels of docosahexaenoic (22:6n-3) and other long-chain polyunsaturated fatty acids (LCPUFA) in blood lipids of patients with all types of RP (1–7). One of the most dramatic reductions in LCPUFA is in X-linked RP, where a positive correlation between blood LCPUFA levels and severity of RP was reported (7). Bazan *et al.* (8) found lower blood levels in persons with Usher's syndrome, a disease that includes loss of both vision and hearing. We (9) recently confirmed this finding and expanded it to show lower LCPUFA in persons with Usher's Type I (more profound vision and hearing loss), but not in persons with Usher's Type II (milder and slower loss of vision and hearing).

We reported that the blood level of 22:6n-3 is reduced in dogs (10) with progressive rod cone degeneration (*prcd*), an animal model of inherited retinal degeneration that closely resembles human RP (11). Interestingly, in the *prcd*-affected dog, there was also reduced 22:6n-3 levels in retinal rod outer segments (ROS) membrane lipids (12). Since there was no concomitant increase in 22:5n-6 in these membranes, the reduction was not due to n-3 deprivation. We thought at first that the primary defect in the dogs was a mutation in the gene encoding a protein involved in LCPUFA synthesis. However, we showed that affected dogs could synthesize 22:6n-3 from n-3 precursors (13). Recently, we analyzed ROS from transgenic pigs with a Pro247Leu rhodopsin mutation (Anderson, R., Maude, M., Petters, R., and Wong, F., unpublished results) and *rds* heterozygous mice with a peripherin mutation (Anderson, R., Maude, M., and Bok, D., unpublished results) and found reduced ROS levels of 22:6n-3 without an increase in 22:5n-6. Thus, in three animal models of RP, two of which have known mutations, there is a selective reduction in 22:6n-3 in the ROS membranes.

Is there a causal relationship between low-blood LCPUFA levels and retinal degeneration? The answer to this question appears to be "no." Our attempts to prevent retinal degeneration in *prcd*-affected dogs by supplementing young adults with fish oil were not successful (12). In that study, blood and ROS levels of 22:6n-3 were significantly elevated in affected

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Abbreviations: LCPUFA, long-chain polyunsaturated fatty acids; RD, retinal degeneration; ROS, rod outer segment; RP, retinitis pigmentosa.

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and normal dogs given fish oil. However, after 5 mon, the 22:6n-3 levels in ROS of normal dogs given safflower oil (controls) were lower than those of the affected dogs given fish oil, yet the affected dogs lost photoreceptors and the normal dogs did not. In other studies, reduction in blood and ROS 22:6n-3 in rats (14,15), monkeys (16,17), and guinea pigs (18,19) can be achieved by feeding an n-3-deficient diet. However, this change is always accompanied by an elevation in n-6 LCPUFA (20). To our knowledge, there have been no reports that such fatty acid changes in ROS membranes lead to death of photoreceptor cells. Therefore, reduced ROS and blood levels of 22:6n-3, while found in animals with inherited retinal degenerations, is not sufficient to cause the degeneration.

Recently, it became clear that mutations in a number of genes encoding proteins expressed only in the retina are the cause of rod photoreceptor cell death (see RetNet™ at: http://www.sph.uth.tmc.edu/RetNet). To date, 97 genes associated with some type of retinal degeneration were mapped and 41 were cloned. Mutations were identified in the visual pigment opsin, the beta subunit of cyclic-GMP specific phosphodiesterase, RDS/peripherin, myosin type VIIa, and several other proteins. Also, different mutations within the same gene were found. There are now almost 100 different rhodopsin mutations that lead to retinal degeneration. Low blood levels of 22:6n-3 were found in patients with these known mutations, even though the proteins the genes encode are clearly not involved in lipid metabolism.

Why do humans and animals with several different, known mutations in genes encoding retina-specific proteins have reduced ROS and blood levels of 22:6n-3? To try to address this question, we have developed the metabolic oxidant stress hypothesis. Simply stated, our hypothesis is that mutations in genes encoding retina-specific proteins cause an oxidant stress that ultimately leads to the death of photoreceptor cells. Given the very high levels of 22:6n-3 in ROS membranes, the high flux of oxygen through the ROS membranes from the choriocapillaris circulation, and the constant bombardment of the ROS by light, conditions certainly exist in these membranes for lipid peroxidation. These postmitotic cells respond to the oxidant stress by undergoing a series of biochemical and morphological adaptations designed to upregulate their antioxidant defenses. This would include reducing the level of 22:6n-3, elevating the level of antioxidant protective molecules and enzymes, and perhaps reducing the ability of the cells to capture photons by either shortening their ROS or reducing the rhodopsin concentration in ROS membranes.

To achieve such changes would require a high degree of plasticity in retinal photoreceptor cells. Previous studies in our laboratory showed that rat photoreceptor cells indeed are capable of such dynamic actions (21–24). Albino rats stressed by rearing in relatively bright cyclic light from birth undergo profound structural and biochemical changes compared to animals raised in dim cyclic light. In these studies, rats were born into a controlled environment of 5, 300, or 800 lux intensity of cyclic light (12 h on/12 h off) and maintained in this environment for up to 12 wk. Compared to animals raised in 5 lux cyclic light, the following differences were found in rats raised in 800 lux cyclic light: (i) dramatic reduction in 22:6n-3 in ROS membranes without concomitant increase in 22:5n-6; (ii) relative reduction in phosphatidylethanolamine and phosphatidylserine, and increase in phosphatidylcholine; (iii) reduction in the amount of the visual pigment rhodopsin relative to phospholipid in the lipid bilayer; (iv) increase in the enzymatic activities of glutathione peroxidase, glutathione reductase, and glutathione S-transferase; (v) increase in the amounts of vitamins E and C; and (vi) shortened outer segment length and moderate disorganization of ROS disk packing.

These adaptive changes are for the most part reversible (24). If animals raised for 9 wk in 800 lux cyclic light are placed for 3 wk in 5 lux cyclic light, the level of ROS 22:6n-3 increases, the activities of the glutathione enzymes decrease, the levels of vitamins E and C decrease, and the morphological appearance of the ROS disks returns to that normally seen for these organelles. The only change that is not reversible is the loss of photoreceptor cell nuclei in the animals raised in the 800 lux cyclic light. The chronic stress of bright cyclic light rearing caused a slow loss (30%) of photoreceptor cells over a 12-wk period of time.

Our hypothesis is that mutations in photoreceptor-specific proteins cause an actual or perceived oxidant stress similar to that discussed above for albino rats raised in a bright cyclic light environment. To deal with such a stress, the retina adapts by reducing the level of 22:6n-3 in ROS membranes and upregulating the levels of antioxidant enzymes and small molecules. While this may afford some protection against retinal degeneration, the effect of the chronic stress is a slow loss of photoreceptor cells, much like that observed for the rats raised in 800 lux cyclic light.

In humans with RP and dogs with *prcd*, the initial biochemical observation was of lower blood levels of 22:6n-3 compared to controls. These results were confirmed in many different laboratories, in patients with all modes of inheritance of RP, so there is little question of their validity. We do not understand how mutations in so many different genes encoding proteins thought to be expressed primarily in the retina can lead to the systemic low-22:6n-3 phenotype. It is unlikely that each mutated gene product acts independently to affect some aspect of systemic fatty acid synthesis, transport, or uptake. We suggest that the oxidant stress caused by these different mutations induces the retina to release some signal to the liver that results in downregulation of 22:6n-3 incorporation into lipoproteins, as suggested by Bazan and Rodriguez de Turco (25), and possibly upregulation of the enzymatic oxidation of n-3 fatty acids. Alternatively, such a signal may be generated by the pineal gland, which also is capable of expressing some proteins otherwise found only in photoreceptor cells.

In order to design therapeutic strategies for RP patients, it is important to determine if their reduced blood level of 22:6n-3 is due to an adaptive response to an oxidant stress or to some other cause. If the reduction is an adaptation to an ox-

idative challenge, then supplementing RP patients with n-3 fatty acids may in fact exacerbate the loss of photoreceptor cells. On the other hand, if the lower blood levels are due to n-3 deficiency, then supplementation with LCPUFA may be of some benefit.

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Rats with Low Levels of Brain Docosahexaenoic Acid Show Impaired Performance in Olfactory-Based and Spatial Learning Tasks

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ABSTRACT: Studies were carried out to determine if decreased levels of central nervous system docosahexaenoic acid (DHA), a result of consuming an n-3-deficient diet, had an effect on learning- and memory-related behaviors in adult male rats. Females were reared on an n-3-deficient or n-3-adequate diet beginning at 21 d of life. Their male pups, the F2 generation, were weaned to the diet of the dam and tested at 9–12 wk of age. An olfactory-based discrimination and Morris water maze task were used to assess performance. Whole brain was collected after the behavioral experiments and central nervous system fatty acid content was analyzed in olfactory bulb total lipid extracts. F2 generation male rats consuming the n-3-deficient diet had an 82% decrease in DHA compared to rats consuming the n-3-adequate diet. The n-3-deficient animals made significantly more total errors in a 7-problem, 2-odor discrimination task compared to the n-3-adequate group. Furthermore, the escape latency in the Morris water maze task was significantly longer for the n-3-deficient rats compared to the n-3-adequate rats. These results indicate that rats with decreased DHA levels in the central nervous system perform poorer in these tasks compared to rats with higher DHA levels and suggest the presence of learning deficits in these animals.

The importance of n-3 fatty acids for the development and function of the central nervous system, particularly the visual system, has been well-established (1–3). Rats and guinea pigs maintained on diets deficient in n-3 fatty acids have abnormal electroretinograms (4–6). Human preterm infants and infant rhesus monkeys fed formulas without docosahexaenoic acid (DHA; 22:6n-3) also have abnormal electroretinograms as well as decreased visual acuity compared to infants fed formulas containing 22:6n-3 (7–10). DHA not only is found in high levels in the retinal rod outer segments but also is found in extremely high concentrations in brain synaptic membranes, suggesting it may play an important role in higher level neural function.

Previous literature describing the effects of decreased levels of brain 22:6n-3 on learning and memory in rodents has been inconsistent (11–16). In these studies, oils low in n-3 fatty acids (safflower, sunflower) were used in diets to achieve low dietary intakes of n-3 fatty acids and thus low levels of 22:6n-3 in the central nervous system. Several generations of animals maintained on the deficient diet are usually required to deplete neural 22:6n-3 (1,2). Bourre *et al.* (5) studied 60-d-old, third-generation male rats that had consumed either a sunflower oil- (low 18:3n-3) or soybean oil-based (adequate 18:3n-3) diet and found learning impairments in the safflower oil-fed rats in a shuttle box test. However, in second-generation adult mice, Wainwright found no significant differences in the Morris water maze between the n-3-deficient group and two other groups, a saturated fat group and adequate n-3 fatty acid group (17). Significantly lower rates of learning were reported in the saturated fat group (n-3 and n-6 fatty acid-deficient) compared to the n-3-adequate group. Criticisms of the animal learning and memory experiments range from improper use of statistics and experimental units to inadequate controls that would rule out sensory or motivational factors that could effect outcomes (18).

The purpose of the present experiment was to determine if decreased levels of brain 22:6n-3, achieved by an n-3-deficient diet, affected the performance of rats in an olfactory-based cognitive task and in the Morris water maze task. Rats have an exceptional ability to learn complex problems when odors are used as discriminative stimuli (19,20). Thus olfactory-based tasks may provide sensitive tests of learning and memory in the n-3-deficient rat.

MATERIALS AND METHODS

Animals. Twenty-one-day-old female, Long-Evans rats (Charles River, Portage, MI) were received at our animal facility, randomized into two groups and began consuming one of two experimental diets at this time. At 8 wk of age, these

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Abbreviation: DHA, docosahexaenoic acid.

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females (F1 generation) were mated with chow-fed males. Their offspring, the F2 generation, were weaned to the diet of the dam and maintained on these diets throughout the study. Behavioral training of F2 generation males began when they were 68 d of age, and tissues were collected for fatty acid analysis at 12 wk of age. All animal procedures were approved by the National Institute on Alcohol Abuse and Alcoholism Animal Care and Use Committee.

Dietary treatments. The two experimental diets (Table 1), deemed "n-3-deficient" and "n-3-adequate," were based on the AIN93 diet (21) with modifications to reduce n-3 fatty acids in the basal diet components (Dyets, Bethlehem, PA). Vitamin-free casein replaced unprocessed casein, and the majority of cornstarch, which contains essential fatty acids, was replaced by maltose-dextrin. The diets were designed to contain only one primary fatty acid variable, α-linolenic acid (18:3n-3). Safflower oil contributed adequate and identical amounts of linoleic acid (18:2n-6) to both diets. A small amount of flaxseed oil, the only variable, was added to the n-3-adequate diet to provide adequate n-3 fatty acids. The fatty acid composition of the diets is shown in Table 2.

Olfactomter. A multichannel olfactometer (Model LD8; Knosys Ltd., Bethesda, MD) was used. The unit was similar in design to that described by Slotnick and Risser (22). Briefly, the unit controls air flow in eight independent channels, allowing any one of eight different odors to be presented. A special feature of the olfactometer was the use of pinch valves to control odor flow. By replacing tubing controlled by the valves when new odors were used, potential valve contamination was eliminated.

The operant conditioning chamber was similar to that described by Lu *et al.* (23). The output of the odor generator was connected to a vertically oriented 25-mm diameter glass tube attached to one wall of the chamber. A 20-mm diameter hole in the tube allowed the rat to sample odors. Snout insertions into the tube were detected by a photocell. A 13-gauge stainless steel drinking tube was located above and 50 mm to one side of the opening in the odor sampling tube. Contacts with the drinking tube were detected by a sensitive circuit. The drinking tube was connected *via* a normally closed solenoid to a water reservoir. Operation of the solenoid dispensed 0.04 mL water.

TABLE 1
Diet Composition

	g/100 g diet	
Casein, vitamin-free	20	
Carbohydrate	60	
Cornstarch	15	
Sucrose	10	
Dextrose	19.9	
Maltose-dextrin	15	
Cellulose	5	
Salt mix[a]	3.5	
Vitamin mix[a]	1	
L-Cystine	0.3	
Choline bitartrate	0.25	
TBHQ	0.002	
Fat	10	
Fat sources	n-3 deficient	n-3 adequate
Coconut oil[b]	8.1	7.75
Safflower oil	1.9	1.77
Flaxseed oil	—	0.48

[a]As detailed in Reference 19.
[b]Hydrogenated. TBHQ, tertiary butylhydroquinone.

TABLE 2
Fatty Acid Composition of the Diets (% of total fatty acids)[a]

Fatty acid[b]	n-3 Deficient	n-3 Adequate
8:0	0.8	1.9
10:0	3.8	3.8
12:0	39.7	36.5
14:0	16.7	15.8
16:0	9.8	9.6
18:0	9.7	9.4
20:0	0.2	0.2
22:0	0.1	0.1
24:0	0.1	0.1
Total saturated	80.8	77.2
16:1n-7	0.03	0.03
18:1n-9	3.5	3.9
18:1n-7	0.3	0.3
20:1	0.1	0.1
22:1	0.01	0.02
Total monounsaturated	3.9	4.3
18:2n-6	15.1	15.3
20:2n-6	0.05	n.d.
Total n-6	15.1	15.3
18:3n-3	0.04	3.1
Total n-3	0.04	3.1
18:2n-6/18:3n-3	345	5
n-6/n-3	346	5
Total PUFA	15	18

[a]n.d. = Not detected.
[b]The following fatty acids were analyzed and were not present at detectable levels: 12:1, 14:1, 20:3n-6, 20:4n-6, 20:5n-3, 22:5n-3, 22:6n-3. PUFA, polyunsaturated fatty acids.

Olfactory training and test procedures. At approximately 7.5 wk of age, F2 males were placed on a 7.0 mL/day water schedule, and beginning 12 d later, rats were trained using standard operant procedures to respond (lick) at the water delivery when it detected an odor in the odor sampling tube. The go, no-go odor discrimination procedure was described in detail (22). Testing began at about 9.5 wk of age. Each day, for 7 d, all rats were tested on a novel two-odor discrimination problem. Odors (McCormick, Hunt Valley, MD) were paired and ordered randomly. Briefly, rats were presented with two different odors each day, one designated S+ (positive stimulus) and one designated S− (negative stimulus). One hundred trials (50 S+ trials and 50 S− trials) were presented in problems 1–5, and 60 trials were presented in problems 6 and 7. Over time, rats learned that the S+ odor was associated with a water reward and the S− odor was not associated with a water reward, and subsequently the rat would only respond to the water tube after sampling the S+ odor. The endpoint was total number of errors (responding to the S− odor or not responding to an S+ odor) at the end of seven problems. Because training procedures and

discrimination tests are completely automated (computer-controlled), experimental bias was eliminated (19,20,22).

Water maze task. The Morris water maze is a more traditional method for testing spatial cognitive abilities. The water maze consists of a circular pool (4 ft diameter by 2 ft deep) containing tap water maintained at 20°C and an escape platform. The maze was in the middle of a room and was surrounded by visible cues (posters, table, door, etc.). All animals were tested two times/day in the Morris water maze. Initially, they were subjected to a visible platform test (training trial) in which the animals were placed into the water maze and allowed a maximum of 90 s to locate the visible platform. The visible test was used to test vision (sensory) and swimming ability of the animals. If the platform was not found, the rats were guided to the platform and allowed to remain there for 30 s. The following day was the first day of testing (learning trial), and a transparent (invisible) platform replaced the visible platform. The rats were allowed a maximum of 90 s to reach the platform in each trial. The times to reach the platform in both trials (escape latency) were summed to result in a total escape latency for each animal (maximal time = 180 s).

Fatty acid analysis. All rats were euthanized by decapitation and tissues removed for fatty acid analysis. Lipids were extracted by the method of Bligh and Dyer and fatty acid methyl esters prepared by a modified method of Morrison and Smith and quantified by gas chromatography as previously described (24). The fatty acid methyl ester 23:0 was used as an internal standard, and peaks were identified by comparison with standard mixtures of fatty acids (Nu-Chek-Prep, Elysian, MN).

Statistical analysis. Data are expressed as the mean plus or minus the standard error for the fatty acid values and behavioral measures. Each animal used in the olfactory testing was from a separate litter (25). Student's *t*-test was used to test for differences between the two dietary groups, and a $P < 0.05$ was considered significant. For the Morris water maze task, the Mann-Whitney U-test was used to determine significant differences between the two dietary groups.

RESULTS

F1 generation female rats began consuming an n-3-adequate or n-3-deficient diet at weaning and were mated at 8 wk of age. F2 generation pups were weaned at approximately 21 d of life. The diets had no effect on body weight of the F1 females (Fig. 1) or the F2 males (data not shown).

Fatty acid analysis. F2 generation male rats were fed the n-3-adequate or n-3-deficient diet for their entire lives. At 9.5 wk of age, rats were tested in one of several behavioral tests and subsequently euthanized, and tissues were collected for fatty acid analysis. Olfactory bulb fatty acid composition was significantly altered by the n-3-deficient diet (Table 3). DHA levels were 82% lower in the total lipid extract in the n-3-deficient olfactory bulbs compared to the n-3-adequate olfactory bulbs. The 22:6n-3 decrease was matched by an 8.4-fold increase in docosapentaenoic acid (22:5n-6) in the deficient group to maintain 22-C levels in the olfactory bulb. Brain levels of arachidonic acid (20:4n-6) and 22:4n-6 were also significantly higher, 15 and 35% respectively, in the n-3 deficient group.

TABLE 3
Fatty Acid Composition of the Olfactory Bulb (total lipid extract)[a]

Fatty acid	n-3 Deficient	n-3 Adequate
14:0	1.4(0.1)	1.1(0.2)
16:0	24.2(0.5)	24.4(0.4)
18:0	15.9(0.4)	15.7(0.4)
20:0	0.3(0.03)	0.3(0.03)
22:0	0.4(0.04)	0.4(0.02)
24:0	0.5(0.1)	0.5(0.1)
Total saturated	42.8(0.3)	42.4(0.4)
16:1n-7	0.49(0.01)	0.53(0.02)*
18:1n-9	12.2(0.1)	13.1(0.2)*
18:1n-7	3.2(0.1)	3.1(0.1)
20:1	0.6(0.04)	0.7(0.04)
22:1	0.07(0.01)	0.07(0.01)
24:1	1.2(0.2)	1.6(0.1)
Total monounsaturated	17.8(0.3)	19.1(0.3)*
18:2n-6	0.5(0.02)	0.48(0.02)
18:3n-6	n.d.	n.d.
20:3n-6	0.2(0.01)	0.3(0.01)*
20:4n-6	9.0(0.3)	7.8(0.1)*
22:4n-6	2.7(0.1)	2.0(0.1)*
22:5n-6	14.1(0.4)	1.5(0.1)*
Total n-6	26.6(0.4)	12.1(0.1)*
18:3n-3	n.d.	n.d.
20:5n-3	n.d.	n.d.
22:5n-3	0.007(0.01)	0.1(0.002)*
22:6n-3	2.7(0.02)	15.5(0.5)*
Total n-3	2.8(0.02)	15.6(0.5)*
20:3n-9	0.09(0.01)	0.08(0.003)
22:5n-6/22:6n-3	5.1(0.2)	0.09(0.003)*
22:5n-6 + 22:6n-3	16.8(0.3)	16.9(0.6)
n-6/n-3	9.7(0.2)	0.8(0.02)*
n-6 + n-3	29.3(0.4)	27.7(0.5)*

[a]Percentage of total fatty acids is expressed as the mean (standard error). Values may not equal 100% due to unidentified peaks. * = $P < 0.05$; n.d. = not detected.

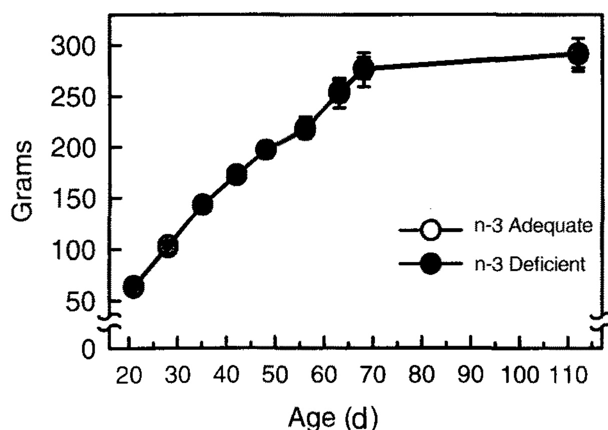

FIG. 1. The change in body weight of F1 female rats in the n-3-deficient and n-3-adequate groups over the experimental period (body weight ± SE). Only one set of data is apparent because the weights are similar at all time points.

Olfactory-based learning task. The n-3-adequate and n-3-deficient animals were tested on seven different two-odor, olfactory discrimination tasks over 7 d. Initially, all animals were testing at chance meaning 50% correct responses as they responded to both the positive and negative odor. However, there was a general trend of decreasing numbers of errors (decreases in responses to the negative odor) each day over the testing period (data not shown). The n-3-deficient rats made significantly more errors in the first two odor discriminations and more errors in all problems than did the n-3-adequate rats. However, both groups showed a gradual and progressive improvement in performance over the seven problems. The n-3-deficient animals had a significant 77% increase in total errors for the seven problems compared to the n-3-adequate group (Fig. 2). Total errors include responding to a negative odor (S−) and not responding to a positive odor (S+); however, most errors were responses to the negative odor.

Water maze task. Rats were placed into the water maze from one of two points every day and allowed 90 s to find a hidden platform (escape latency). The endpoint was total number of seconds to find the platform in two trials in the first session (day). In the first session of the learning test with the transparent platform, only 30% of the n-3-deficient animals reached the platform on both trials compared to 70% of the n-3-adequate animals. Using nonparametric statistical analysis (Mann Whitney U-test), the escape latency of the n-3-deficient group was significantly longer compared to the n-3-adequate group (Fig. 3).

DISCUSSION

In the F2 generation, the n-3-deficient brain contained 82% less 22:6n-3 compared to the n-3-adequate brain. This is a remarkable decrease in 22:6n-3 by the second generation. Many studies have reported a much less impressive decrease in this fatty acid in the second or third generation of rodents, probably due to the use of protein, carbohydrate, and fat sources that contain small, but important amounts of n-3 fatty acids (12,26). Extensive analyses were performed when preparing the diets to ensure the lowest possible n-3 content while maintaining diets that were still nutritious and palatable. This was achieved by using vitamin-free casein, a product designed to contain negligible amounts of fat-soluble vitamins, which also has a lower overall fat content compared to unprocessed casein. Some of the cornstarch was replaced with maltose-dextrin to further reduce the n-3 and n-6 fatty acids in the base diet. This is the first study to show such a marked decrease in 22:6n-3 in the second generation, using only dietary manipulations and indicating that brain 22:6n-3 levels can be lowered with diet in dam-reared rats, nearly to the extent observed in artificially-reared rats (27).

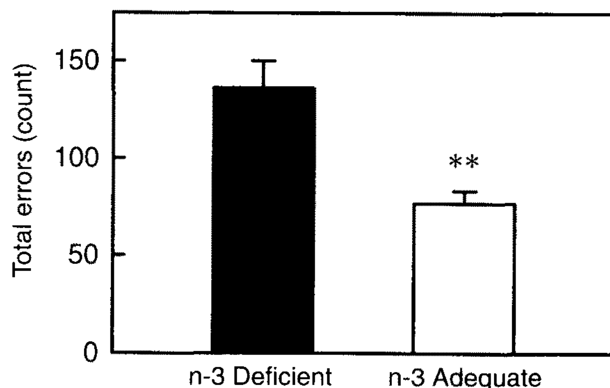

FIG. 2. Effect of the n-3-deficient and n-3-adequate diet on the total number of errors in an olfactory-based learning task (total errors ± SE), $*P < 0.02$.

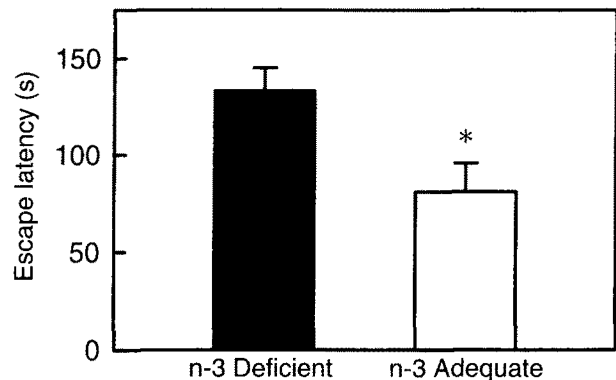

FIG. 3. Effect of the n-3-deficient and n-3-adequate diet on the escape latency in the first session of the Morris water maze task (escape latency ± SE), $*P < 0.005$.

An olfactory-based learning task was chosen to address some of the criticisms concerning rat learning experiments. Previous research in this area focused mainly on the visual system in rats. While this modality is certainly important in primates, rats are macrosmatic and rely primarily on olfactory cues. Thus olfaction may be the most appropriate modality to employ when using rats in learning experiments involving higher level neural functions. The present results indicate that n-3-deficient rats learn odor discrimination tasks more slowly than n-3-adequate rats. Additional studies will be required to determine whether this represents a sensory deficit or a deficit in making stimulus-response associations. Interestingly, rats are able to acquire a learning set when odor cues are used, and the procedures employed in the current study could easily be extended to assess learning set performance. This would be of considerable interest because acquisition of a learning set requires the subject to demonstrate a strategy or rule for responding (e.g., "win-stay, lose-shift"), and the test is widely used to assess higher-level cognitive performance (19–20,22).

The results from the Morris water maze support the results from the olfactory-based learning task. The n-3-deficient animals took longer to locate the hidden platform compared to the n-3-adequate animals, suggesting learning deficit in these animals. Both groups of animals received the same amount of

training (visible platform test), but the n-3-deficient animals had a significantly longer escape latency in session one.

These studies were conducted on adult rats (9.5–12 wk of age), and therefore, differences seen in task performance using these methods endure into maturity. Many visual deficits associated with n-3 fatty acid deficiency were reported to disappear over time and therefore could be mistakenly considered unimportant. In some cases, the behavioral task used became insensitive later in neurodevelopment. We did not examine the performance of young rats in this study, but learning differences between the n-3-adequate and n-3-deficient groups were detected in the adult rat and are thus unlikely to disappear over time. This is important when discussing the effects of n-3 deficiency on the central nervous system and demonstrates that all deficits associated with low levels of 22:6n-3 are not transient.

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Low Serum Docosahexaenoic Acid Is a Significant Risk Factor for Alzheimer's Dementia

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Docosahexaenoic acid (DHA) is the major fatty acid of neurological and retinal membranes. It makes up more than 30% of the structural lipid of the neuron and is particularly enriched in synaptosomal membranes. A number of seemingly unrelated neuropathological conditions including depression, attention deficit hyperactivity disorder, and dementia have a common association in that patients exhibit a subnormal DHA status. Clinical studies in patients with adrenoleukodystrophy, long chain hydroxyacyl CoA dehydrogenase deficiency, dyslexia, and Alzheimer's dementia (AD) demonstrated improvements in visual or neurological deficits by elevating circulating DHA levels through a dietary intervention. In this study, we assessed the circulating DHA status of 1,188 elderly American subjects (mean age of 75 yr) using serum phosphatidylcholine (PC) as the biomarker. Frozen plasma samples obtained in 1985 were extracted with chloroform and methanol, and the phospholipids were separated by high-performance liquid chromatography. Purified PC fractions were collected, transesterified with methanolic base, and the fatty acid methyl esters were separated by capillary column gas chromatograpy.

Most of the serum PC fatty acids exhibited a normal frequency distribution, but there was a nonnormal distribution of the serum PC-associated DHA and eicosapentaenoic acid (EPA). This skewed distribution reflects the influence of preformed DHA and EPA in the diet, likely from fish consumption, in some members of the population. In fact, there was a significant positive correlation between serum PC-EPA and PC-DHA levels. Following the analysis of the blood samples, we undertook a blinded prospective analysis of the clinical outcomes [both AD diagnosis as well as scores on the Minimental State Exam (MMSE)] of the patients over the next 10 yr. Of those individuals who had clinically diagnosed AD at the time the blood sample was taken, there was a two-fold higher frequency (11 vs. 5) of AD in subjects from the lower half of the DHA distribution. Subjects whose serum PC-DHA was in the lower half of the distribution, but who had no AD at the time the blood sample was taken had a 67% greater ($P < 0.05$) likelihood of developing AD in the subsequent 10 yr of life. It is well known that individuals with at least one copy of the serum apolipoprotein E4 allele have a greater risk of AD, and women who had at least one apolipoprotein E4 allele had a fourfold greater risk of low scores on the MMSE in the subsequent 10 yr if they were also in the lower half of the DHA distribution. No similar relationships could be found between serum levels of other n-3 fatty acids, such as EPA or n-3 docosapentaenoic acid, and dementia.

These data suggest that low levels of circulating PC-DHA may be a significant risk factor for low scores on the MMSE, and in the development of AD in elderly patients. The inability to maintain a high level of DHA may be due to a reduced ability to synthesize DHA late in life as the result of a reduction in Δ6-desaturase activity. Moreover, the data suggest that maintaining adequate levels of serum DHA through the consumption of fish or other dietary supplements rich in DHA may be particularly important for the elderly. Intervention trials are now underway to determine the effect of dietary DHA in a triglyceride form from an algal source on the development of AD. This source of DHA was chosen because of its safety and efficacy profile, and because it contains no EPA, which may be contraindicated in otherwise healthy elderly patients.

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Abbreviations: AD, Alzheimer's dementia; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; MMSE, Minimental State Exam; PC, phosphatidylcholine.

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Ethyl Docosahexaenoic Acid Administration During Intrauterine Life Enhances Prostanoid Production and Reduces Free Radicals Generation in the Fetal Rat Brain

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Recently we demonstrated that a single intra-amniotic injection of ethyl (Et) docosahexaenoic acid (Et-DHA, 22:6n-3) reduced by 39% the ability of fetal brain slices to form Fe^{2+}-mediated lipid peroxides (LPO) measured by thiobarbituric acid reactants (TBAR), suggesting an antioxidant role for DHA (1). Under these conditions, arachidonic acid (AA) was preferentially lost from major phospholipids, suggesting a relatively greater loss of AA compared to DHA after stress (Fig. 1) in animals, 3 d following Et-DHA administration.

Since AA is the immediate precursor for most prostanoids, we examined the production of thromboxane B_2 (TxB_2) and prostaglandin E_2 (PGE_2) under these conditions. Slices taken from Et-DHA-treated and control animals were incubated in the presence of 0.1 mM Fe^{2+} and O_2. After 30 min at 37°C, medium was collected and the levels of TxB_2 and PGE_2 were determined by a radioimmunoassay technique. As shown in Figure 2A, DHA-treated slices produced more PGE_2 (182%) and TxB_2 (218%) compared to untreated ones. In contrast the levels of TBAR were lower by almost 50% compared to untreated brains. The data suggest that enhanced production of prostanoids may be an alternative pathway by which damaging hydroxyl radicals (OH˙) may be diverted to a lesser harmful metabolic route. Studies in progress are aimed to evaluate a possible activation of phospholipase A_2 and cyclooxygenase as part of this derouting process enhanced by Et-DHA administration.

An alternative mechanism by which DHA could exert its antioxidant capability against lipid peroxidation is by subserving as a direct or indirect free radical scavanger. To explore this possibility, brain homogenates and total brain lipid extracts obtained from Et-DHA-treated and control fetuses were incubated with 0.1 M DMPO (5,5-dimethyl-1-pyrroline-*N*-oxide) spin trap in the presence of 1 mM Fe^{2+} and 2 mM H_2O_2, and the changes in the electron paramagnetic resonance were examined. The appearance of DMPO-OH adducts in crude lipid extracts prepared from Et-DHA pretreated fetuses, was reduced by almost 40% in comparison to untreated fetuses (Fig 2B). No changes were found in the lipid-soluble low molecular weight antioxidant (i.e., α-tocopherol) as determined by cyclic voltammetry. Silicic acid chromatography

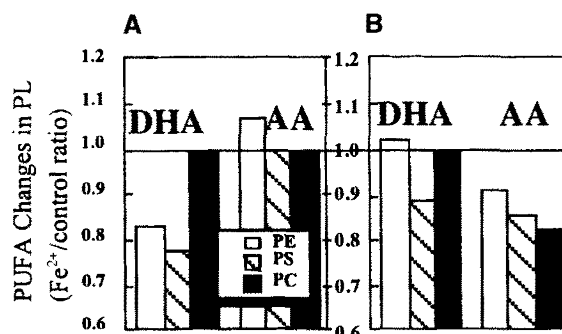

FIG. 1. Gas chromatography analysis of arachidonic acid (AA) (20:4) and docosahexaenoic acid (DHA) (22:6) content in phosphatidylethanolamine (PE), -choline (PC), and -serine (PS) after Fe^{2+}-mediated oxidative stress in fetal brain slices from (A) untreated and (B) ethyl (Et)-DHA-treated (3 d) animals. Values of each of the polyunsaturated fatty acids (PUFA) were normalized to non stress conditions. PL, phospholipids.

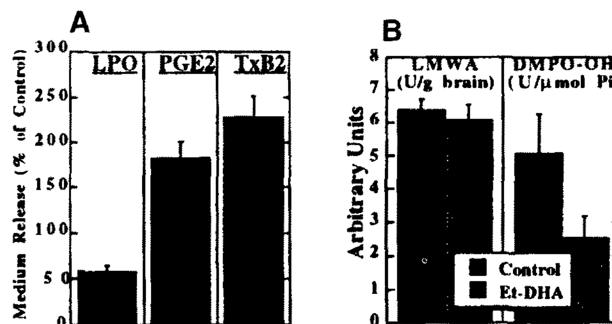

FIG. 2. Effect of Et-DHA administration on production of (A) prostaglandin E_2 (PGE_2), thromboxane B_2 (TxB_2), and lipid peroxides (LPO) and (B) free radicals (DMPO-OH) scavengers and low molecular weight antioxidant (LMWA, Fig. 2B) levels in fetal rat brain slices subjected to Fe^{2+} mediated oxidative stress. Values shown in Figure 2A are normalized to untreated brain slices and those in Figure 2B are expressed as relative units. See Figure 1 for other abbreviation.

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Abbreviations: AA, arachidonic acid; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; Et-DHA, ethyl docosahexaenoic acid; LPO, lipid peroxides; PGE_2, prostaglandin E_2; TBAR, thiobarbituric acid reactants; TXB_2, thromboxane B_2.

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of lipid extracts prepared from Et-DHA-treated fetal brains revealed a better capacity of an enriched phospholipid fraction to reduce, in a concentration-dependent manner, the production of DMPO-OH adducts. The antioxidant property acquired by a single intra-amniotic injection of Et-DHA may be a combined result of free radicals scavenging at the plasma membrane level and stimulation of AA release to produce prostanoids.

Acknowledgment. Supported by a grant from the Gulton Foundation, New York.

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The Role of Docosahexaenoic Acid (22:6n-3) in Neuronal Signaling

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While it is believed that docosahexaenoic acid (22:6n-3) is essential for proper neuronal function, the mechanism underlying its essentiality is not clearly understood. The involvement of free arachidonic acid (20:4n-6) or phosphatidylserine (PS) in signal transduction has been demonstrated (1,2). Therefore, we hypothesized that 22:6n-3 participates in neuronal signaling through the release from the brain cells in response to neurotransmitters and/or through the modulation of the PS accumulation. The release of 22:6n-3 was investigated in comparison to 20:4n-6, using two major types of brain cells, astroglia and neuronal cells. We observed that the rat brain synaptosomes and primary cultures of hippocampal neurons did not release 22:6n-3 under the conditions where 20:4n-6 release had been established (3). However, we found that astroglia released 22:6n-3 as favorably as 20:4n-6, under the basal condition and also in response to various neurotransmitters (3,4). These data suggested that the release of 22:6n-3 may not be the mechanism for intracellular signaling in neuronal cells. One of the major functions of astroglia is to supply trophic factors to neurons for their survival. The ready release of 22:6n-3 from astroglia suggests that one of the supporting roles of astroglia may be providing 22:6n-3 to neuronal cells, probably as a trophic factor. In order to test this possibility, 22:6n-3 was added to neuronal cell culture, and apoptotic cell death induced by serum deprivation was examined using DNA fragmentation as an indicator. Neuro-2A cells exposed to 22:6n-3 for 5 h did not have any effect on cell death induced by serum deprivation. After enrichment for 48 h, however, 22:6n-3 protected Neuro-2A cells from apoptosis (Fig. 1). After 5 h of incubation, 22:6n-3 was mainly incorporated in triglycerides and phosphatidylcholines, while after 48 h, this fatty acid was primarily incorporated in aminophospholipids, phosphatidylethanolamine, and PS, suggesting that 22:6n-3 as aminophospholipids in membranes may play an important role in the survival of neuronal cells. The 22:6n-3 fatty acid is particularly enriched in PS. And PS does participate in cell signaling such as protein kinase C (2) and Raf-1 translocation (5). Since we learned that 22:6n-3 as aminophospholipids in membranes plays an important role in the survival of neuronal cells, we subsequently hypothesized that 22:6n-3 plays a role in accumulating PS, and this may be an important mechanism supporting the essentiality of 22:6n-3. Using two approaches including an n-3 deficiency animal model and a 22:6n-3 supplemented cell system, we found that PS biosynthetic activity was inhibited during n-3 deficiency but significantly increased with 22:6n-3 supplementation (6). The accumulation of PS was also significantly reduced by n-3 deficiency in rat brain microsomes (6) and pineal (7). Based on these data, we concluded that PS accumulation as well as its biosynthesis in brain is sensitive to changes of the docosahexaenoate levels in phospholipids. We postulate that 22:6n-3 may be a modulator of PS biosynthesis, which in turn, can affect neuronal signaling.

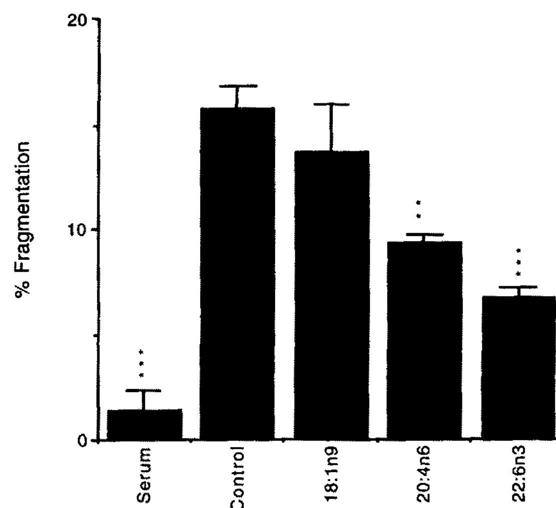

FIG. 1. DNA fragmentation affected by the enrichment of Neuro-2A cells with various fatty acids (25 μM in 0.1% fetal bovine serum) for 48 h prior to serum deprivation. *Indicates that values are significantly different from control (**, $P < 0.01$; ***, $P < 0.001$).

Lipids 34, S249–S250 (1999).

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Abbreviations: PS, phosphatidylserine.

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n-3 Polyunsaturated Fatty Acid Deficiency and Dopamine Metabolism in the Rat Frontal Cortex

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Brain lipids are very rich in polyunsaturated fatty acids (PUFA), in particular arachidonic and docosahexaenoic acids, derived, respectively, from linoleic and α-linolenic acid. As these fatty acids are totally provided by the diet, their effects on brain functions can be studied through dietary manipulations in animal models.

It is known that a chronic dietary deficiency in α-linolenic acid in rats induces several changes, including modifications in the fatty acid composition of brain membranes, visual perturbations, and behavioral disturbances consisting of abnormal responses in several learning tests. This last finding raises the question about the mechanisms which mediate the effects of n-3 PUFA deficiency on behavior. These behavioral changes can be related to the function of the mesocorticolimbic dopaminergic systems. We therefore proposed that chronic n-3 PUFA deficiency could act on the dopaminergic functions. In order to validate this hypothesis, we used a rat model of chronic α-linolenic acid deficiency. These animals received peanut oil as an exclusive source of lipids for three generations, whereas control animals received a mixture of peanut and rapeseed oil providing both linoleic and α-linolenic acids. The male rats of the third generation, receiving the same diet as their dams, were studied at 2–3 mon of age. On this animal model, we previously measured in the prefrontal cortex a considerable decrease in the amount of n-3 PUFA, compensated for by an increase in n-6 PUFA. In addition, we found specifically in this region that the endogenous levels of dopamine were 35–40% lower in deficient rats compared to controls (1,2).

We therefore used the method of cerebral microdialysis in order to define the neurochemical mechanisms involved (3). Microdialysis allows a dynamic approach of neurotransmission processes *via* implantation of a probe in the frontal cortex on alive rats. Basal levels of dopamine released into cortical dialysates of awake rats were similar in both groups. By contrast, we measured significantly higher metabolite basal levels [dihydroxyphenyl acetic acid (DOPAC) and homovanillic acid (HVA)] in deficient rats compared to controls: DOPAC was 74 ± 37 fmol/μL of dialysate samples vs. 17 ± 10 fmol/μL, and HVA was 64 ± 13 fmol/μL vs. 26 ± 11 fmol/μL in deficient and control rats, respectively (n = 8:8). On anesthetized rats, KCl perfusion (200 mM), known to induce release of newly synthesized dopamine localized in the cytoplasmic compartment, led to a similar increase in dialysate dopamine content in deficient and control rats (n = 8:8). By contrast, tyramine perfusion (200 μM), known to release dopamine from the vesicular stores, resulted in an increase in dialysate dopamine levels three times lower in deficient rats compared to controls (n = 8:8).

These findings confirm the hypothesis of an involvement of the dopamine systems in the effects of n-3 PUFA deficiency on behavior. Our results suggest that the cortical dopamine metabolic pathway could be increased in deficient rats. The dopaminergic storage compartment could be decreased in deficient rats and then, most of the newly synthesized dopamine is not protected in vesicles from degradation by monoamine oxidase, this is in agreement with the increased metabolite production. The modification of storage compartment could be caused by lower dopamine internalization or by modifications of the structure or the number of vesicles. This inadequate storage of newly synthesized dopamine might not be enough for the maintenance of high release during stimulated cognitive processes and so could explain disturbances in cognitive performance previously observed.

In order to support our hypothesis, the study of cortical monoamine vesicles, in particular their number and structure, is now in progress. The modulatory effects of the cortical dopaminergic system on the nucleus accumbens, involved in several components of learning tasks, are also investigated.

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Abbreviations: DOPAC, dihydroxyphenyl acetic acid; HVA, homovanillic acid; PUFA, polyunsaturated fatty acid.

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Amount and Type of Unsaturated Aldehydes in Chicken Plasma and Tissues Depend More on Dietary Lipids Than on Vitamin E Status

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Free radicals are linked to a variety of neurological disorders in humans and animals. In the chicken, the feeding of n-6 fatty acids together with a vitamin E-deficient diet (1) leads to nutritional encephalomalacia (NE). Only the cerebellum is affected, leading to ataxia, prostration, and death. The subject of our research was the possible involvement of unsaturated aldehydes in the pathogenesis of experimental NE in broiler chicken. This disease serves as a model for the consequences of dietary fatty acid type on lipid peroxidation events.

Laying hens received a diet low in vitamin E. Resulting chicks were assigned to four groups fed with either linoleic (C18:2n-6, 55%) or linolenic acid (C18:3n-3, 54%) together with 1 or 50 ppm vitamin E; 9 d post-hatching NE occurred in the vitamin E-deficient group fed linoleic acid. With each chick showing NE, a healthy one from all four groups was sacrificed. Malondialdehyde (MDA), pentenal, hexenal, OH-hexenal, heptenal, octenal, and OH-nonenal were determined in plasma, liver, cerebrum, and cerebellum after derivatization with methylhydrazine (2). The aldehyde hydrazones were separated by gas–liquid chromatography (GLC) and detected with a thermoionic-specific detector (3).

Up to day 12, mortality was 50%. Nine animals showed the typical clinical signs of NE. In the brain tissues, differences in aldehyde pattern were small. An effect of the vitamin E supply was not observed. Furthermore, cerebellar damage due to NE was not accompanied by increased levels of unsaturated aldehydes.

In plasma, OH-hexenal was the most prominent aldehyde, followed by MDA and OH-nonenal in the case of linoleic acid feeding. For MDA (analysis of variance: $P = 0.0002$) and OH-nonenal (analysis of variance: $P = 0.0280$), we observed a significant increase in vitamin E deficiency. Diseased animals had high values of total aldehydes mainly due to OH-hexenal. Means were significantly different between diseased animals and those fed linoleic acid with 5 ppm vitamin E or linolenic acid.

In the liver, the concentration of total aldehydes was higher than in brain tissues. The portion of aldehydes from n-3 fatty acids was considerably lower than in the brain. MDA and OH-nonenal were the dominating aldehydes. Linoleic acid feeding resulted in a significantly higher content of total aldehydes in this tissue. Vitamin E deficiency increased the levels of pentenal ($P = 0.0379$) and hexenal ($P = 0.030$). Diseased animals in addition had increased aldehydes from n-3 fatty acids (total n-3, pentenal, hexenal, $P < 0.01$) in comparison to healthy ones from the same group.

Although NE dramatically affects the cerebellum macroscopically and microscopically, tissue aldehydes were not elevated in diseased animals. This observation is not expected in the light of earlier results on the influence of vitamin E on lipid peroxidation (4). However, in rats comparable results on the oxidative resistance of brain membranes were reported (5).

NE only occurs in animals when n-6 fatty acids like linoleic acid are fed. Evidence exists that n-6 fatty acids have a genuine prooxidative-toxic effect. Linoleic acid intake seems to be associated with increased oxidation susceptibility (6). Moreover the higher oxidizability of n-6 in comparison to n-3 fatty acids *in vitro* was reported recently (7). According to our study, the link between linoleic acid feeding and occurrence of NE is the increased concentration of lipid peroxidation products in liver and plasma when linoleic acid was fed. In plasma, we found the expected increase of aldehydes in response to vitamin E deficiency, namely OH-nonenal and MDA. Metabolism of aldehydes is supposed to be negligible in plasma, so these aldehydes may be lipid peroxidation products delivered into the plasma from tissues other than the brain.

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Abbreviations: MDA, malondialdehyde; NE, nutritional encephalomalacia.

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Interactions Between Lipid Metabolism and Schizophrenia: The Biochemical Changes Which May Have Made Us Human

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There are many aspects of human evolution which require explanation in biochemical terms. Some of the most important differences between humans and great apes are as follows: (i) the increase in brain size which began around 2.5 million yr ago and reached modern values between 0.5 and 1.0 million yr ago; (ii) the presence of subcutaneous fat, buttocks, and breasts; (iii) the surprising absence of obvious major cultural changes accompanying the increase in brain size; and (iv) the cultural explosion which began somewhere between 50,000 and 100,000 yr ago with the emergence of art, music, religion, and warfare.

The brain, like subcutaneous fat, is particularly rich in lipids, and there may be changes in lipid metabolism which differentiate humans from the great apes. The growth in the size of the brain and the development of subcutaneous fat and associated breasts and buttocks may have occurred because of changes in proteins which regulate the rate of delivery of fatty acids to tissues. Two candidate classes are lipoprotein lipases and the fatty acid-binding and transport proteins. Changes in the expression of brain fatty acid-binding proteins are known to be associated with the seasonal changes in size of part of the brain in songbirds.

Creativity is not simply related to brain size. Autistic people and Neanderthals both exhibit brain sizes greater than those of normal modern humans. Creativity is more likely to be related to the richness of connectivity between neurons. Neuronal microconnectivity is largely regulated by phospholipid synthesizing, remodeling, and degrading enzymes which are highly expressed whenever synaptic remodeling is taking place. These enzymes are therefore candidates for an association with creativity.

There is increased evidence that schizophrenia is associated with abnormalities of phospholipid breakdown and synthesis. These changes will lead to changed synaptic connectivity. Schizophrenia entered humanity prior to the separation of the races because it is found with approximately the same prevalence in all human races. Family and adoption studies show that relatives of schizophrenics have a high risk of developing schizophrenia and other psychiatric disorders. But the same studies also show that there is also an increased probability of high creativity, leadership qualities, achievements in many fields, high musical skills, and an intense interest in religion. These are precisely the qualities that entered the human race around 100,000 yr ago and ended tens of thousands of generations of cultural near-stagnation. The changes in phospholipid metabolism which are associated with schizophrenia may also be responsible for those features characteristic of our humanity.

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n-3 Fatty Acids and Human Lipoprotein Metabolism: An Update

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OVERVIEW OF LIPID-ALTERING EFFECTS OF n-3 FATTY ACIDS

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. To gain a perspective on the overall effects of n-3 FA on serum lipids, a meta-analysis was conducted based on 72 placebo-controlled human trials, at least 2 wk in length and providing 7 g or less of n-3 FA/d (1,2). Trials with normolipidemic subjects (triglycerides < 2.0 mM) were compared to those studying hypertriglyceridemic patients (triglycerides ≥ 2.0 mM). In the normal subjects, mean triglyceride levels decreased by 25% ($P < 0.0001$), and total cholesterol (C) levels increased by 2% ($P < 0.009$) owing 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 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. There was a clear dose–response relationship when all studies were examined together, and the triglyceride-lowering effects of n-3 FA persisted even out to more than 2 yr in placebo-controlled trials. Thus, it is well-established that n-3 FA are hypotriglyceridemic when taken in doses of 3–4 g/d.

LOW DOSE STUDIES

In stark contrast to our earliest studies with n-3 FA in which up to 25 g of eicosapentaenoic acid (EPA) + docosahexaenoic acid (DHA) were fed daily (3), we recently completed a trial comparing 1.7 vs. 3.4 g (provided as 2 vs. 4 g) of Omacor (Pronova Biocare, Oslo, Norway) in patients with severe hypertriglyceridemia (triglyceride levels between 5.65 and 22.6 mM (500 and 2,000 mg/dL). Following the completion of a 4-mon, placebo-controlled, randomized, double-blind trial of 4 g of Omacor (4), 28 patients meeting the inclusion criteria were randomized to take either 2 or 4 capsules of Omacor per day for an additional 6 mon. Compared to original baseline values, Omacor 4 g significantly reduced mean triglyceride concentrations by 45% ($P < 0.0001$), C by 15% ($P < 0.001$), very low density lipoprotein-C by 32% ($P < 0.0001$), and C/HDL-C ratio by 20% ($P = 0.0013$); and it raised HDL-C by 13% ($P = 0.014$) and LDL-C by 31% ($P = 0.0014$). The lower dose (2 g) lowered mean triglyceride levels by 25% ($P = 0.05$) and raised HDL-C by 14% ($P = 0.015$) but had no significant impact on other lipid parameters. Thus, Omacor demonstrated a dose-dependent triglyceride-lowering effect, with only two capsules per day producing a clinically meaningful, statistically significant reduction.

ANIMAL STUDIES

As discussed above, in humans, n-3 FA exert a significant hypotriglyceridemic effect that is dose-dependent and persistent. Effects on other lipoprotein fractions are relatively minimal. No animal model has yet been developed which mimics these effects (5). In several species triglyceride levels do not fall; in others, reduced atherosclerotic disease is found with n-3 FA feeding despite "adverse" changes in HDL-C and LDL-C levels (6,7). Accordingly, extreme care must be taken with these models so as to avoid inappropriate extrapolations to humans.

CLINICAL INTERVENTION STUDIES WITH n-3 FA AND CORONARY HEART DISEASE

There have been only two prospective, randomized clinical trials with n-3 FA having "hard" end points, i.e., cardiac events, and mortality: the Diet and Reinfarction Trial (8) and the Indian Experiment of Infarct Survival (9). The former study randomized 2,033 male survivors of a myocardial infarction to receive (or not receive) advice to increase their intake of oily fish by about 200–400 g/wk. This raised their EPA + DHA intakes to approximately 500–700 mg/d. Those patients randomized to the fish advice group experienced 29% fewer deaths in the ensuing 2 yr. This was the first trial to show that increasing fish oil intake could actually protect against heart attacks.

The Indian Experiment (9), although smaller in size, was designed to address more directly the question of whether marine- and/or plant-derived n-3 FA could reduce cardiac events in a secondary prevention population. Approximately 360 patients presenting to the emergency room with a suspected my-

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Abbreviations: C, cholesterol; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; HDL, high density lipoprotein; LDL, low den-

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ocardial infarction were randomized to either 2 g of EPA + DHA (6 capsules of Maxepa, Seven Seas, Ltd., Haeld, England), 2.9 g of α-linolenic acid (20 g of mustard seed oil), or placebo (capsules containing 100 mg of the inert compound aluminum hydroxide). Patients were started on one of these three treatments within 18 h of admission, and were followed for the next 12 mon for clinical events. The authors reported that total cardiac mortality was significantly decreased in both n-3 FA groups, with marked reductions in new acute myocardial infarctions, the development of new angina pectoris, arrhythmias, and heart failure. These finding provide the most compelling evidence to date that n-3 FA can have clinically important impact in preventing coronary artery disease. Additional trials are clearly called for to confirm these findings.

In summary, there is extensive and consistent documentation of the ability of marine n-3 FA to reduce serum triglyceride levels in humans by 20–30% at doses of 3–4 g per day. Smaller doses over longer periods of time may also prove clinically useful. The extent to which alterations in serum lipids and lipoproteins play a role in the new-well-documented antiatherogenic effects of n-3 FA remains to be seen.

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Long-Chain n-3 Polyunsaturated Fatty Acids and Triacylglycerol Metabolism in the Postprandial State

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ABSTRACT: Elevated plasma triacylglycerol (TG; triglyceride) concentrations, especially in the postprandial state, have been associated with an increased risk of coronary heart disease (CHD). Postprandial lipemia represents a complex series of reactions which occur following the ingestion of a meal containing fat and is associated with a number of adverse metabolic events including the production of atherogenic chylomicron remnants, the formation of the highly atherogenic small dense low density lipoprotein particles, a reduction in the concentration of the cardioprotective high density lipoprotein fraction and the activation of coagulation factor VII. Fish oils are a rich source of the long-chain n-3 polyunsaturated fatty acids (PUFA), eicosapentaenoic acid and docosahexaenoic acid. Long chain n-3 PUFA are effective hypotriglyceridemic agents, lowering both fasting and postprandial TG concentrations. There is a large body of evidence which shows that n-3 PUFA reduces plasma TG concentrations through reduced endogenous very low density lipoprotein production. This in turn may account for the reduced postprandial lipemic response following n-3 PUFA supplementation. However, this does not preclude a contribution of enhanced chylomicron clearance, which may be mediated through altered chylomicron size, structure or chemical composition, or altered lipoprotein lipase metabolism in terms of enzyme concentration, activity, or affinity for chylomicrons. However the precise biochemical nature of this effect remains to be established. The reduction of postprandial plasma TG concentrations by n-3 PUFA may partly explain why n-3 PUFA intake is inversely related to CHD mortality.

POSTPRANDIAL LIPID METABOLISM

Postprandial triacylglycerol (TG) metabolism refers to the series of metabolic events which occur following the ingestion, digestion, and absorption of a meal containing fat (1,2). Dietary fat is principally composed of TG, which, after digestion and absorption, stimulates the production of chylomicrons within the enterocyte (3–5). Chylomicrons are TG-rich lipoproteins (TRL) which transport exogenous dietary TG within the circulation, causing an increase in plasma TG concentrations during the postprandial response. Chylomicrons are identified by virtue of their density as well as their unique apoprotein, apo B48. Figure 1 illustrates a typical postprandial TG response to 40 g of fat (6), showing a significant transient increase in plasma TG concentrations and plasma apo B48 concentrations.

The removal of chylomicron TG is catalyzed by the enzyme lipoprotein lipase (LPL) which is present on the surface of capillary endothelial cells of adipose tissue (7). LPL is also present in other tissues with a high fatty acid requirement, including skeletal muscle, cardiac muscle, and the mammary gland (8). In the postprandial state, adipose tissue LPL is exclusively activated by insulin, which inhibits LPL activity in the other tissues. LPL is the rate-limiting hydrolytic enzyme which controls TRL removal from the circulation, and therefore LPL has a regulatory effect on the extent and duration of postprandial lipemia. During postprandial lipemia there is a significant increase in very low density lipoprotein (VLDL) TG. This occurs later in the postprandial phase (4–6 h) (9). Chylomicrons are preferentially hydrolyzed by LPL which leads to delayed VLDL removal and postprandial VLDL accumulation (10–12). The hydrolysis of chylomicron TG by LPL generates monoacylglycerol and nonesterified fatty acids (NEFA). These hydrolytic products are rapidly transported across the endothelium to parenchymal cells, where they are reesterified for storage in adipose tissue. An appreciable quantity (up to 50%) of NEFA escapes into the circulation (13), and plasma NEFA concentrations return to preprandial levels. The release of NEFA into the circulation during chylomicron lipolysis stimulates hepatic TG synthesis, thereby promoting VLDL synthesis and secretion, which in turn leads to an increase in VLDL concentrations during the postprandial response (9). The resultant delipidated chylomicron, now termed a "chylomicron remnant," is catabolized by the liver. The postprandial lipemic response lasts approximately 4 to 8

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Abbreviations: apo, apoprotein; ALP, atherogenic lipoprotein phenotype; CE, cholesteryl ester; CHD, coronary heart disease; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; LPL, lipoprotein lipase; NEFA, nonesterified fatty acids; PUFA, polyunsaturated fatty acids; TG, triacylglycerol; TRL, triacylglycerol-rich lipoprotein; VLDL, very low density lipoprotein

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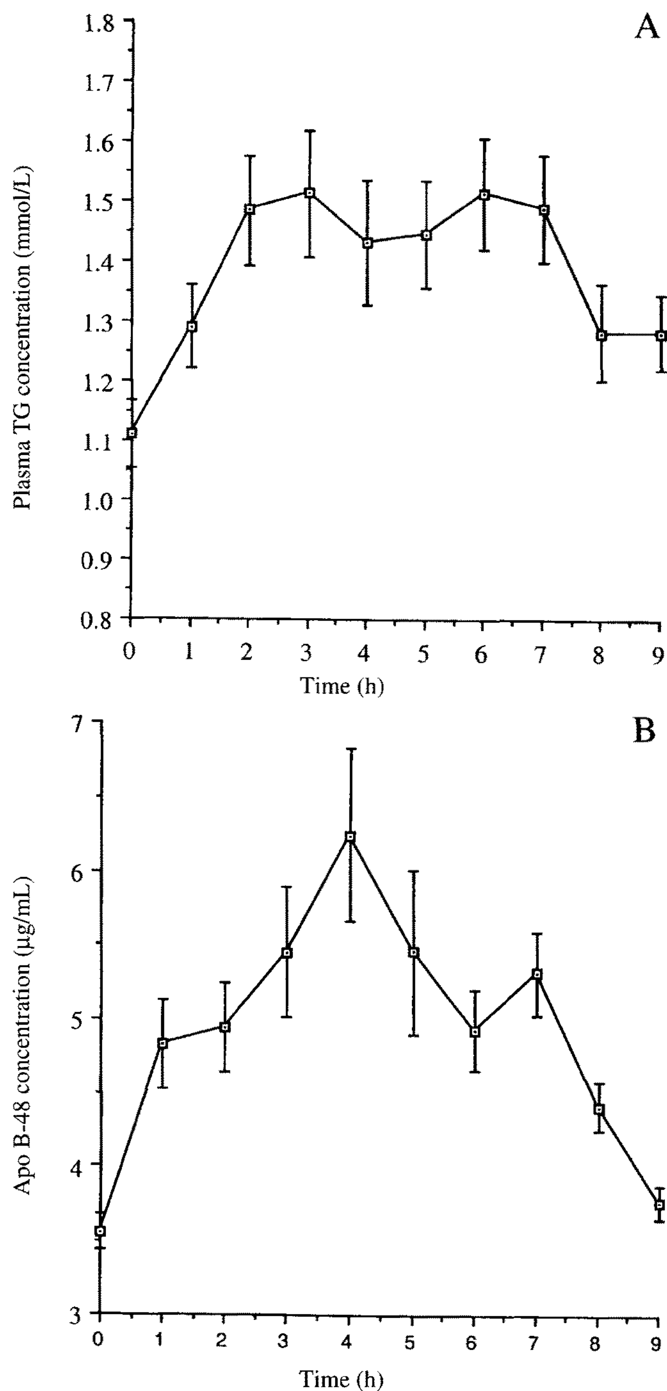

FIG. 1. Postprandial triacylglycerols (TG) and apoprotein (apo) B48 concentrations following the ingestion of a 40-g fat meal.

h, the magnitude and duration of which is determined by a number of physiological factors, such as age, gender, body weight and nutritional factors, which have been extensively reviewed elsewhere (1).

PLASMA TG AS A RISK FACTOR FOR CHD

Traditionally, fasting plasma TG concentrations have not been recognized as an independent risk factor for coronary heart disease (CHD) (14), but recent data suggests that the relative importance of TG as a risk factor may have been underestimated (15). The importance of TG as a risk factor for CHD has been underestimated because of the high degree of variability in plasma TG concentrations, the strong negative association which exists between plasma TG and high density lipoprotein (HDL) cholesterol concentrations, or the influence of concomitant conditions such as diabetes. Several authors have proposed that the negative correlation between plasma TG and HDL cholesterol, which reflects the physiological relationship between the lipoproteins, may explain why TG does not emerge as an independent risk factor in epidemiological studies and why low HDL cholesterol concentrations may only be a marker of plasma TG metabolism and not an independent risk factor of CHD (16,17). This hypothesis is supported by results of the Lipid Research Clinics 12-yr follow-up study (18), the Framingham Study (19) and the Caerphilly and Speedwell Collaborative Heart Disease Study (20). The results of these studies showed that high plasma TG concentrations were risk factor for CHD when HDL cholesterol concentrations were also low.

Plasma TG concentrations are highly variable, both within and between individuals. This high degree of variability leads to a low statistical power to detect significant associations in multivariate analysis. Recently, a large meta-analysis of 17 population-based prospective studies showed that increased plasma TG concentrations were associated with a significant increase in risk of CHD (21). The analysis of this large cohort (n = 57,277) showed that plasma TG concentrations were an independent risk factor for CHD, particularly in women, whereby 1 mmol/L increase in plasma TG concentration was associated with an increased cardiovascular risk of 32% in men and 76% in women (21).

A growing body of evidence shows that nonfasting or postprandial plasma TG concentrations play an important role in relation to CHD. The Physicians Heart Health Study provided prospective evidence that nonfasting serum TG concentration was a significant predictor of future myocardial infarction (22). Numerous clinical studies showed that the magnitude of postprandial lipemic response is related to the presence and progression of CHD. Patients with CHD demonstrate an elevated and delayed postprandial lipemic response compared to controls (23,24). Furthermore, Karpe *et al.* (25) showed that the concentration of postprandial chylomicron remnant apo B48 was directly related to the rate of progression of coronary lesions in postinfarction male patients.

POSTPRANDIAL TG METABOLISM AND CHD

The precise mechanism whereby postprandial TG concentrations affect the pathogenesis and progression of CHD has not been elucidated, but at least four possible mechanisms were identified. Firstly, while TG itself is not a component part of the atherosclerotic lesion, elevated postprandial TG concentrations may exert their adverse effect by promoting the production of

atherogenic chylomicron remnants (26). The "Zilversmit Hypothesis" was the first to propose that cholesteryl ester-rich chylomicron remnants were as atherogenic as low density lipoprotein (LDL) (27). An elevated postprandial response promotes the formation of small cholesteryl ester (CE)-enriched chylomicron remnants. These remnants share with LDL the ability to mediate cholesterol influx into the arterial wall intima in humans (26), thereby promoting atherogenesis.

Importantly, the postprandial lipemic response not only represents the flux of dietary TG within the circulation but it also represents a very significant period during which the composition of the cholesterol-rich lipoproteins, LDL and HDL, is changed. The extent of lipoprotein remodeling which occurs during postprandial lipemia is positively related to the magnitude and duration of the postprandial triacylglycerolemia (28). These compositional changes in turn affect the metabolic fate of HDL and LDL. Plasma TG has a major metabolic influence in the physicochemical properties of LDL; elevated postprandial lipemia stimulates the formation of small dense LDL (29). This highly atherogenic LDL subfraction has been associated with an increased risk (four- to sixfold) of CHD (30). The formation of small dense LDL represents the second mechanism underlying pathogenesis postprandial lipid metabolism.

Thirdly, elevated postprandial TG concentrations reduce the concentration of the cardioprotective HDL fraction (31). During postprandial lipemia, HDL become enriched with TG at the expense of CE, an exchange mediated by the enzyme cholesterol ester transfer protein (CETP). Pronounced postprandial triacylglycerolemia allows greater interaction and excessive lipid exchange between chylomicrons and HDL_2 (32,33). Hepatic lipase delipidates these TG-rich HDL_2 particles, converting them to HDL_3 particles, thus lowering the concentration of the cardioprotective and metabolically active HDL_2 subfraction. Finally, elevated postprandial plasma TG concentrations adversely affect the coagulation system. Postprandial TRL have the ability to activate coagulation factor VII (34). Therefore, elevated postprandial lipemia represents a proatherogenic and prothrombotic metabolic state.

Syndrome X and the atherogenic lipoprotein phenotype (ALP) are two very common metabolic syndromes of altered plasma TG metabolism which are associated with increased risk of CHD (35,36). Both refer to a cluster of metabolic abnormalities which are associated with an increased risk of CHD, including raised plasma TG concentration, low HDL cholesterol concentration, and an increased proportion of small dense LDL, raised plasma glucose concentration, and insulin resistance. Therefore, therapies which improve plasma TG metabolism could reduce the incidence of these common dyslipidemias which are associated with increased risk of CHD.

Fish oils, TG, and CHD. Eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) are the principal long chain n-3 polyunsaturated fatty acids (PUFA) derived from fish oils. Epidemiological studies have demonstrated that the incidence of CHD is inversely associated with consumption of n-3 PUFA (37,38). The DART trial provided prospective evidence that relatively low doses of n-3 PUFA reduce the risk of secondary coronary events (39). A number of biochemical mechanisms explain the cardioprotective effect of n-3 PUFA, and these include antithrombotic, antiarrhythmic, and antiinflammatory mechanisms, as well as improved plasma lipid and lipoprotein concentrations (40–44). The remainder of this review will focus on the effect of n-3 PUFA on plasma TG metabolism in the fasted and postprandial state.

In humans, n-3 PUFA exert a consistent hypotriglyceridemic effect, which is dose-dependent and persistent. In a recent review of 72 placebo-controlled human studies which supplemented a range of 1.0 to 7.0 g EPA and DHA daily for at least 2 wk, plasma TG concentrations were consistently reduced by 25–30 % (45). Several studies showed that n-3 PUFA have a dose-dependent hypotriglyceridemic effect on fasting plasma TG concentrations (46–48). When the data from these studies are pooled (Fig. 2), it emerges that the change (percentage baseline concentration) in fasting plasma TG concentration (ΔT) is related to the dose of n-3 PUFA intake (g n-3 PUFA) according to the equation $\Delta T = -7.67 - 3.05(\text{g n-3 PUFA})$ ($R^2 = 0.874$). Notably, this equation relates to doses of n-3 PUFA (1–9 g/d), which were supplemented for a relatively short period of time (4–12 wk).

The duration of supplementation is also an important factor which determines the hypotriglyceridemic efficacy of n-3 PUFA. Lower doses of n-3 PUFA supplemented for a longer time have an equivalent hypotriacylglycerolemia effect as short-term, high-dose n-3 PUFA supplementation trials. Investigations by this research group demonstrated that a low dose of fish oil (1 g/d n-3 PUFA) supplemented for 16 wk significantly reduced fasting plasma TG concentration by 21.2%, a level much greater

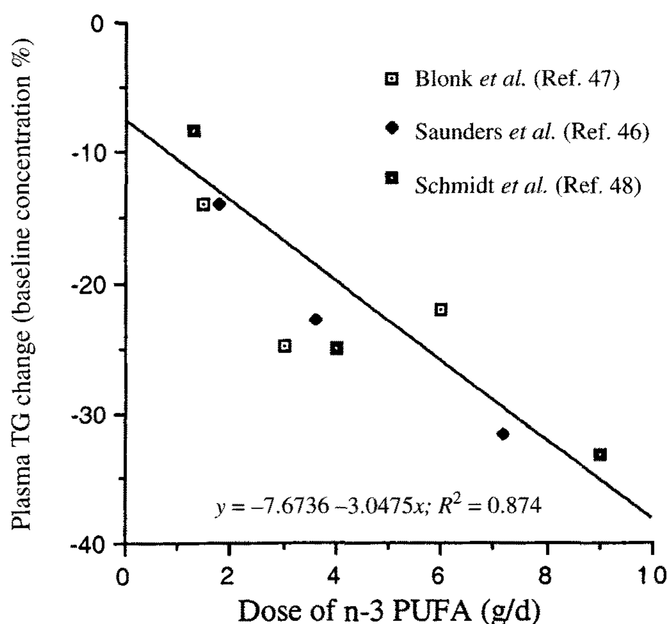

FIG. 2. The dose-dependent hypotriacylglycerolemic effect of n-3 polyunsaturated fatty acid (PUFA) supplementation. See Figure 1 for other abbreviations.

than that predicted from the above equation (–10.7%) (49). The importance of the duration of the supplementation period is supported by the results of other long-term studies. Schmidt *et al.* (50) demonstrated that daily supplementation with 4 g of n-3 PUFA was associated with a time-related reduction of plasma TG concentration during a 9-mon supplementation period (50). Saynor and Gillott (51) showed continued reduction of plasma TG levels following supplementation with 3 g of n-3 PUFA per day for 4 yr of the 7-yr intervention trial. Therefore it is reasonable to conclude that long-term n-3 PUFA supplementation is an effective hypotriglyceridemic agent.

The postprandial TG response is also significantly reduced by n-3 PUFA supplementation. A number of studies have investigated the effects of a wide range of n-3 PUFA intakes on postprandial lipemia. A summary of the results of these studies is presented in Table 1. Briefly, Harris *et al.* (52) and Weintraub *et al.* (53) demonstrated that a background diet rich in n-3 PUFA significantly reduced the postprandial response to a saturated fat test meal, compared to the postprandial response following a saturated fat diet. Williams *et al.* (54) and Agren *et al.* (55) showed that n-3 PUFA supplementation, using lower doses of n-3 PUFA for a longer time, achieved an equivalent hypotriacylglycerolemic effect. Noticeably, the long-term studies of 15 and 16 wk demonstrated the most effective reduction of postprandial TG concentrations per unit of n-3 PUFA supplemented (49,55). Therefore, the maximal hypotriglyceridemic effect on postprandial TG metabolism may be also achieved through long-term supplementation with low doses of n-3 PUFA.

Interestingly, in the same study Agren *et al.* (55) demonstrated that plasma TG concentrations were significantly reduced in another study group who received DHA-rich oil, which was free of EPA (1.68 g DHA, daily), although to a lesser extent than that shown by the group who received fish oil (1.33 g EPA and 1.95 g DHA, daily). This lesser suppression may be due to the lower dose of n-3 PUFA or a difference in the hypotriacylglycerolemic effect of EPA vs. DHA. Childs *et al.* compared EPA-rich pollock oil with DHA-rich tuna and salmon oil blends (56) and it was demonstrated that both fatty acids had equivalent hypotriacylglycerolemic effects. Conversely, another study demonstrated that DHA had no hypotriglyceridemic effect compared to EPA, when 3 g/d of either fatty acid was supplemented in humans for 3 wk. Studies in rats have shown that EPA but not DHA inhibited VLDL secretion, and this was attributed to increased mitochondrial β-oxidation of fatty acids which would reduce the supply of fatty acids for VLDL TG synthesis (58). Clearly the relative effects of both fatty acids on plasma TG metabolism need to be resolved.

The exact biochemical basis of the hypotriglyceridemic effect of n-3 PUFA on postprandial TG metabolism has not been elucidated, but it must be due to either reduced TRL synthesis, increased TRL removal, or a combination of both. Reduced chylomicron synthesis is unlikely because there is no evidence that fish oil supplementation reduces fat absorption (59). Furthermore, studies in rats demonstrated that n-3 PUFA supplementation had no effect on postprandial chylomicron synthesis and secretion (60). Therefore, it is unlikely that reduced exogenous TRL concentrations can explain the attenuated postprandial lipemic response which occurs following n-3 PUFA supplementation. However, diminished endogenous TRL, or VLDL, production does represent a possible mechanism. Animal studies showed that n-3 PUFA inhibit TG synthesis, through the inhibition of 1,2-diacylglycerol transferase (61, 62). *In vitro* studies similarly showed that the incubation of Hep G2 cells with EPA reduced VLDL output (63). Furthermore, kinetic studies in humans fed a background diet rich in n-3 PUFA, in which the protein moiety of VLDL was radio-labeled, demonstrated that n-3 PUFA inhibited endogenous VLDL synthesis and secretion (64). Chylomicrons and VLDL compete for LPL-mediated removal from the circulation (10,65). Therefore, when VLDL synthesis is diminished, chylomicron particles would have a greater opportunity to interact with LPL, thereby allowing more efficient clearance of postprandial TRL.

Enhanced TRL clearance represents another way in which n-3 PUFA exert their hypotriglycerodemic effect on postprandial TG metabolism. However, the evidence in relation to this aspect of n-3 PUFA and TG metabolism is less well understood. As described previously, LPL is the rate-limiting hydrolytic enzyme which controls TRL removal from the circulation; therefore any changes in LPL activity will affect the magnitude and duration of postprandial lipemia (7). However there is a relative paucity of information, and there are conflicting data in relation to n-3 PUFA and LPL activity. Two of the studies cited in Table 1 which investigated the effect of n-3 PUFA on postprandial TG metabolism showed that post-heparin LPL activity was not significantly affected by the presence of n-3 PUFA in the background diet (52,53). However, another study has since demonstrated that fish oil feeding

TABLE 1
Comparison of Postprandial TG n-3 PUFA Intervention Studies[a]

Study	Dose n-3 PUFA (g/d)	Duration (wk)	Fasting TG concentration (mmol/L)			TG AUC % change
			Initial	Final	% change	
Harris *et al.* (Ref. 52)	28.0	3	0.91	0.51	–43.7	–44.1
Weintraub *et al.* (Ref. 53)	7.0	3.5	1.09	0.63	–42.7	–54.0
Williams *et al.* (Ref. 54)	2.7	6	1.05	0.79	–24.8	–43.5
Agren *et al.* (Ref. 55)	2.3	15	1.21	0.89	–26.5	–31.6
Roche and Gibney (Ref. 49)	1.0	16	0.82	0.65	–21.2	–31.8

[a]TG AUC, area under the curve of the postprandial response; PUFA, polyunsaturated fatty acids; TG, triglycerides.

caused a small but significant increase in post-heparin LPL activity (66). It is important to realize that the post-heparin LPL assay measures the amount of LPL that is released from the tissue, rather than the actual physiological level of LPL activity *in vivo*. Therefore, it is unknown whether the analysis of post-heparin LPL is sensitive enough to detect any real changes in LPL activity in those studies. Recently, it was demonstrated that endogenous nonheparin-stimulated LPL activity was significantly increased following n-3 PUFA supplementation (67). Again it is unknown whether endogenous LPL activity reflects LPL activity *in vivo*. Nevertheless, the lack of a specific information in relation to LPL activity does not rule out the possibility that n-3 PUFA increase chylomicron clearance.

A number of issues in relation to n-3 PUFA and chylomicron metabolism remain unresolved. Firstly, chronic n-3 PUFA may lead to the production of chylomicrons which have a different size, structure or chemical composition, which in turn may affect chylomicron lipolysis and/or remnant disposal. Secondly, n-3 PUFA supplementation causes marked changes in membrane phospholipid fatty acid composition, which in turn could affect the affinity of membrane-bound LPL for chylomicrons. Thirdly, n-3 PUFA may increase postprandial blood flow through adipose tissue, thereby affecting the interaction between postprandial chylomicron metabolism and LPL activity. In relation to the first possibility, this hypothesis is supported by *in vitro* investigations which showed that chylomicron TG, produced following a n-3 PUFA meal, are hydrolyzed at a greater rate by LPL, than saturated fatty acid-rich chylomicron TG. Furthermore, the fatty acid composition of the acute test meal affects the composition and clearance of postprandial TRL, the degree of unsaturation of fat in the test meal was inversely related to the magnitude of the postprandial response (59). In that study, post-heparin LPL activity was significantly greater following the n-3 PUFA test meal compared to the saturated fatty acid-rich meal. Therefore, it was proposed that chylomicron TG composed of highly unsaturated fatty acids were more readily hydrolyzed by LPL. Since acute fat composition affects chylomicron removal, it is plausible that chronic or background dietary fat composition also affects the composition and catabolism of chylomicrons; however, this needs to be investigated. Furthermore, chronic n-3 PUFA consumption may affect the apoprotein composition of this chylomicron, which in turn would influence the chylomicron metabolism. Other factors which affect chylomicron lipolysis such as the geometry of the lipolytic site need to be resolved. Dietary n-3 PUFA may affect the size of the chylomicon, and TRL size affects LPL activity, whereby LPL preferentially hydrolyzes larger more TAG-rich lipoproteins (7). Clearly there is a requirement to investigate the precise effects of chronic n-3 PUFA supplementation on chylomicron metabolism.

There is a relative paucity of information in relation to the effect of n-3 PUFA on the amount, affinity, and accessibility of membrane-bound LPL for chylomicrons. Rat studies showed that fish oil leads to significantly higher expression of adipose tissue LPL mRNA (68) which suggests that n-3 PUFA supplementation increases the amount of LPL available to mediated TRL clearance. The physiological relevance of this effect and the extent to which this applies to TG in humans needs to be resolved. The n-3 PUFA chylomicrons lead to very extensive changes in membrane phospholipid composition, which in turn may affect LPL metabolism, but this effect was not investigated.

In summary, a large body of evidence shows that n-3 PUFA reduces plasma TG concentrations through reduced endogenous VLDL production. This in turn probably accounts for reduced postprandial lipemic response following n-3 PUFA supplementation; however, this does not preclude a contribution of enhanced chylomicron clearance as well; however, the precise biochemical nature of this effect remains to be solved.

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Triglyceride as a Risk Factor, Epidemiology

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In recent years, triglyceride (TG) has emerged as an important risk factor for coronary artery disease (CAD). While earlier studies reported a positive association between TG and CAD, these effects were often attentuated after adjustment for other powerful covariates. However, a meta-analysis summarizing population-based prospective studies demonstrated a significant association between TG and CAD risk, independent of high density lipoprotein (HDL-C). For each 1 mmol/L rise in TG, CAD increased by 14% in men (n = 22,293) and 37% in women (n = 6,345) (1). A more recent follow-up of the Copenhagen Male Study reported that fasting levels of TG were independent predictors of initial cardiovascular events, even in subjects at the highest stratum of HDL-C (2).

One prevailing issue in assessing coronary risk is whether there is a designated level (or range) of TG above which CAD rates increase. For total cholesterol (TC), this level has been designated as 200 mg/dL (5.2 mmol/L). The rationale for this cutpoint is based on epidemiologic data; in Multiple Risk Factor Intervention Trial, CAD rates increased curvilinearly as TC levels exceeded 200 mg/dL (3).

A similar cutpoint for TG was established by a National Institutes of Health Consensus Panel in 1993 and subsequently adopted by the National Cholesterol Education Program (NCEP). However, there is little data to support a similar TG cutpoint for the following reasons. First, epidemiologic studies have not supported a curvilinear relationship between TG and CAD; at high TG (>1,000 mg/dL), large predominating very low density lipoprotein- and chylomicron particles are less permeable across the endothelium and consequently less atherogenic. In contrast, partially degraded and highly penetrable remnant particles that accompany mild to moderate hypertriglyceridemic states are associated with premature CAD (e.g., Type IV phenotype). Secondly, while reducing TC, and in particular LDL-C, was demonstrated to reduce CAD event rates, there were no studies evaluating the potential impact of selective TG lowering on CAD event rate. Finally, the median TC in the United States is 200 mg/dL with acceleration of CAD rates above this level. The median TG however is only 100 mg/dL, raising the possibility that TG cutpoints established by NCEP may be too high.

To explore this issue, we followed 350 men and women with arteriographic CAD for 18 yr. During this period, there were 199 new events, including CAD death, nonfatal myocardial infarction, and coronary revascularization. After controlling for other co-variates including HDL-C, an age-adjusted Cox proportional hazards model demonstrated that TG >100 mg/dL (1.13 mmol/L) was an independent predictor of new CAD events (relative risk 1.5, 95% confidence interval 1.1–2.1) (4). These data suggest that lower TG levels than previously considered (100– 199 mg/dL) may pose additional risk in CAD patients. Further studies evaluating the effect of TG lowering on CAD event rate may refine our understanding of the importance attributed to TG in atherothrombosis.

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Abbreviations: CAD, coronary artery disease; HDL-C, high density lipoprotein-C; NCEP, National Cholesterol Education Program, TC, total cholesterol; TG, triglyceride.

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Triglyceride-Lowering Effect of n-3 Long Chain Polyunsaturated Fatty Acid: Eicosapentaenoic Acid vs. Docosahexaenoic Acid

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It is widely accepted that fish oil decreases serum triglycerides. Eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) are believed to be the active components. By far the majority of animal experiments and human trials investigating the effect of n-3 long chain polyunsaturated fatty acid on serum triglyceride levels were carried out with fish oil concentrates containing both EPA and DHA. Little information exists on the effect of EPA or DHA on serum triglyceride levels as a single agent. This paper reviews the results of human studies which investigated the effect of EPA and DHA on serum triglycerides.

The results of human intervention studies investigating the effects of purified EPA and DHA on plasma triglycerides clearly demonstrate that EPA as well as DHA, given as a single agent, significantly and consistently lowers plasma triglyceride levels. In the papers reviewed, the triglyceride-lowering effect of 2.7–4.0 g EPA per day varied between 16 and 33%. A daily dose of 1.25–2.5 g of DHA resulted in a triglyceride reduction of 17–21%. Only a few studies have been published so far which were designed to investigate the effect of purified EPA vs. purified DHA on fasting triglycerides. Two recently published clinical trials investigated the triglyceride-lowering effect of 4.0 g EPA vs. 4.0 g DHA in healthy young men. These studies revealed a serum triglyceride decrease of 12 and 18% in the EPA group and of 17 and 20% in the DHA group, respectively. A study which investigated the effect of a supplementation with EPA vs. DHA on the postprandial triglyceride surge reported a marked decrease of the postprandial triglyceridemia in both the EPA and the DHA group, though this effect was more pronounced in the people who received DHA. The effect of EPA and DHA on serum lipids such as total cholesterol, low density lipoprotein-cholesterol, and high density lipoprotein, however, appears to be less consistent.

In summary, the available evidence points to the fact that both EPA and DHA have a marked hypotriglyceridemic effect in humans. As it regards the effect of purified EPA and DHA on lipoprotein subfractions, there may be some differences that warrant further clarification.

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Abbreviations: DHA, docosapentaenoic acid; EPA, eicosapentaenoic acid.

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Fish Oil in Hypertriglyceridemia: Safety and Recommendations

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Fish and humans have consumed fish oil for thousands of years. The fact that these populations have survived and flourished suggests the relative safety of fish oils, which are now given to patients to prevent and treat various disease conditions. Nonetheless, certain concerns have arisen about their safety. These include excessive bleeding, thrombocytopenia, increased incidence of stroke, enhanced oxidation of fatty acids, a worsening of glucose control in diabetics, and gastrointestinal side effects such as increased gas. From an examination of extensive literature about fish oil, there is no evidence of significant toxicity. Patients undergoing elective vascular surgery and who have been consuming up to 16 g of fish oil per day have not had enhanced bleeding. The reduction in platelet count has been minimal. While early studies showed some deterioration of diabetic glucose control after fish oil, the more recent and better-controlled studies by meta-analysis showed no such effects. The benefit achieved in diabetics by lowering very low density lipoprotein and triglyceride concentrations in the plasma is considerable. Since vitamin E is included in all fish oil capsules, increased oxidation of fatty acids has not occurred. Perhaps no other therapeutic substance has undergone so much testing over the past two decades without there being significant evidence for toxicity.

This being the case, recommendations for the use of fish oil and its constituent fatty acids, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), in the treatment of hypertriglyceridemia states are both logical and practical. This is particularly so for the more purified preparations of fish oil which contain 50% or more of the fatty acids EPA and DHA. This may bring the dose as low as 2 to 4 g per day of the purified components vs. 6 or more grams of the usual fish oil preparation. The use of fish oil in hypertriglyceridemia is especially important because thrombosis is a complication of this disorder, perhaps through activation of factor VII. By several actions, the EPA of fish oil is antithrombotic. The most important action is to reduce the synthesis of thromboxane A2 in platelets.

In types IV and V hyperlipidemia, fish oil can be safely utilized with other therapeutic measures such as a low-fat diet and fibrate drugs. In combined hyperlipidemia, fish oil is very helpful when given concurrently with a statin drug. Fish oil not only reduces fasting plasma triglyceride levels, but also reduces postprandial lipemia, which is both atherogenic and thrombotic. In summary, fish oil is a useful and safe therapeutic modality in the treatment of hypertriglyceridemic states, both primary and secondary.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid.

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Regulation of Cellular 15-Lipoxygenase Activity on Pretranslational, Translational, and Posttranslational Levels

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ABSTRACT: In mammalian cells, enzymatic lipid peroxidation catalyzed by 12/15-lipoxygenases is regulated by pretranslational, translational, and posttranslational processes. In rabbits, rats, and mice induction of experimental anemia leads to a systemic up-regulation of 12/15-lipoxygenases expression. In addition, interleukins-4 and -13 were identified as strong up-regulators of this enzyme in human and murine monocyte/macrophages and in the lung carcinoma cell line A549, and the interleukin-4(13) cell surface receptor as well as the signal transducer and activator of transcription 6 (STATG) appears to be involved in the signal transduction cascade. On the level of translation, 15-lipoxygenase synthesis is blocked by the binding of regulatory proteins to a characteristic gurnine-cytosine-rich repetitive element in the 3′-untranslated region of the rabbit 15-lipoxygenase mRNA, and the formation of such 15-lipoxygenase mRNA/protein complexes was identified as molecular reason for the translational inactivity of the 15-lipoxygenase mRNA in immature red blood cells. However, proteolytic breakdown of the regulatory proteins which were recently identified as hnRNP K and hnRNP E1 overcomes translational inhibition during later stages of reticulocyte maturation. For maximal intracellular activity, 12/15-lipoxygenases require a rise in cytosolic calcium concentration inducing a translocation of the enzyme from the cytosol to cellular membranes as well as small amounts of preformed hydroperoxides which act as essential activators of the enzymes. 12/15-Lipoxygenases undergo irreversible suicide inactivation during fatty acid oxygenation, and this process may be considered an element of down-regulation of enzyme activity. Suicide inactivation and proteolytic breakdown may contribute to the disappearance of functional 12/15-lipoxygenase at later stages of erythropoiesis.

Lipid peroxidation is generally considered a deleterious process which leads to the destruction of subcellular organelles and eventually to cell death (1,2). However, if lipid peroxidation proceeds in a controlled manner and when it is restricted to certain cellular compartments, it may have beneficial effects for cells and for the entire organism. Local destructive processes are an integral part of normal cellular metabolism. In particular, cell differentiation and/or maturation is characterized by a breakdown of certain cell structures and by remodeling of cellular membranes (3). Membrane remodeling and membrane fusion require local destabilization of the membrane structure, in particular a disturbance of the hydrophobic lipid–lipid and lipid–protein interaction within the lipid bilayer. Introduction of a hydrophilic peroxyl group into the hydrophobic fatty acid chains may contribute to local membrane destabilization. Membrane destabilization may not be restricted to cells which undergo differentiation and/or maturation but may also occur in cells which fulfill special tasks in the body. Phagocytosis, degranulation, antigen presentation, receptor-mediated ligand uptake etc. are among the processes associated with membrane remodeling and/or membrane fusion.

An essential precondition for a potential beneficial effect of local lipid peroxidation appears to be a tight regulation. There must be the possibility to turn on lipid peroxidation when it is needed and to switch it off when it starts to do harm to the cells. However, nonenzymatic lipid peroxidation is an autocatalytic process which is difficult to regulate. Although cells contain antioxidants and/or antioxidative enzymes which are designed to protect cells from oxidative damage, this may not be considered real regulation. In contrast, several elements of up- and down-regulation of enzymatic lipid peroxidation have been described (Table 1) and there may be others which have not yet been identified.

In this paper the recent advances in regulation of enzymatic lipid peroxidation by 12/15-lipoxygenases (LOX) are summarized, and the potential impact of enzymatic lipid peroxidation on cell structure and function will be discussed.

EXPERIMENTAL PROCEDURES

Materials and preparations. The 15-LOX was prepared from reticulocyte-rich blood cell suspensions of rabbits by fractionated ammonium sulfate precipitation and consecutive hy-

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Abbreviations: EPR, electron paramagnetic resonance spectroscopy; 15-HPETE, 15-hydroperoxy-5*Z*,8*Z*,11*Z*,13*E*-eicosatetraenoic acid; HPLC, high-performance liquid chromatography; 13*S*-HPODE, 13*S*-hydroperoxy-9*Z*,11*E*-octadecadienoic acid; IL-4(13), interleukin-4(13); LOX, lipoxygenase; MHC, major histocompatibility complex; PCR, polymerase chain reaction; PH-GPx, phospholipid hydroperoxide glutathione peroxidase; STAT, signal transducer and activator of transcription.

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TABLE 1
Characteristics of Lipid Peroxidation[a]

Parameter		LOX reaction	Nonenzymatic lipid peroxidation
K_M	(fatty acids)	low (2–20 μM)	Usually high (system dependent)
	(oxygen)	low (2–5 μM)	Usually high (system dependent)
Stability		Labile (denaturation) Suicide inactivation	Stable
Specificity		High regioselectivity High enantioselectivity	No regioselectivity No enantioselectivity
Regulation		Pretranslational (see text) Translational Posttranslational	No real regulation (interference by antioxidants)

[a]LOX, lipoxygenase.

drophobic interaction and anion exchange chromatography (4). Experimental anemia was induced in rabbits or mice by repeated subcutaneous injection of a neutralized phenylhydrazine solution at a dose of 7 mg/kg body weight (rabbits) or 14 mg/kg body weight (mice). Development of anemia was followed by measuring the hematocrit, and reticulocytes were counted after staining with brilliant cresyl blue. Phospholipid hydroperoxide glutathione peroxidase (PH-GPx) was purified from pig hearts as described in Reference 5. Monocytes (human and murine) were prepared from peripheral blood by density gradient centrifugation and adherence to plastic dishes (6,7). Cells were cultured *in vitro* in the presence of recombinant interleukins for 3–5 d (5,8), and expression of the 15-LOX was tested by activity assays, immunoblotting, and quantitative reverse-transcriptase polymerase chain reaction (RT-PCR). Transgenic mice which overexpress interleukin-4 (IL-4) under the control of major histocompatibility, complex (MHC) I regulatory elements (9) were kindly provided by Dr. A. Schimpl (Würzburg). Homozygous signal transducer and activator of transcription (STAT) 6-deficient mice (10) and corresponding inbred control animals with identical genetic background were obtained from Dr. J. Ihle (Memphis).

Assay systems. The activity of 12/15-LOX was assayed either spectrophotometrically measuring the increase in absorbance at 235 nm or by high-performance liquid chromatography (HPLC) quantification of specific LOX products after incubation of a cell homogenate with exogenous arachidonic acid (100 μM). PH-GPx activity was assayed with the coupled optical test (5) using 1-palmitoyl, 2-hydroperoxylinoleyl phosphatidylcholine as substrate. Immunoblots were carried out using a polyclonal anti-rabbit-15-LOX antibody (Ig G fraction of Mono-Q FPLC). Reverse-transcriptase polymerase chain reaction (RT-PCR) of the 15-LOX mRNA was performed as described before (8): Briefly, total RNA (3 μg) was reverse-transcribed, and 2 μL of this sample was used for PCR. For amplification of the 15-LOX mRNA, the primer sequences 5′-GGGGCTGGCCGACCTCGCTATC-3′ (up) and 5′-TCCTGTGCGGGGCAGCTGGAGC-3′ (down) were selected from regions displaying minimal homology to the sequences of the human platelet-type 12- and the 5-LOX. The primers for amplification of the glyceraldehyde-3-phosphate dehydrogenase mRNA were 5′-TCGGAGTCAACGGATTTGGTCGTA-3′ (up) and 5′-ATGGACTGTGGTCATGAGTCCTTC-3′ (down). After initial denaturation for 3 min at 94°C, PCR was carried out for 23–35 cycles, and each cycle consisted of a denaturing period (40 s at 94°C), an annealing phase (30 s at 66°C for glyceraldehyde-3-phosphate dehydrogenase and at 71°C for 15-LOX) and an extension period (30 s at 72°C). The reaction mixture was a 10 mM Tris-HCl buffer, pH 8.3, containing 50 mM KCl, 2 mM $MgCl_2$, 0.1 mg/mL gelatin, 6 pmol of primer sets, 100 μM each of dNTP, 100 μg/mL albumin, and 2.5 U Taq DNA polymerase. PCR products were separated by 2% agarose gel electrophoresis. For quantification, DNA was stained with ethidium bromide and analyzed densitometrically. Densitometric data were corrected for molar equivalence and log/log plotted as function of internal standard-derived PCR products. HPLC analyses of the lipoxygenase products were carried out on a Shimadzu instrument coupled with a Hewlett-Packard diode array detector 1040 A. Reverse-phase HPLC was performed on a Nucleosil C-18 column (Macherey/Nagel, Düren, Germany; KS-system, 250 × 4 mm, 5 μm particle size). A solvent system of methanol/water/acetic acid (85:15:0.1, by vol) and a flow rate of 1 mL/min were used. Chromatograms were quantified by peak areas. The fractions containing the oxygenated polyenoic fatty acids were pooled, the solvent was evaporated, the residues were reconstituted in a mixture of *n*-hexane/2-propanol/acetic acid (100:2:0.1, by vol) and injected to straight-phase HPLC analysis which was carried out on a Zorbax SIL column (Macherey/Nagel, KS-system, 250 × 4 mm, 5 μm particle size) with a solvent system consisting of *n*-hexane/2-propanol/acetic acid (100:2:0.1, by vol) and a flow rate of 1 mL/min. For detection of the primary LOX products, the ultraviolet absorbance at 235 nm was recorded.

RESULTS AND DISCUSSION

Pretranslational regulation of 15-LOX expression. Erythrocytes of various species do not express a 12/15-LOX. However, after induction of experimental anemia in mice, rabbits (Table 2), and rats (data not shown) a 12/15-LOX activity can be measured. Experimental anemia was induced in mice and rabbits by subcutaneous injection of a neutralized phenylhydrazine solution at four consecutive days. Rabbits were treated with a dose of 7 mg phenylhydrazine/kg body weight, whereas

TABLE 2
Lipoxygenase Activity of Red Blood Cells Before and During Experimental Anemia

	LOX activity (μg HETE formation/mL of packed red cells × 15 min)	
Species	Before phenylhydrazine injection	After phenylhydrazine injection
Mouse	0[a]	210 (12*S*-HETE)[b]
Rabbit	0	91 (15*S*-HETE)

[a]In young mice we detected occasionally a 12-LOX activity in the red cells even when the animals had not been treated with phenylhydrazine.
[b]Measured 17 d after the first phenylhydrazine injection. In parentheses the chemical structure of the major product is given (see Fig. 1).

mice received 14 mg/kg body weight, 14 d after the first injection blood was withdrawn and the red cells were prepared. After washing twice with isotonic saline the cells (100 μL of packed cells suspended in 1 mL of phosphate buffered saline) were incubated for 15 min with 100 μM arachidonic acid. The reaction was stopped by addition of sodium borohydride. After acidification to pH 3, the lipids were extracted twice with 1 mL of ethyl acetate. The solvent was removed under vacuum, the lipids were reconstituted in 0.1 mL of methanol, and aliquots were injected to reverse-phase HPLC analysis with the solvent system methanol/water/acetic acid (80:20:0.1, by vol). HPLC analysis revealed that 15*S*-hydroxy-5*Z*,8*Z*,11*Z*,13*E*-[H(P)ETE] was the major product of arachidonic acid oxygenation by rabbit reticulocytes. In contrast, mainly 12-H(P)ETE was found when murine reticulocytes were incubated with arachidonic acid (Fig. 1). More detailed structural analysis indicated that rabbit reticulocytes formed a 95:5 mixture of 15*S*-H(P)ETE/12*S*-H(P)ETE, whereas this ratio was inverted for murine reticulocytes (3:97). The latter finding was quite surprising since murine peritoneal macrophages form a 12*S*-H(P)ETE/15*S*-H(P)ETE ratio of about 70:30 (11,12). If the formation of 12/15-LOX products by murine reticulocytes and murine macrophages would be catalyzed by one and the same enzyme, a more similar product pattern would have been expected. On the other hand, it was shown before that the positional specificity of the LOX reaction is not an absolute enzyme property but may depend on the reaction conditions. Thus, product analysis alone is not sufficient to answer the question of whether or not in mice two different 12/15-LOX isoforms may be expressed in a tissue-specific manner. Experiments with transgenic mice in which the gene coding for the macrophage 12-LOX was disrupted may contribute to answer this question (13).

Anemia-induced LOX expression is not restricted to the reticulocytes but was also detected in peripheral monocytes, in lung, spleen, kidney and liver, but hardly in skeleton muscle and various parts of the brain (14). At present, the mechanism of 12/15-LOX induction during experimental anemia is not known. Possibly, experimental anemia leads to the secretion of various cytokines which in turn may switch on systemic expression of 12/15-LOX.

A second element of pretranslational regulation is the induction of the 12/15-LOX by interleukins (6,15,16). Human and murine peripheral monocytes as well as the human lung carcinoma cell line A 549 do not express a 12/15-LOX. However, when the cells were cultured in the presence of IL-4 or IL-13, a functional LOX is expressed as indicated by Northern-blot analysis, RT-PCR, immunoblotting, and by activity assays. Other cytokines such as IFNγ, IL-6, IL-10, GM-CSF etc. did not exhibit this stimulatory effect. No up-regulation of 12/15-LOX expression was observed when permanent human (HL 60, THP 1, U 937, Mono-Mac 6) or murine (J 774, P 388.D1) monocytic cell lines were tested (8). These data were rather surprising since all of these cells express the IL-4 cell surface receptor. The reasons for this differential behavior of peripheral monocytes and monocytic cell lines are not clear, but it may be speculated that a high degree of maturation may be required. Most of the permanent monocytic cell lines represent rather immature precursor cells, and this may be the rea-

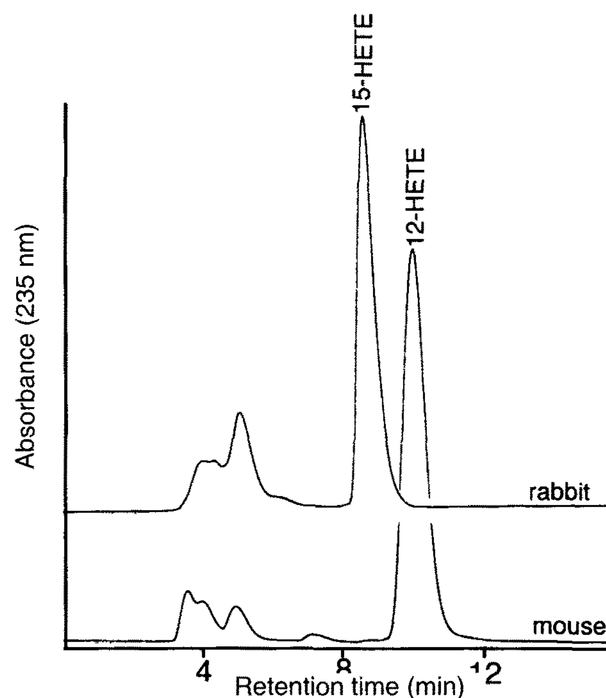

FIG. 1. Formation of specific lipoxygenase (LOX) products by rabbit and murine reticulocytes. Induction of experimental anemia, reticulocyte preparation, activity assays, and reverse-phase high-performance liquid chromatography analysis were carried out as described in the legend to Table 2. The retention times of the authentic standards are indicated above the traces. 12-HETE, 12-hydroxy-5*Z*,8*Z*,10*E*,14*Z*-eicosatetraenoic acid; 15-HETE, 15-hydroxy-5*Z*,8*T*,11*Z*,13*E*-eicosatetraenoic acid.

son for their unresponsiveness. Human alveolar macrophages (16) and murine peritoneal macrophages (7) constitutively express a 12/15-LOX. When cultured in the presence of IL-4, the level of expression is up-regulated by a factor of 2–5 (7,16). Although the mechanism of IL-4-induced up-regulation of 12/15-LOX synthesis is not completely understood, several constituents of the signal transduction cascade were identified. Competition assays with IL-4 receptor antagonists (IL-4 mutant Y124D) suggested the involvement of the IL-4(13) cell surface receptor (8). This IL-4 mutant, which is capable of binding to the receptor but does not induce the intracellular signal transduction cascade (17), inhibited the up-regulation of 15-LOX expression (8) as indicated by activity assays and immunoblot analysis.

For B-cells, IL-4 constitutes a signal for switching immunoglobulin synthesis toward immunoglobulin synthesis toward E formation, and the transcription factor STAT 6 was shown to be involved. After IL-4 binding to its cell-surface receptor, STAT 6 is phosphorylated and translocates as homodimer into the nucleus where it binds to STAT 6-responsive elements in the promoter region of IL-4-sensitive genes to activate transcription (18). In order to find out whether STAT 6 may also be involved in IL-4-induced up-regulation of 12/15-LOX expression, experiments with peritoneal macrophages were carried out which were prepared from transgenic mice in which the gene coding for STAT 6 had been inactivated by targeted gene disruption (10). We found that in these cells the expression of the 12/15-LOX was not up-regulated when cultured in the presence of IL-4 (7).

In the promoter region of the human 15-LOX gene, STAT 6 responsive elements with the consensus sequence TTC NNN(N) GAA (19) were identified, but their functionality is still unclear. Thus, the question of whether the 15-LOX gene may belong to the family of immediate early genes switched on by IL-4 cannot be answered at this time. However, the time course of 12/15-LOX expression following IL-4 stimulation (6,8) strongly suggests that the 12/15-LOX gene may not constitute an immediate early gene of IL-4 response. RT-PCR data on A 549 cells indicated that transcription of the 15-LOX gene started about 24 h after IL-4 stimulation, and expression of the functional enzyme was maximal after 3–5 d. Such a long time interval between stimulation and expression may not be typical for immediate early genes. Recently, it was reported that in human monocytes, IL-4 stimulation may induce the expression of a novel transcription factor (transcription factor X) which is capable of binding to the promoter region of the human 15-LOX gene (20). In Figure 2 the potential sequence of events involved in IL-4-induced up-regulation of 12/15-LOX expression is shown.

Translational regulation of 15-LOX expression. In young rabbit reticulocytes, the 15-LOX mRNA is present, but no functional enzyme is expressed (21). As mechanistic reasons for these biological dynamics, two regulatory proteins were identified which are capable of binding to repetitive sequences in the 3′-untranslated region of the 15-LOX mRNA which were called differentiation control elements (DICE). Protein binding to DICE prevents translation of the 15-LOX mRNA as indicated by *in vitro* translation studies (22). The regulatory proteins were purified from a lysate of rabbit reticulocytes and were identified as hnRNP K and E_1 (23). Transfection of hnRNP K and hnRNP E_1 into HeLa cells specifically silenced the translation of reporter mRNA, bearing the repetitive element of the rabbit 15-LOX mRNA in their 3′-untranslated region (23). Silenced LOX mRNA of rabbit reticulocytes specifically coimmunoprecipitated with hnRNP K. Addition of recombinant hnRNP K and/or hnRNP E_1 causes inhibition of 80S ribosome assembly on the 15-LOX mRNA. Moreover, both proteins can control cap-dependent and internal ribosome entry site-mediated translation by binding to the differentiation control elements (DICE). These data suggest a specific cytoplasmic function for hnRNP as translational regulatory proteins. In later stages of red cell maturation, these regulatory proteins may be degraded proteolytically, and the functional 15-LOX will be expressed.

Posttranslational regulation of 15-LOX activity. 12/15-LOX are not a subject of major posttranslational modification. Major glycosylation was not detected (24,25), and there are no signs for myristoylation or isoprenylation (25). Moreover, there is no experimental evidence for a regulatory phosphorylation/dephosphorylation cycle of the enzyme.

Nevertheless, 12/15-LOX require activation in order to exhibit their catalytic activity. When highly purified polyenoic fatty acids are used as LOX substrate, the reaction starts with a kinetic lag-phase which can be abolished by addition of hydroperoxy fatty acids (Fig. 3). Although the chemical nature of this activation process is not well understood, oxidation of the ferrous nonheme iron to a ferric form is involved (26). In order to find out whether such activation is also required for the oxygenation of complex substrates, we investigated the impact of a lipid peroxide-reducing enzyme on 12/15-LOX-catalyzed oxygenation of biomembranes (27). When mitochondrial membranes were incubated with the purified 15-LOX, the unsaturated phospholipids present in the membranes were oxygenated as indicated by the formation of specific LOX products. However, when the membranes were pre-incubated with phospholipid hydroperoxide glutathione peroxidase (PH-GPx), an enzyme capable of reducing esterified hydroperoxy lipids to their corresponding hydroxy derivatives, the formation of these products was prevented (27). These data may be explained by the fact that PH-GPx depletes the membranes from hydroperoxides, and thus enzyme activation becomes impossible. However, when an excess of hydroperoxy fatty acids was added, the LOX reaction did start immediately (27).

Nitric oxide also appears to be of regulatory importance for the 12/15-LOX pathway (28,29). Short-term incubations of the enzyme in the presence of nitric oxide lead to a prolongation of the kinetic lag-period, suggesting a reversible inhibition of the enzyme (29). However, after long-term incubations, the kinetic lag-phase was considerably shortened. These data suggest that during NO/15-LOX interaction an oxidation of the ferrous nonheme iron may have taken place. To

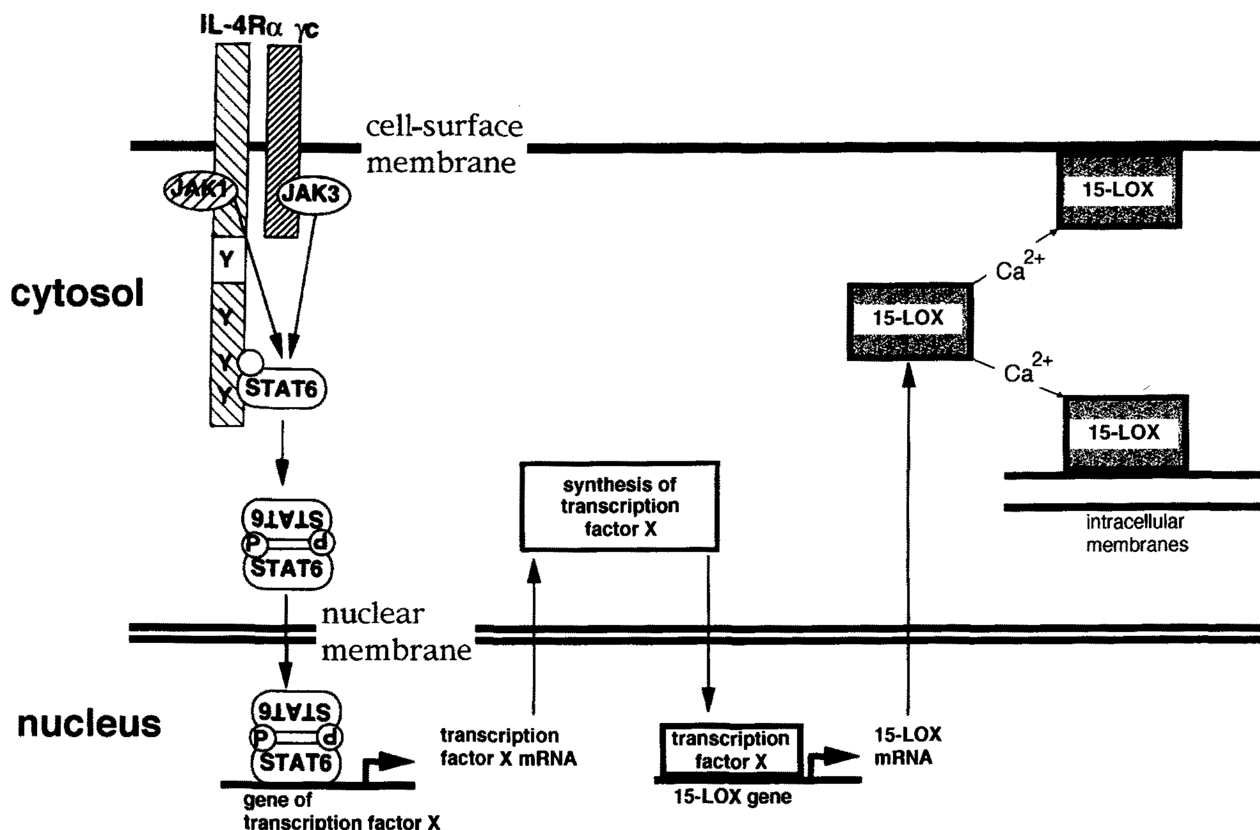

FIG. 2. Possible sequence of events involved in interleukin-4-induced up-regulation of 15-LOX expression. See Figure 1 for abbreviation.

obtain direct evidence for such an oxidation, X-ray absorption studies and electron paramagnetic resonance spectroscopy measurements were carried out (28), and with both methods we obtained evidence for the formation of ferric LOX species during 15-LOX/NO interaction.

As third element of posttranslational regulation a calcium-dependent membrane association was reported. When the purified rabbit 15-LOX is incubated with biomembranes in the presence of calcium ions, it binds to membranes as indicated by immunoblotting (30). This membrane association, which is a necessary precondition for the oxygenation of the membrane phospholipids, is paralleled by an up to 10-fold stimulation of the fatty acid oxygenase activity. Removal of calcium (complexation by EDTA) dissociates the enzyme from the membranes and prevents the oxygenation of the membrane lipids, suggesting reversibility of the membrane binding.

Many years ago it was shown that most mammalian LOX are irreversibly inhibited during oxygenation of polyenoic fatty acids, but the mechanism of this suicide inactivation is still unclear. The purified rabbit 15-LOX is rapidly inactivated when incubated with hydroperoxy linoleic acid (13*S*-HPODE), the primary product of LOX-catalyzed oxygenation of linoleic acid. Comparison of the circular dichroism spectra of the native and the 13*S*-HPODE-inactivated enzyme did not reveal major dif-

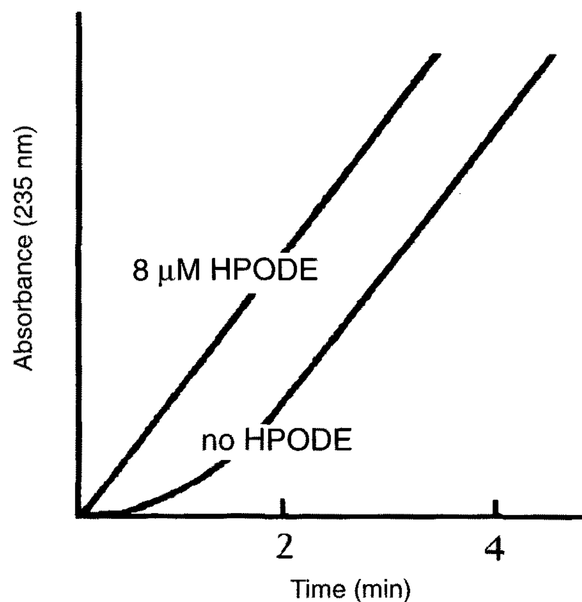

FIG. 3. Peroxide activation of rabbit 15-LOX. The rabbit 15-LOX was incubated with 0.25 mM linoleic acid in the absence and presence of hydroperoxy linoleic acid (13*S*-HPODE), and the increase in absorbance at 235 nm was assayed as a measure of linoleic acid oxygenation. See Figure 1 for other abbreviation.

ferences, suggesting that suicide inactivation may not be due to major changes in the secondary structure (Ludwig, P., unpublished data). Thus, a rather specific mechanism of suicide inactivation was proposed. Later on, it was found that suicidal inactivation was paralleled by a selective oxidation of one methionine residue to methionine sulfoxide (31). More detailed protein chemical studies identified met590 as the target amino acid (32). However, when this methionine was mutated to an oxidation-resistant leucine, the mutant enzyme species also underwent suicidal inactivation, suggesting that oxidation of met590 may not be the major molecular reason of suicide inactivation (32).

More recently the inactivation of the porcine leukocyte 12-LOX by 15-HPETE was studied (33). When radioactive 15*S*-HPETE was used as inactivating agent, the enzyme was labeled radioactively during the inactivation process. It was hypothesized that 15-HPETE is converted by the enzyme to a reactive 14,15-epoxy leukotriene intermediate which in turn reacts with the LOX protein to form a covalent adduct (33), and recent experiments with 14,15-leukotriene A_4 appear to support this hypothesis. Similar data were also obtained with the rabbit 15-LOX (Weisner, R., and Kühni, H., unpublished data).

During the publication process of this paper, it was reported (34) that IL-13-induced expression of the 15-LOX in human monocytes requires tyrosine phosphorylation of JAK2 and TYK2. These data implicate JAK and TYK kinases as additional members of the IL-4/IL-3 induced signal transduction cascade which leads to 15-LOX expression.

ACKNOWLEDGMENT

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Fatty Acid Ethyl Esters: Nonoxidative Ethanol Metabolites with Emerging Biological and Clinical Significance

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Despite the fact that alcoholism is a major cause of disease in society, little is known about the mechanism by which ethanol abuse induces organ damage. One increasingly compelling hypothesis is that fatty acid ethyl esters (FAEE), esterified products of fatty acids and ethanol, are at least partly responsible for the observed pattern of organ damage in alcoholics. FAEE were discovered and rediscovered on multiple occasions in different cells and tissues through the 1960s and 1970s. In the last several years *in vitro* and *in vivo* evidence emerged that FAEE contribute to ethanol-induced organ damage, with a variety of different mechanisms proposed for mediation of this toxic effect. Multiple enzymatic activities associated with FAEE formation have now been described. Independent of its role in mediating cell injury, it was very recently shown that FAEE are useful short- and long-term serum markers of ethanol intake, given their appearance in the blood rapidly after ethanol ingestion and their presence when ethanol is no longer detectable.

THE TOXIC EFFECTS OF FAEE

In 1986, a hypothetical connection was established between FAEE and ethanol abuse. Tissues obtained postmortem from humans acutely intoxicated at the time of death were tested and the organs commonly damaged by ethanol abuse, pancreas, liver, heart and brain, were found to have high levels of enzyme activity for the synthesis of FAEE and the highest concentrations of FAEE among many different organs and tissues tested (1). Adipose tissue was also found to have an accumulation of FAEE and measurable levels of FAEE synthetic activity. The organs not typically damaged by ethanol abuse showed little or no FAEE and correspondingly little ethyl ester synthase activity. FAEE were implicated as mediators of ethanol toxicity because FAEE and the enzyme(s) responsible for their synthesis were distributed primarily in organs damaged by ethanol abuse. However, there was no causal effect demonstrated between FAEE and cytotoxicity in this report.

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Abbreviations: FAEE, fatty acid ethyl esters; LDL, low density lipoproteins; rLDL, reconstituted low density lipoprotein; WBC, white blood cell.

Prior to the 1986 autopsy study, the possibility that FAEE mediate ethanol-induced organ damage had been raised. Using FAEE solubilized in emulsions and isolated heart muscle mitochondria from rabbits, a report in 1983 indicated that FAEE inhibited mitochondrial function by uncoupling oxidative phosphorylation (2). It was speculated that FAEE are hydrolyzed in cells in the mitochondrial membrane, resulting in a high concentration of free fatty acids, which impair mitochondrial function.

In 1993, Haber *et al.* (3) demonstrated that rat pancreatic lysosomes incubated for 20 min with ethyl oleate become unstable and leak their enzymes into the surrounding medium. The hypothesis was raised that increased pancreatic lysosomal fragility mediated by FAEE is associated with ethanol abuse.

Studies from my research group by Szczepiorkowski *et al.* (4) provided the first demonstration that FAEE in a physiologic particle, low density lipoprotein (LDL), exert toxic effects on an intact cell. We previously observed in human subjects that FAEE are present within LDL following ethanol ingestion (5). Therefore, in the toxicity studies, to deliver FAEE to intact cells, we synthesized radiolabeled FAEE and incorporated them into human LDL particles, which bind to LDL receptors (6). In these studies, ethyl palmitate and ethyl oleate were incorporated into LDL, yielding molar ratios of FAEE to LDL particle of 1614 ± 187 and 3154 ± 303 ($n = 6$, mean $\pm$ SE), respectively. LDL reconstituted with FAEE (rLDL) was not oxidatively modified, and native LDL markedly decreased the uptake of ethyl oleate in rLDL by HepG2 cells. Cultured HepG2 cells were incubated with LDL containing FAEE (either ethyl oleate or ethyl arachidonate), and cell proliferation was measured by [methyl-^{3}H]thymidine incorporation, and protein synthesis was determined using L-[^{35}S]methionine. Incubation of cells with 600 μM ethyl oleate or 800 μM ethyl arachidonate, which permitted us to achieve intracellular FAEE concentrations found *in vivo* in the 1986 autopsy study (1), decreased [methyl-^{3}H]thymidine incorporation into HepG2 cells by 30 and 35%, respectively. rLDL with ethyl oleate (400 μM) decreased protein synthesis in intact HepG2 cells by 40%. Electron microscopy revealed signifi-

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cant changes in cell morphology, with accumulation of intracellular lipids and a distortion of the intracellular lipids and a distortion of the nuclear membrane. FAEE delivered in rLDL were rapidly hydrolyzed, and the fatty acids reesterified into phospholipids, triacylglycerols and cholesteryl esters, with preference for triacylglycerols. These findings provided evidence that FAEE are toxic for intact human hepatoblastoma cells and that they or their metabolites may be a causative agent in ethanol-induced liver damage.

Gubitosi-Klug and Gross (7) examined the effect of FAEE on a human brain potassium channel in SF9 cells expressing the recombinant channel. They found that physiologically relevant FAEE concentrations accelerated the kinetics of activation of the channel, and raised the possibility that this *in vitro* observation may be one of the pathologic consequences of ethanol abuse in the central nervous system.

The *in vitro* toxicity studies still left unanswered the question of whether FAEE are toxic *in vivo*. This led us to perform experiments to assess the toxicity of FAEE in rLDL *in vivo* in rats (8). In these studies, rats received FAEE in rLDL at FAEE concentrations which are physiologically attainable (10–30 μM) after ethanol ingestion. The FAEE were delivered as a bolus and then by continuous infusion for 1 h through a cannula placed in the carotid artery and advanced through the aorta to the superior mesenteric artery. This placement was made to maximize the likelihood for observing FAEE-induced cytotoxicity in the pancreas. The rats were sacrificed 3–24 h after infusion of FAEE, and biochemical markers of organ damage were measured. Histologic analysis was performed for multiple organs, and the wet/dry ratio of the pancreas determined to assess pancreatic edema. Control rats received LDL which was reconstituted with cholesterol ester. The wet/dry ratio for the rats receiving the LDL containing FAEE was significantly higher than that of the rats receiving rLDL with cholesterol ester. In addition by 3 h, the level of trypsinogen-activating peptide, a biochemical marker for pancreatic cell damage, was threefold higher in the animals receiving the rLDL relative to controls.

Thus, at this time many *in vitro* and *in vivo* studies show that FAEE have significant capacity to induce cell injury.

ENZYMES ASSOCIATED WITH FAEE SYNTHESIS

In 1984, an FAEE synthase enzyme was purified to homogeneity from rabbit myocardium (9).

In a 1987 study involving FAEE synthesis using a postmortem human brain (10), FAEE synthase activity was found in 10 different anatomic locations in human brain, with gray matter sites containing approximately twice the activity of white matter sites.

A study published in 1990 investigated the synthesis and degradation of FAEE by rat hepatoma cells exposed to ethanol in tissue culture (11). FAEE synthesis by cultured hepatoma cells was found to be linearly associated with the concentration of ethanol added to the culture medium. In addition, the enzymes responsible for the synthesis of ethyl esters were shown to be primarily membrane-bound and concentrated in the microsomal fraction of the hepatocytes, rather than in the cytoplasm. It was also observed in this study that FAEE can be hydrolyzed to free fatty acids and ethanol by membrane-bound enzymes in the microsomal and mitochondrial-lysosomal fractions.

A 1996 report by Treloar *et al.* (12) provided additional information on the cytosolic and microsomal forms of FAEE synthase. The cytosolic FAEE synthase activity, which uses ethanol and free fatty acid as substrates for FAEE synthesis, was designated as FAEE synthase, and the microsomal FAEE synthase activity, which uses ethanol and fatty acyl-CoA as substrates, was referred to as acyl-CoA:ethanol acyltransferase. The data indicated that the activities/g liver of these two FAEE syntheses are comparable.

Several enzymes known to catalyze other reactions were also shown to catalyze FAEE synthesis. These include carboxylesterase from adipose tissue (13) and pancreas (14), lipoprotein lipase (15,16), pancreatic triglyceride lipase (17), and possibly cholesterol esterase (18).

We reported that white blood cells (WBC) are capable of synthesizing FAEE (19). This finding is significant because it demonstrates that a blood sample rather than a biopsy can be used to quantitate FAEE synthase activity in populations. We determined that the lymphocyte–monocyte population of WBC was particularly high in FAEE synthase activity and that the natural killer cells had the highest activity among the lymphocytes. This result correlated with a report published at the same time, showing that delivery of ethanol to rats previously injected with tumor cells permitted metastasis by inhibition of natural killer cell function (20). This observation raises the possibility that, upon ethanol ingestion, FAEE synthesis in natural killer cells is a causative factor in the inhibition of immunologic activity and the promotion of tumor metastasis.

We also demonstrated in this study that FAEE synthase activity could be induced nearly twofold in the WBC fraction of humans ingesting 2 ounces of Scotch whisky for 6 d (19). This supports the conclusion that FAEE synthase is regulated to some extent by the presence of ethanol. The enzyme activity returned to baseline levels despite ingestion of 2 ounces of Scotch whisky for an additional 3 d. This report also showed that alcoholic individuals have approximately half the WBC FAEE synthase activity detected in normal controls. The lower enzyme activity observed in the WBC of alcoholics in a detoxification center may be the result of years of ethanol abuse, or it may be that alcoholics congenitally have low levels of FAEE synthase. If the latter is true, this finding may explain in part the genetic predisposition of many alcoholic individuals to ethanol abuse.

FAEE AS FATTY ACID SUPPLEMENTS

There is substantial clinical interest in fatty acid supplements as treatments for a variety of diseases. Oral preparations of FAEE are available for fatty acid supplementation. Ethyl esters and triglycerides were shown to be equally well absorbed from the gastrointestinal tract in human subjects (21). In

1992, Yamazaki and Hamazaki (22) infused emulsions of ethyl eicosapentaenoate and found an increase in the eicosapentaenoate content in the phospholipids of a variety of organs.

We recently tested the hypothesis that orally ingested supplemental FAEE are rapidly degraded in the gastrointestinal tract and blood to explain the lack of toxicity (23). Using rats given FAEE as an oil directly into the stomach or within LDL particles directly into the circulation, we demonstrated that FAEE hydrolysis in the gastrointestinal tract and blood is rapid and extensive. The fate of the fatty acid from FAEE hydrolysis was highly dependent on the organ or tissue presented with the FAEE.

FAEE AS MARKERS OF ETHANOL INTAKE

In a 1994 study with five volunteers investigating ethanol, FAEE were shown to be bound to lipoproteins and albumin in serum (5). A higher percentage of saturated fatty acids was found in the FAEE pool than in the serum free fatty acid or triacylglycerol pools. In this study, we also demonstrated that when FAEE are isolated by density gradient ultracentrifugation from the sera of intoxicated emergency room patients, 68.3% of the FAEE associate with the $d > 1.21$ fraction, which contains albumin. To assess whether FAEE in serum, like nonesterified fatty acids, are bound to albumin, sera from several emergency room patients with detectable blood ethanol were pooled, and the serum albumin in the sample was immunoprecipitated. Lipids from the immunoprecipitate were extracted, and FAEE were found in the precipitates. These data provide evidence to support the conclusion of earlier density gradient studies that a significant fraction of serum FAEE is bound to albumin.

In a series of nuclear magnetic resonance-based studies, we analyzed FAEE binding to small unilamellar phospholipid vesicles, human LDL, and bovine serum albumin by ^{13}C nuclear magnetic resonance spectroscopy using ethyl (1–^{13}C, 99%) oleate (24). The conclusions from this study were that addition of ethyl oleate to isolated human LDL resulted in ethyl oleate in the core of the lipoprotein, that albumin has a much greater affinity for oleic acid than for ethyl oleate, and that ethyl oleate rapidly transfers between LDL and phospholipid vesicles. In our report by Doyle *et al.* (5), we found that albumin transports the majority of FAEE in plasma. The accumulated observations suggest that despite its low affinity for FAEE, albumin transports most of the FAEE in the blood because it has a much greater plasma concentration than lipoprotein particles. In a separate study, we reported that as

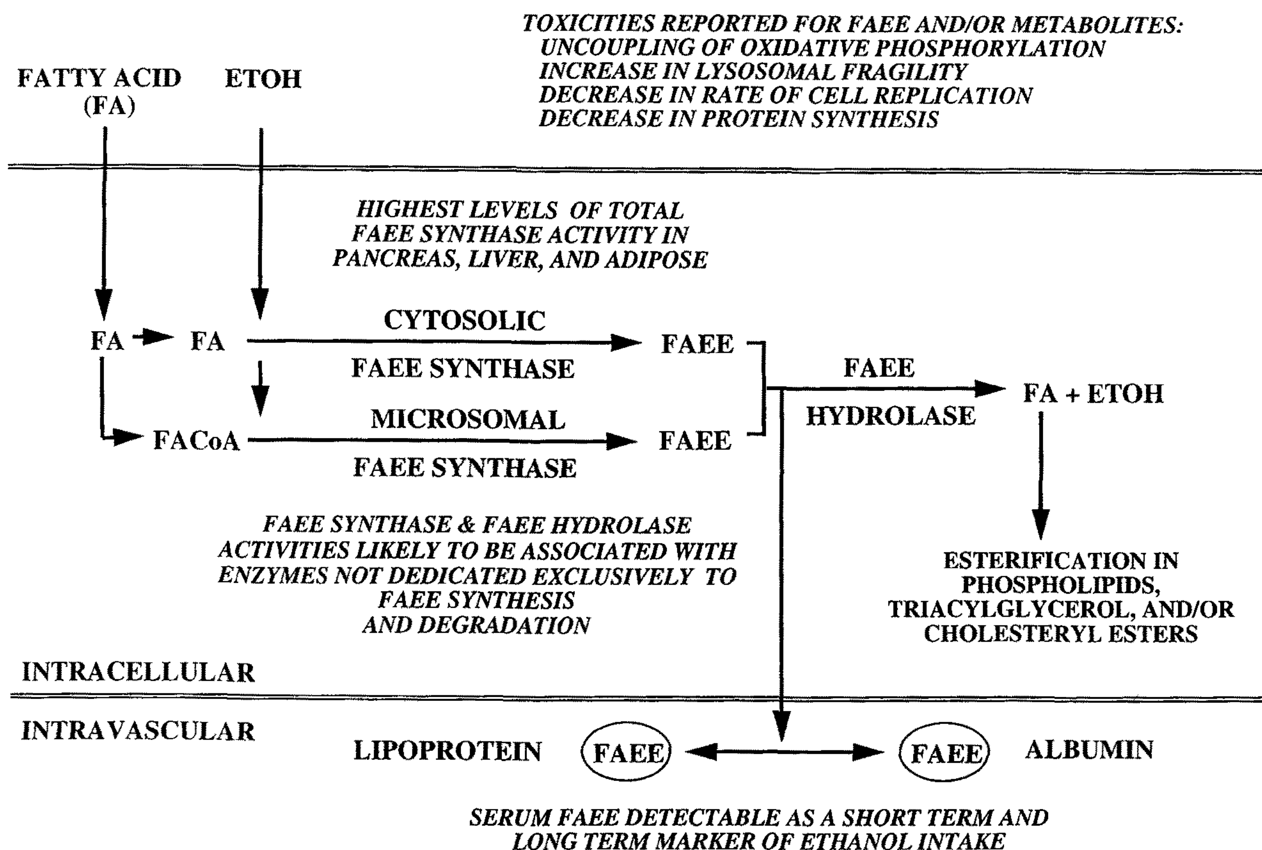

FIG. 1. A working hypothesis for the synthesis and degradation of fatty acid ethyl esters. FA, fatty acid; FACoA, fatty acyl-CoA; FAEE, fatty acid ethyl esters; ETOH, ethanol. Reprinted from *Addiction Biology*, (1998) *3*, 5–14. CARFAX Publishing Ltd., Abingdon, Oxfordshire, OX14 3UE, United Kingdom.

the serum FAEE concentration rises, the percentage of FAEE associated with lipoproteins increases and correspondingly the percentage associated with albumin decreases (25).

FAEE may be clinically important, independent of whether FAEE induce cytotoxicity, because they can serve as a marker for ethanol intake. We performed a study to determine the clinical utility of FAEE in the blood as a short-term confirmatory marker for ethanol intake and as a long-term marker for ethanol intake after ethanol is no longer detectable (26). To isolate FAEE from plasma for quantitation by gas chromatrography–mass spectrometry in these clinical studies, we developed a two-step method using solid-phase extraction with a recovery of 70 ± 3%, using ethyl oleate as a recovery marker (27).

The design for the study was a controlled clinical trial with seven healthy subjects. The subjects ingested a known amount of ethanol at a fixed rate. The concentration of FAEE in the blood after ethanol intake was determined for up to 24 h. FAEE disappearance from the blood followed a decay curve which initially resembled the decay curve for blood ethanol. However, because of a very slow secondary elimination phase, the FAEE persisted in the blood for at least 24 h after ethanol intake was completed. Also included in this report was a blinded comparison involving 48 samples that were either positive, negative, or equivocal for blood ethanol. All 20 samples positive for ethanol were positive for ethyl esters; 7 of 7 samples equivocal for ethanol and classified as negative because the amount was too low for accurate quantitation were positive for ethyl esters; and 21 of 21 samples negative for ethanol were negative for ethyl esters. These data allowed us to conclude that FAEE in the blood can serve as an excellent short-term confirmatory test for ethanol intake as well as a long-term marker of ethanol ingestion.

A summary of many of the major observations in the field is shown in Figure 1 and described in two recent reviews (28,29). The last several years have seen rapid growth of our understanding of FAEE synthesis and degradation, FAEE-induced organ damage, and the monitoring of ethanol intake with serum FAEE measurements. The years ahead could bring a mechanistic explanation for FAEE-induced cell injury and a robust clinical assay for detecting ethanol intake.

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The Effects of Cigarette Smoking on the Metabolism of Essential Fatty Acids

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Free radicals generated in cigarette smoke are known to deplete antioxidants and may result in increased lipid peroxidation which leads to decreased concentrations of long chain polyunsaturated fatty acids (LC-PUFA). The effect of smoking on the conversion of 18-carbon essential fatty acids to LC-PUFA has never been studied yet there is evidence that smoking alters plasma fatty acids. A stable isotope gas chromatography–mass spectrometry (GC–MS) method was used to investigate the metabolism of d_5-18:2n-6 to d_5-20:4n-6 and d_5-18:3n-3 to d_5-22:6n-3 in the plasma of smokers (n = 10) and controls (n = 10) maintained on a controlled diet. The isotopic enrichments of the plasma fatty acids pools, d_5-18:2n-6, d_5-20:3n-6, and d_5-20:4n-6, from controls and smokers were compared over 168 h. A multiple compartment model of the essential fatty acid kinetics was used to predict the percent conversion of d_5-18:2n-6 to d_5-20:3n-6 and d_5-20:3n-6 to d_5-20:4n-6 in both smokers and controls (Fig. 1). In addition, sensitive and specific GC–MS procedures were used to determine the concentration of the 4-hydroxyalkenals and 8-isoprostane-F2-α in the plasma of subjects.

METHODS

Ten subjects (five female and five male) who smoked between one and two packs of cigarettes per day and 10 volunteers (five male and five female) were placed on a controlled diet for 3 wk. The diet was low in LC-PUFA in that no fish was taken and only lean cuts of beef were eaten. In addition the primary vegetable oil used was olive oil. Beginning the third week, subjects were given a 1-g oral dose of d_5-18:2n-6 with their breakfast. Blood was collected at 0, 8, 24, 48, 72, 96, and 168 h subsequent to dosing, and the plasma was extracted and analyzed for the fatty acids and the labeled compounds by flame-ionization detector and GC–MS (1), respectively. Plasma from both groups was also subjected to analysis for 4-hydroxyalkenals and 8-isoprostane PGF2-α using appropriate derivatization procedures and GC–MS techniques (2).

The results demonstrated that the rates of disappearance of the labeled precursors from the blood were similar in both groups. Smokers had a highly significant ($P < 0.004$) increase

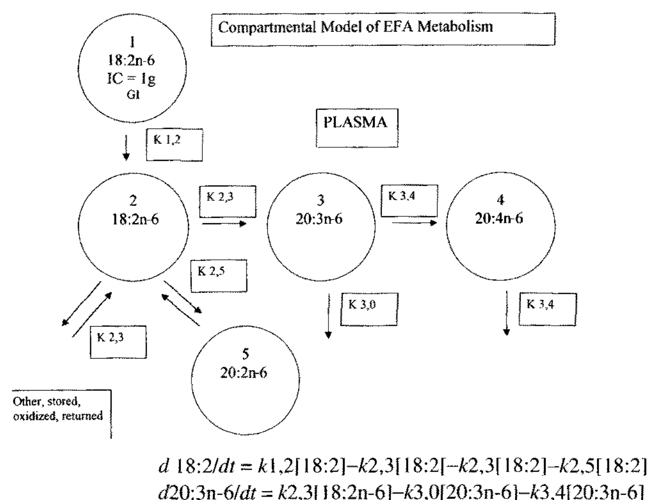

FIG. 1. Compartmental model of essential fatty acid metabolism.

(150%) in the isotopic enrichment of plasma 20:3n-6 compared to controls; however, there was no difference in the plasma pool enrichments of 20:4n-6 between the two groups. As a result, there appeared to be a 37% decrease in the conversion of 20:3n-6 to 20:4n-6. The model assessment of the essential fatty acid kinetics predicted a nearly twofold increase in the conversion of 18:2n-6 to 20:3n-6 in smokers than controls. Lipid peroxidation determinations demonstrated that there was a significantly greater concentration of 8-isoprostane-F2-α ($P < 0.001$) in the plasma of the smokers (224 ± 61 pmol/L) compared to controls (117 ± 23) and 4-hydroxynonenal ($P < 0.02$). This study showed that that cigarette smoking, which causes an oxidation in lipids, also inhibits the production of PUFA through some as yet unknown mechanism.

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Abbreviations: GC–MS, gas chromatography–mass spectrometry; LC PUFA, long chain polyunsaturated fatty acids.

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Impairment of Antioxidant Defense Mechanisms in Elderly Women Without Increase in Oxidative Stress Markers: "A Weak Equilibrium"

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The free radical theory of aging suggests that progressive defects in protection against free radicals allow tissue damage to occur. Concordant data have been observed in cell cultures or in animal models of aging. However, previous findings on antioxidant levels in human blood are somewhat conflicting according to the inclusion criteria (age, intercurrent diseases, habitus).

Oxidative metabolism was investigated in 198 elderly healthy women more than 75 yr old (81.6 ± 3.7; 75–99) living at home. Oxidative stress was evaluated by determining: the end product of lipid peroxidation, malondialdehyde (TBARS), and plasma and red blood cell (RBC) lipid content in polyunsaturated fatty acids (PUFA); nonenzymatic antioxidant system: plasma and RBC α-tocopherol (pl Vit. E and RBC Vit. E), RBC glutathione (GSH); enzymatic antioxidant: RBC superoxide dismutase (SOD) and plasma glutathione peroxidase (GPx); and trace elements (copper, zinc, selenium). Nutritional status was monitored by body mass index, caloric intake, protein and lipid markers of malnutrition (albumin, prealbumin, retinol-binding protein, cholesterol, and triglycerides). Results were compared to a control group of 50 healthy young women (age range 20–45 yr).

Despite reduced caloric intake (average 1582 kcal, range 697–3352), elderly women did not have any clinical (body mass index: 25 kg/m^2) or biological criteria of malnutrition: albumin (43.9 ± 3.4 vs. 40.5 ± 3.1 g/L; $P < 0.001$), prealbumin (0.34 ± 0.17 vs. 0.29 ± 0.1 g/L; $P < 0.01$), retinol-binding protein (44.8 ± 10 vs. 43.7 ± 11 mg/L), cholesterol (6.13 ± 1.1 vs. 5.12 ± 0.95 mmol/L; $P < 0.001$), and triglycerides (1.18 ± 0.56 vs. 0.99 ± 0.45 mmol/L) were similar or increased in elderly vs. young women, respectively. When compared to healthy volunteers, SOD activity (1.16 ± 0.23 vs. 1.28 ± 0.25 U/mg Hb, $P < 0.01$) and RBC GSH (4.32 ± 1.10 vs. 4.69 ± 1.06 nmol/mg Hb, $P < 0.05$) were significantly decreased, whereas pl Vit. E, RBC Vit. E, and GPx remained unchanged; copper (1.17 ± 0.21 vs. 1.31 ± 0.38 mg/L; $P < 0.001$) and zinc levels (0.86 ± 0.16 vs. 0.94 ± 0.16 mg/L; $P < 0.01$) were decreased; by contrast selenium levels were unaffected (75 ± 22 vs. 80 ± 17 µg/L). Despite these impairments in antioxidant defense mechanisms, we did not observe any increase in TBARS level (1.26 ± 0.36 vs. 1.34 ± 0.37 mmol/L) or a decrease in PUFA content in plasma and RBC phospholipids.

In healthy, well-nourished elderly populations, the impairment in antioxidant system did not result in an oxidative injury but in a precarious balance. This weak equilibrium could result in an increased susceptibility to reactive oxygen species and could be an underlying event for age-related diseases.

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Abbreviations: GPX, glutathione peroxide; GSH, glutathione; PUFA, polyunsaturated fatty acids; RBC, red blood cell; SOD, superoxide dismutase; TBARS, malondialdehyde.

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A High Linoleic Acid Diet Increases Oxidative Stress *in Vivo* and Affects Nitric Oxide Metabolism in Humans

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Oxidative stress is considered to be involved in the initial stages of atherosclerosis. A hypothesis based on *in vitro* findings suggests that polyunsaturated fatty acids are a potential source of oxidative stress in humans (1–3). Oxidation products, in turn, may have an inhibitory effect on NO production (4,5) and thus on endothelium-dependent vasodilation. To study whether high-linoleic acid (LA) diets cause oxidative stress *in vivo* in a way that affects endothelial function, we measured the urinary excretion of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) and urinary NO metabolites as well as the plasma levels of intercellular adhesion molecule (ICAM)-1 from healthy subjects participating a controlled dietary intervention with either high-LA or high- oleic acid (OA) diets.

Subjects. Thirty-eight healthy volunteers (20 women,18 men; mean 26.6 yr) were selected from university students and employees. Exclusion criteria were serum cholesterol > 7.0 mmol/L, hypertension, anemia, glycosuria, and proteinuria. Furthermore, 13 control subjects were selected for the study. They had their habitual diets throughout the study but gave the same samples as the participating subjects.

The experiment lasted 8 wk. During the first 4 wk the subjects were given butter and advised to use other foods rich in dairy fat. For the second 4 wk 19 subjects were given a high-LA diet and 19 subjects a high-OA diet in a controlled manner. Ninety percent of daily energy intake was provided. The two diets were designed to contain similar proportions of energy from fats, carbohydrates, and proteins, similar proportions of saturated fatty acids, and similar amounts of cholesterol. They differed only in their proportions of LA and OA. At the end of each diet period the subjects gave two blood samples and collected five 24-h urine samples.

Methods. The urinary excretion of 8-iso-$PGF_{2\alpha}$ was analyzed using a newly developed radioimmunoassay (6). Concentrations of urinary NO metabolites and soluble ICAM-1 (sICAM-1) in plasma were measured with respective immunoassays (Cayman Co., Ann Arbor, MI, and R&D Systems, Minneapolis, MN).

Results. The LA diet significantly increased the excretion of 8-iso-$PGF_{2\alpha}$ (from 1.7 to 2.4 ng/μmol creatinine, $P = 0.04$) and decreased levels of urinary NO metabolites (270 vs. 166 μg/μmol creatinine, $P = 0.03$) (Table 1). No significant changes were found after the OA diet. In the control group the excretion of nitrate slightly increased ($P = 0.05$) during the study. The plasma concentration of sICAM-1 and

TABLE 1
Plasma α-Tocopherol, Plasma Soluble ICAM-1, Urinary 8-Iso-$PGF_{2\alpha}$, and Urinary Nitrate Metabolites of 38 Subjects and 13 Controls Who Consumed High-Linoleic Acid (LA) or High-Oleic Acid (OA) Diets After a Saturated Fat Baseline Diet

	LA-group (n = 19)		OA-group (n = 19)		Controls (n = 13)	
	Baseline	Experimental	Baseline	Experimental	Baseline	Experimental
α-Tocopherol (mg/L)	10.1 (2.2)	10.6 (2.8)	10.0 (0.9)	10.3 (2.2)	10.5 (3.4)	10.2 (2.6)
8-Iso-$PGF_{2\alpha}$ (ng/μmol creatinine)	1.7 (1.0)	2.4 (1.2)*	1.5 (0.7)	1.8 (0.8)	1.6 (0.9)	1.3 (0.6)
NO metabolites (μg/μmol creatinine)	270 (263)	166 (147)*	307 (253)	253 (244)	204 (169)	374 (294)**
sICAM-1 (ng/mL)	288 (102)	277 (91)	297 (83)	294 (92)	305 (89)	298 (67)

[a]Values are mean (SD); *P < 005, **P = 0.05 denote significance of difference from the preceding baseline diet. Abbreviations: sICAM-1, soluble intercellular adhesion molecule; 8-iso-$PGF_{2\alpha}$, 8-isoprostaglandin $F_{2\alpha}$.

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Abbreviations: 8-iso-$PGF_{2\alpha}$, 8-isoprostaglandin $F_{2\alpha}$; LA, linoleic acid; OA, oleic acid.

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α-tocopherol stayed unaltered both during the dietary treatments and in the control group.

Discussion. The possibility raised by *in vitro* experiments that a high intake of LA would increase oxidative stress in the body is supported by the results of our strictly controlled human experiment: urinary excretion of 8-oxo-$PGF_{2\alpha}$ was significantly increased in these healthy subjects although their intake and plasma levels of antioxidants were within recommendations. Our results also indicate that both the synthesis of F_2-isoprostanes and NO may be modulated by dietary fatty acid composition. Whether the decrease in urinary NO metabolites in the present study was due to an increase in reactive oxygen metabolites capable of inhibiting NO cannot be answered. Dysfunction of the endothelium is unlikely due to the short duration of the study and the health status of our subjects. This is also supported by the unchanged sICAM levels. Increased levels of circulating ICAM-1 have been found in several inflammatory diseases involving leucocyte activation (7).

Although endothelial dysfunction after this short period is unlikely, even a slight decrease in NO for a longer period may be of consequence to human health.

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Vitamin E Inhibition of $O_2^{\bullet -}$ Production in the Promonocyte Cell Line THP-1 Is Essentially Due to *RRR*-δ-Tocopherol

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According to the oxidative theory of atherosclerosis, vitamin E—a potent peroxyl radical scavenger—prevents oxidation of low-density lipoprotein (LDL) which possibly explains the decrease in the risk of cardiovascular disease reported by epidemiological studies and nutritional intervention. Vitamin E also mediates cell actions. In particular, vitamin E decreases monocyte $O_2^{\bullet -}$ production that is involved in LDL oxidation. We previously found that α-tocopherol (α-Toco)-containing LDL (the main circulating form of vitamin E) decreases monocyte $O_2^{\bullet -}$ production by a cell-mediated action in a manner dependent on the LDL α-Toco content. In addition, we recently established that α-Toco impairs the assembly of the NADPH oxidase complex through cell events involving a decrease in protein kinase C (PKC)-mediated phosphorylation of $p47^{phox}$. We also showed that in monocytes vitamin E is able to decrease the membrane activity but not the membrane translocation of PKC.

We already know that antioxidant properties of vitamin E are not bound to the molecular stereostructure. Because of cell event involvement in the decrease of the monocyte $O_2^{\bullet -}$ production, we suspected that this phenomenon was stereospecifically related to the molecular structure of vitamin E. It is the reason why we decided to evaluate the inhibition potency of either a mixture of the different forms or the different forms separately of *RRR*-stereoisomers, and to more specifically compare *RRR*-α-Toco and *RRR*-δ-Toco to all-*rac*-α-Toco and all-*rac*-δ-Toco, respectively.

To do so, we chose to use the THP-1 promonocyte cell line after a 72 h differentiation through the 10^{-6} M retinoic acid, 10^{-7} M 1,25-di-OH-calciferol, and 100 U/mL interferon γ treatment. Once differentiated, cells were pre-incubated in the presence of the molecule(s) to be tested at the concentration of 20 mg/L (a physiological concentration) for 24, 48, or 72 h. They were then activated for 90 min in the presence of 0.1 μM phorbol myristate acetate. All these operations were carried out at 37°C. Lucigenin-enhanced bioluminescence was finally assessed by means of an LKB Wallac 1251 luminometer. The luminescence signal was counted for 10 s every 20 s. Results corresponded to the maximal signal value recorded with the tested molecules after a 90-min incubation in the presence of phorbol myristate acetate and expressed as percentage of the value obtained without tested molecules (but with hydrogenated soy oil as vehicle). It was verified that superoxide dismutase was able to completely depress the luminescence signal and that the tested molecules were unable to diminish the signal in a noncellular system represented by the enzyme–substrate mixture xanthine oxidase/xanthine. Measures were always carried out at least in triplicate, each incubation time on separate cell preparations.

We found that the mixture of *RRR*-α-Toco, *RRR*-β-Toco, *RRR*-γ-Toco, and *RRR*-δ-Toco (Covi-ox T-70 from Henkel, 12/2/60/21 on a weight basis, determined by high-performance liquid chromatography) depressed the THP-1 $O_2^{\bullet -}$ production during the incubation time period in a time-dependent manner, with a 54% inhibition after 72 h. *RRR*-δ-Toco was the most efficient (≈80% inhibition), whereas *RRR*-α-Toco unexpectedly had no effect. We established that *RRR*-δ-Toco had a higher inhibition potency than the all-*rac*-δ-Toco after 24-h incubation (46 vs. 17%) but not after 72-h incubation (73 vs. 72%). On the contrary, all-*rac*-α-Toco was found without effect as was *RRR*-α-Toco.

This suggests that an initial recognition step takes place in the NADPH oxidase inhibition process in these cells, involving at least one asymmetric center (possibly the C2 position). This is in accordance with the vitamin E cell transporter-receptor hypothesis. However, we totally ignore if this is a membrane or cytosolic event. Given our recent findings regarding the vitamin E inhibition of $O_2^{\bullet -}$ production in monocytes, it would be interesting first to confirm the general and physiological significance of this phenomenon and then to examine the respective effect of stereoisomers on the membrane-PKC activity and on the cytosolic-phosphorylation of $p47^{phox}$

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Abbreviations: LDL, low density lipoprotein; PKC, protein kinase C; α-Toco, α-tocopherol.

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Stimulation of Platelet Aggregation in Response to Arachidonic Acid Hydroperoxide *via* Phospholipase Activation

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Considerable interest exists concerning the role played by lipid peroxides in cell damage and in the development of cardiovascular diseases. Indeed, an exacerbated platelet activation, as observed in aging, was associated with an increased overall lipid peroxidation and an increased peroxidation of arachidonic acid (AA). The platelet antioxidant defenses were altered, namely, the vitamin E level and the activity of glutathione-peroxidase (GSH-Px) (1), a key enzyme reducing lipid hydroperoxides. A transient accumulation of the 12-lipoxygenase-derived product of AA, 12-hydroperoxy-eicosatetraenoic acid (12-HPETE), resulting from the decreased GSH-Px activity, is likely to activate the dioxygenases and therefore to lead to platelet hyperactivation. Considering that the biological functions of lipid hydroperoxides are not well-defined, it is of interest to determine whether low concentrations of 12-HPETE may induce platelet aggregation. Human platelets were incubated in the presence or absence of 12-HPETE for 1 min at 37°C. A subthreshold concentration (STC) of collagen—defined as the highest concentration of collagen that did not induce any platelet aggregation—was then added to the platelet suspension for another minute. Although 12-HPETE alone had no effect on platelet aggregation, the addition of nanomolar concentrations of 12-HPETE (from 10 to 100 nM) together with an STC of collagen significantly induced platelet aggregation. It was closely associated with an increased formation of thromboxane B_2, the stable catabolite of thromboxane A_2. Notably the concentration of 12-HPETE required to potentiate platelet aggregation was 50-fold lower than the one required to prime platelet aggregation in response to AA *via* an activation of the cyclo-oxygenase (2). As the activation of platelets by collagen involves the release of AA from platelet phospholipids, the concentration of unesterified AA was determined by gas chromatography. The addition of 12-HPETE to platelets co-incubated with an STC of collagen resulted in a threefold increase in the amount of unesterified AA. As it is known that the cytosolic phospholipase A_2 ($cPLA_2$) of 85 kDa is involved in the release of AA from membrane phospholipids, platelets were preincubated with a potent and selective inhibitor of the $cPLA_2$, arachidonoyl trifluoromethyl ketone ($AACOCF_3$). $AACOCF_3$ prevented the increase in the amount of unesterified AA induced by 12-HPETE and an STC of collagen, suggesting the involvement of the $cPLA_2$. In order to determine the origin of released AA, the molecular species of platelet phospholipid subclasses were measured by reversed-phase high-performance liquid chromatography (HPLC) (3). After lipid extraction, phospholipid classes were separated by thin-layer chromatography (TLC), hydrolyzed by phospholipase C, and corresponding diradylglycerols were derivatized with 3,5-dinitrobenzoyl chloride. Phospholipid subclasses were separated by TLC, and various molecular species were quantified by HPLC with ultraviolet detection at 240 nm. Among the arachidonoyl-containing molecular species of 1,2-diacyl-glycerophosphocholine (GPC), 16:0/AA and 18:0/AA species decreased in platelets incubated with 12-HPETE whereas 18:1/AA did not change significantly. Concerning the molecular species of the 1-alkyl-2-acyl-GPC subclass, 18:1/AA, 16:0/AA, and 18:0/AA tended to decrease in response to 12-HPETE. On the contrary, no difference was observed between control platelets and 12-HPETE-treated platelets in the 1,2-diacyl-glycerophosphoethanolamine pool.

In conclusion, physiologically relevant concentrations of 12-HPETE stimulate the aggregation of platelets co-incubated with nonaggregating concentrations of collagen which could be mediated *via* an activation of the $cPLA_2$. These results enlighten the physiological importance of 12-HPETE in controlling the unesterified AA level and therefore the formation of eicosanoids.

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Abbreviations : AA, arachidonic acid; $AACOCF_3$, arachidonoyl trifluoromethylketone; $cPLA_2$, cytosolic phospholipase A_2; GPC, glycerophosphocholine; GSH-Px, glutathione-peroxidase; HPLC, high-performance liquid chromatography; 12-HPETE, 12-hydroperoxy-eicosatetraenoic acid; STC, sub-threshold concentration; TLC, thin-layer chromatography.

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New Products from the Agri-Food Industry: The Return of n-3 Fatty Acids into the Food Supply

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ABSTRACT: The meat from animals and fish in the wild, chicken eggs produced under complete natural conditions, and wild plants contain higher amounts of n-3 fatty acids compared to domesticated or cultivated ones. The composition of meats, fish, and eggs is dependent on animal feed. Fish-meal, flax, and n-3 from algae in animal feeds increase the n-3 fatty acid content of egg yolks and lead to the availability of n-3 fatty acid-enriched eggs in the marketplace. Research is ongoing for the production of n-3 fatty acid-enriched products from poultry, beef, lamb, pork, milk, bakery products, etc. In the case of n-3 fatty acid-enriched eggs, the egg under complete natural conditions (Greek or Ampelistra egg) can serve as a guide for proper composition. Otherwise, the amount of n-3 fatty acids is determined by the organoleptic properties of the products. It is essential in the process of returning the n-3 fatty acids into the food supply that the balance of n-6/n-3 fatty acids in the diet that existed during evolution is maintained. Clinical investigations confirm the importance of n-3 fatty acids for normal function during growth and development and in the modulation of chronic diseases. The availability of n-3 fatty acid-enriched products should lead to improvements in the food supply. Pregnant and lactating women and infants should benefit since their diet is deficient in n-3 fatty acids, especially for the vegetarians among them. Studies with n-3-enriched eggs lower cholesterol levels, platelet aggregation, and blood pressure. Since cardiovascular disease, hypertension, and autoimmune, allergic, and neurological disorders appear to respond to n-3 fatty acid supplementation, a diet balanced in n-3 and n-6 fatty acids consistent with the diet during human evolution should decrease or delay their manifestation.

The health of the individual and the population in general is the result of interactions between genetics and the environment. Nutrition is an environmental factor of major importance. Furthermore, each disease has medical and cultural components. Western culture has brought about many changes in the nutritional environment of human beings. Major changes have taken place since the agricultural revolution 10,000 yr ago and even more since the industrial revolution 200 yr ago (Fig. 1) (1). Agribusiness and the processed food industry have made it possible to increase the production and shelf life of many products, but at the same time brought about a change in the content and nutrient structure of many foods, i.e., (i) whereas human milk contains arachidonic acid (AA), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), infant formula is devoid of these fatty acids in the United States; and (ii) the hydrogenation process led to structural changes in the double bonds of fatty acid molecules from *cis* to *trans* resulting in an increase in *trans* fatty acids found in margarine, frying oils, salad dressings, and bakery products. The increase in *trans* fatty acids in the food supply has raised concerns about their role in increasing the risk of coronary heart disease and in interfering with the desaturation and elongation of essential fatty acids (EFA) 18:2n-6 and 18:3n-3 (2). Industry is now taking measures to reduce the content of *trans* fatty acids in margarine. In the past decade, science has provided new information on the importance of the balance between n-3 and n-6 fatty acids in health and disease and in growth and development (3,4), which must be taken into consideration for a healthful food supply.

Paleolithic diet. While all these nutritional changes have taken place, our genetic profile has not changed significantly

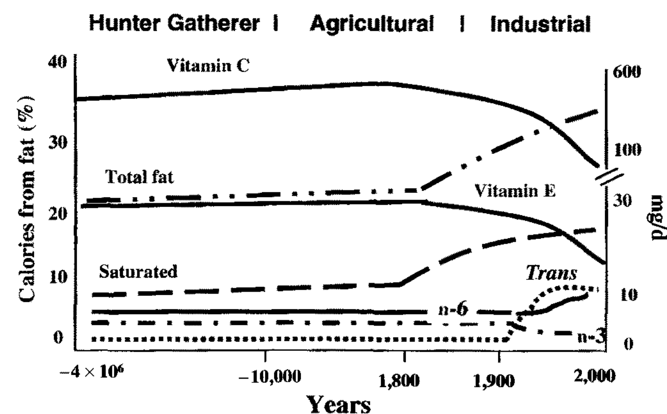

FIG. 1. Hypothetical scheme of fat, fatty acid (n-3, n-6, *trans* and total) intake (as percentage of calories from fat) and intake of vitamins E and C (mg/d). Data were extrapolated from cross-sectional analyses of contemporary hunter–gatherer populations and from longitudinal observations and their putative changes during the preceding 100 yr (Ref. 1).

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Abbreviations: AA, arachidonic acid; DHA, docosahexaenoic acid; DPA, docosapentaenoic acid; EFA, essential fatty acid; EPA, eicosapentaenoic acid; LA, linoleic acid; LNA, linolenic acid.

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TABLE 1
Estimated n-3 and n-6 Fatty Acid Intake in the Late Paleolithic Period (g/d)[a]

Plants		
LA	4.28	
LNA	11.40	
Animals		
LA	4.56	
LNA	1.21	
Total		
LA	8.84	n-6:n-3 = .70
LNA	12.60	
Animal		
AA (n-6)	1.81	n-6:n-3 = 1.79
EPA (n-3)	0.39	
DTA (n-6)	0.12	
DPA (n-3)	0.42	
DHA (n-3)	0.27	

[a]Data from Eaton *et al.* (6). LA, linoleic acid; LNA, linolenic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; DPA, docosapentaenoic acid; DHA, docosahexaenoic acid; DTA, docosatetraenoic acid.

over the past 40,000 yr. The Paleolithic diet is characterized by lower fat and lower saturated fat intake than Western diets; a balanced intake of n-6 and n-3 EFA; small amounts of *trans* fatty acids, contributing less than 2% of dietary energy; lots of green leafy vegetables and fruits providing higher levels of vitamin E and vitamin C and other antioxidants than today's diet; and higher amounts of calcium and potassium but lower sodium intake (1,5–7) (Fig. 1).

Today, the ratio of n-6/n-3 is between 10–20:1 in Western Europe and the United States, whereas during evolution it was 1:1 or even less (Tables 1 and 2) (1,3,5,6). Our current diet (Western diet) is characterized by an increase in total fat, saturated fat, *trans* fatty acids, and the n-6 EFA; a decrease in the n-3 EFA, the antioxidant vitamins C and E, and calcium and potassium; and an increase in sodium intake (1–8) (Fig. 1).

The change in the EFA balance came about because of the indiscriminate recommendation to substitute vegetable oils, i.e., corn oil, safflower, sunflower, and cottonseed oils, for saturated fat since 1960. These vegetable oils are very high in n-6 fatty acids and very low in n-3 fatty acids. Corn oil has a ratio of n-6/n-3 of 60:1 and safflower oil 77:1. In addition, because farm animals are grain-fed, their carcasses contain small amounts of n-3 fatty acids, but they are high in saturated fats and n-6 fatty acids unlike the composition of meat from animals in the wild (9). Eggs and poultry in agriculture, fish in aquaculture, and cultivated plants contain lower amounts of n-3 fatty acids than eggs from free-ranging chickens (Table 3) (10,11), fish in the wild (12), and wild plants (i.e., purslane) (Table 4) (13,14).

Information from archeological findings and studies from modern day hunter–gatherers suggest that the Paleolithic diet is the diet we evolved on and for which our genetic profile was programmed.

The traditional Greek diet. In addition to the information from studies on the Paleolithic diet, the studies on the traditional diet of Greece prior to 1960, and specifically Crete, provide further evidence for the higher content of n-3 fatty acids in eggs, meat, milk, cheese, and edible wild plants.

TABLE 2
Estimated n-3 and n-6 Fatty Acid Intake in Current Western Patterns (g/d)[a,b]

LA		22.5
LNA		1.2
AA		0.6
EPA		0.05
DTA		—
DPA		0.05
DHA		0.08
AA:n-3	= 3.33	
Total n-6:n-3 = 16.74		

[a]Data from Reference 6. See Table 1 for abbreviations.
[b]Assuming an energy intake of 35:65 of animal/plant sources.

TABLE 3
Fatty Acid Levels (mg/g yolk) in Chicken Egg Yolks[a]

Fatty acid	Greek egg	Supermarket egg	fish-meal egg	Flax egg
Saturates				
14:0	1.1	0.7	1.0	0.6
15:0	—	0.1	0.3	0.2
16:0	77.6	56.7	67.8	58.9
17:0	0.7	0.3	0.8	0.5
18:0	21.3	22.9	23.0	26.7
Total	100.7	80.7	92.9	86.9
Monounsaturates				
16:1n-7	21.7	4.7	5.1	4.4
18:1	120.5	110.0	102.8	94.2
20:1n-9	0.6	0.7	0.9	0.5
24:1n-9	—	—	0.1	—
Total	142.8	115.4	108.9	99.1
n-6 Polyunsaturates				
18:2n-6	16.0	26.1	67.8	42.4
18:3n-6	—	0.3	0.3	0.2
20:2n-6	0.2	0.4	0.6	0.4
20:3n-6	0.5	0.5	0.5	0.4
20:4n-6	5.4	5.0	4.4	2.6
22:4n-6	0.7	0.4	0.3	—
22:5n-6	0.3	1.2	0.2	—
Total	23.1	33.9	74.1	46.0
n-3 Polyunsaturates				
18:3n-3	6.9	0.5	4.1	21.3
20:3n-3	0.2	—	0.1	0.4
20:5n-3	1.2	—	0.2	0.5
22:5n-3	2.8	0.1	0.4	0.7
22:6n-3	6.6	1.1	6.5	5.1
Total	17.7	1.7	11.3	28.0
P/S ratio	0.4	0.4	0.9	0.9
M/S ratio	1.4	1.4	1.2	1.1
n-6/n-3 ratio	1.3	19.9	6.6	1.6

[a]Modified from Reference 10. The eggs were hard-boiled, and their fatty acid composition and lipid content were assessed as described elsewhere. Greek eggs, free-ranging chickens; supermarket eggs, standard U.S. Department of Agriculture eggs found in U.S. supermarkets; fish-meal eggs, main source of fatty acids provided by fish-meal and whole soybeans; flax eggs, main source of fatty acids provided by flax flour. P/S = Polyunsaturates/saturates. M/S = Monounsaturates/saturates.

TABLE 4
Fatty Acid Content (mg/g of wet weight) of Plants[a]

Fatty acid	Purslane	Spinach	Red leaf lettuce	Buttercrunch lettuce	Mustard
14:0	0.16	0.03	0.03	0.01	0.02
16:0	0.81	0.16	0.10	0.07	0.13
18:0	0.20	0.01	0.01	0.02	0.02
18:1n-9	0.43	0.04	0.01	0.03	0.01
18:2n-6	0.89	0.14	0.12	0.10	0.12
18:3n-3	4.05	0.89	0.31	0.26	0.48
20:5n-3	0.01	0.00	0.00	0.00	0.00
22:6n-3	0.00	0.00	0.002	0.001	0.001
Other	1.95	0.43	0.12	0.11	0.32
Total fatty acid content	8.50	1.70	0.702	0.60	1.101

[a]Adapted from Reference 13.

Table 5 compares the fatty acid content of various U.S. and Greek cheeses (15). AA, EPA, and DHA are found in the Greek but not in the American cheeses. Since the major source of cooking oil in Greece is olive oil, providing only 6–10% linoleic acid (LA), the n-6 content of the diet is easily balanced with the high fish intake. Furthermore, the n-3 fatty acids were present in each meal, providing a good balance of the n-6 and n-3 EFA in the diet of Crete (15).

The traditional Greek diet (the diet prior to 1960) and, for that matter, the diet of the people in Crete, is characterized by a high monounsaturated fatty acid intake (olive oil), low saturated fat intake, balanced n-6 and n-3 fatty acids, less than 2% *trans* fatty acids of total energy intake, and lots of green leafy vegetables and fruits. The Greek-Crete diet is closer to the Paleolithic diet than the current Western diets (16).

The people of Crete participated in the Seven Countries Study and were shown to have lower rates of heart disease and cancer, including higher longevity, than the populations of Japan, Yugoslavia, Italy, Holland, Finland, and the United States. The people of Crete had the highest amount of 18:3n-3 (α-linolenic acid, LNA) and the lowest amount of 18:2n-6 (LA) in their serum cholesteryl esters than the other six populations (17). Closer examination of the diet of Crete, or the traditional Greek diet, showed that the n-3 fatty acids were present in every meal the people ate, either as LNA (18:3n-3) or EPA (20:5n-3) and DHA (22:6n-3) or all three. The n-3 fatty acids were present in wild plants and other green leafy vegetables, and in meat and fish, eggs, milk, cheese, and all products made from milk and eggs, such as noodles and cookies. Because the people of Crete did not use, in cooking, vegetable oils such as corn oil, safflower, sunflower, or cottonseed oils, all rich in n-6 fatty acids, their n-6/n-3 ratio was just over 1, whereas in Western diets this ratio is usually over 10–20:1 (15).

The importance of a balanced ratio of n-6/n-3, a lower saturated fatty acid intake and lower overall fat (30–33%), along with higher intakes of fruits and vegetables leading to increases in vitamins E and C, was tested in the Lyon Heart study. The Lyon study, based on a modified Crete diet, confirmed the importance of n-3 fatty acids from marine and terrestrial sources, and vitamin E and vitamin C, in the secondary prevention of coronary heart disease (18,19).

In Western diets, the current consumption of total fat, saturated fat, n-6 fatty acids, and *trans* fatty acids is not consistent with the evolutionary aspects of diet. Because health as

TABLE 5
Fatty Acid Content[a] of Various Cheeses[b]

	2% Milk	Cheddar	American	Swiss	Greek Myzithra	Greek feta
Total saturated fat	1.2	21.00	19.69	16.04	9.30	7.20
12:0	<1	.54	.48	.57	—	—
14:0	<1	3.33	3.21	2.70	1.90	1.60
16:0	<1	9.80	9.10	7.19	5.40	3.90
18:0	<1	4.70	3.00	2.60	2.00	1.70
Total monounsaturated fat	1	9.99	8.95	7.05	3.90	3.00
Total polyunsaturated fat	.07	.94	.99	.62	.80	.58
18:2	.04	.58	.61	.34	.38	.29
18:3	.03	.36	.38	.28	.30	.20
Arachidonic acid	—	—	—	—	14 mg	10 mg
Eicosapentaenoic acid	—	—	—	—	18 mg	14 mg
Docosapentaenoic acid	—	—	—	—	31 mg	23 mg
Docosahexaenoic acid	—	—	—	—	5.5 mg	5.1 mg
Total fat	2.27	31.93	29.63	23.71	14.00	10.78

[a]Amount in g/100 g edible portion, except where noted.
[b]*Note*: milk, Cheddar, American and Swiss from U.S. Department of Agriculture Handbook #8; Greek Myzithra and Greek feta from National Institute on Alcohol Abuse and Alcoholism analyses. Reproduced with permission from Reference 15.

well as disease are the result of the interaction of genes and the environment—in this case, genes and nutrients—the presently constituted Western food supply is not appropriate for health, particularly for individuals genetically predisposed to chronic diseases such as coronary heart disease, hypertension, diabetes, arthritis, and possibly cancer. The time has come to return the n-3 fatty acids into the food supply. Human beings need both classes of fatty acids, the n-6 and n-3, and the 18 carbon (LNA) and the 20–22 carbon atoms (EPA and DHA).

The return of n-3 fatty acids into the food supply. There are now a number of products in the market that are enriched with n-3 fatty acids. They fall into the following categories:

(i) Oils. Oils rich in LNA, such as canola, flaxseed, perilla, and soybean. In addition, avoidance of vegetable oils rich in n-6 and the use of oils lower in LA such as olive oil, canola oil, and new vegetable oils rich in monounsaturated oils help bring about an improvement in the LA/LNA ratio (16).

(ii) Bakery products. Flaxseed flour and encapsulated fish oils (20) are used in bakery products, including breads.

(iii) Eggs. Changes in chicken feeds led to enrichment of n-3 fatty acids in eggs. n-3-Enriched eggs are found in many markets around the world. The chicken feeds are enriched with fish-meal, flaxseed, or DHA from algae (10,11,21,22). These eggs have a lower n-6/n-3 ratio and contain significant amounts of AA and DHA and are modeled after the "natural egg" (i.e., the egg from the Ampelistra farm in Greece listed in Table 3 as the Greek egg) which is the egg obtained under completely natural conditions (10,11).

(iv) Infant formula. Human milk contains AA, DHA and EPA, whereas infant formula based on cow milk does not. In Europe and the Far East, infant formula is now enriched with AA and DHA from various sources (23,24). However, in the United States, infant formula does not contain AA and DHA, yet.

(v) Milk. Research shows promising results in increasing DHA in cow milk (25).

(vi) Mayonnaises, margarines, and salad dressings. Hydrogenated fish oils and canola oils are used in the preparation of mayonnaise, margarines, and salad dressings (20).

(vii) Meat and poultry products. Research on how best to titrate the amount of fish oils in animal feeds without affecting stability and organoleptic properties is advancing in many parts of the world. Poultry, cattle, and pigs are being studied, and the consumption of n-3-enriched meats is not far in the future (21,26,27).

(viii) Farmed fish. There is a need to further improve the fatty acid composition of fish in aquaculture (12).

Enriched processed foods are a feasible way to increase EPA and DHA intake and bring about beneficial changes (28). Consumption of the new products modulates tissue fatty acid composition; decreases triglycerides and platelet aggregation; and reduces low density lipoprotein particle density, making it less atherogenic (22).

Issues. Research advances are solving a major problem that is the deficiency of n-3 fatty acids in Western diets. Yet at the same time a number of questions need to be answered. How much n-3 fatty acids should be in each serving? How much LNA and how much EPA, docosapentaenoic acid (DPA, 22:5n-3) and DHA? What should be the ratio of total n-6/n-3, how much LA/LNA, and what should be the proportions of the n-3 fatty acids EPA, DPA, and DHA? What are the best models—animals in the wild, fish in the wild, mother's milk from women on a paleolithic diet or a Greek/Mediterranean diet?

What can we learn from the composition of edible wild plants? We have shown that edible wild plants contain more LNA than LA. But, Guil *et al.* (29) showed that many of the edible wild plants along the Mediterranean contain AA, EPA, DPA, and DHA (Table 6). Table 6 shows that certain plants are higher in AA than EPA, DPA and DHA. The findings of Guil *et al.* are provocative and important. Although their findings need to be confirmed, they point to a need to have precise information on the fatty acid composition of traditional diets. Obviously, there is a need to investigate further edible wild plants in order to determine the terrestrial and marine sources of the 20- to 22-carbon polyunsaturated fatty acids.

Conclusion. n-3 Fatty acids must become incorporated into foods rather than be used solely as dietary supplements, which is a quasi-pharmaceutical approach. Furthermore, the development of a variety of n-3-rich foodstuffs would allow increased dietary intakes with little change of dietary habits.

n-3 Fatty acids maintain preventive and therapeutic properties when packaged in a food other than fish. Efficient use of dietary n-3 fatty acids will require the simultaneous reduction in the food content of n-6 fatty acids and their substitution with monounsaturated oils. Dietary n-3 fats give rise to

TABLE 6
Edible Wild Plants (percentage of fatty acid content)[a]

Species	AA	EPA	DPA (22:5n-3)	DHA
Wild beet (*Beta maritima* L.)	0.52	0.54	0.49	0.65
Hoary cress (*Cardaria draba* L.)	0.56	2.16	0.00	0.00
Goosefoot (*Chenopodium opulifolium* Schrader)	0.00	3.06	0.74	2.30
Goosefoot (*Ch. album* L.)	1.30	0.36	0.00	0.00
Goosefoot (*Ch. murale* L.)	1.01	0.41	0.00	0.00
Rock samphire (*Crithmum maritimum* L.)	0.00	0.76	0.76	0.00
Plantain (*Plantago major* L.)	1.02	1.27	0.00	1.47
Hedge mustard (*Sisymbrium irio* L.)	0.32	0.55	0.21	0.83
Common mallow (*Malva sylvestris* L.)	5.30	0.00	0.00	0.00
Sow-thistle-of-the-wall (*Sonchus tenerrimus* L.)	1.83	0.00	0.38	0.00

[a]Data from Reference 29. See Table 1 for abbreviations.

higher tissue levels of EPA when the "background" diet is low in n-6 fats. Compared to n-6 fatty acids, olive oil increases the incorporation of n-3 fatty acids into tissues.

In the past, industry focused on improvements in food production and processing to increase shelf life of the products, whereas now and in the future the focus will be on nutritional quality in product development (30,31). This will necessitate the development of research for the nutritional evaluation of the various food products and educational programs for professionals and the public (30). The definition of food safety will have to expand in order to include nutrient structural changes and food composition (31). The dawn of the twenty-first century will enhance the scientific base for product development and expand collaboration among agricultural, nutritional, and medical scientists. This should bring about a greater involvement of nutritionists and dieticians in industrial research and development to respond to an ever-increasing consumer interest in the health attributes of foods.

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n-3 Fatty Acids and Coronary Heart Disease—The Urgent Need of Clinical Trials

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Before 1970 there were only scattered reports that dietary n-3 polyunsaturated fatty acids (n-3 PUFA) might protect against coronary heart disease (CHD). However, our studies beginning in 1970 in traditionally living Greenland Eskimos with an extremely high intake of n-3 PUFA drew interest to the potential role for prevention of CHD of n-3 PUFA. Based on five expeditions to Greenland (1–4), we (Bang and Dyerberg) were able to show that this Inuit population had a remarkably low incidence of CHD, and due to their diet, an antiatherogenic lipid pattern, a strongly attenuated platelet reactivity, and favorable indices of platelet vessel–wall interaction (1–6). This made us and other scientists pursue the intriguing hypothesis that seafood, rich in n-3 PUFA, might reduce the risk of CHD.

Now, nearly 30 yr later, much more knowledge about the physiology, biochemistry, potentially preventive mechanisms of action, and other effects of n-3 PUFA has been gained. Thus, lessons from epidemiology, experimental studies, and from the effects of n-3 PUFA on established and new risk factors for CHD in humans have substantiated the existence of a beneficial effect of n-3 PUFA in the prevention and treatment of CHD (7).

In this paper we will deal with the crucial issue of clinical trials and in some detail discuss and suggest new trials and the problems associated with such studies. We will limit ourselves to studies with marine n-3 PUFA in humans and consequently not include α-linolenic acid and only discuss trials directly related to CHD.

CLINICAL TRIALS IN PATIENTS WITH CHD

Stable angina pectoris. Saynor *et al.* (8) in 1984 reported an astonishing effect of low doses (1.8 g/d) of dietary n-3 PUFA supplementation on angina symptoms in patients with stable angina pectoris in an open trial. We were, however, unable to confirm this in a larger controlled trial (9), and other studies on small groups of patients were also negative (6).

Acute myocardial infarction. There is some experimental evidence that n-3 PUFA may be beneficial in acute myocardial ischemia and reduce myocardial infarct size (10). However, no studies have been published in patients admitted with an acute myocardial infarction, unstable angina pectoris, or other acute coronary syndromes. A recent major prospective cohort study demonstrated an inverse association between fish consumption and 30-yr risk of fatal myocardial infarction (11).

Secondary prevention after myocardial infarction (MI). Burr *et al.* (12) in the diet and reinfarction trial (DART) randomized more than 2000 men with a recent MI to various dietary changes including an increased intake of n-3 PUFA by eating fatty fish twice weekly or, alternatively, using fish oil capsules (taken by approximately 20% of the participants) for 2 yr. There was a significant 29% reduction in total mortality and deaths from CHD in those given fish advice and increasing their intake of n-3 PUFA by approximately 1 g/d. However, the number of nonfatal MI increased—nonsignificantly—in the group given fish advice. This indicates that the underlying mechanism of cardioprotection is less likely to be antithrombotic, and the authors suggested an antiarrhythmic effect of n-3 PUFA as an explanation. This study was a landmark trial and still provides the best clinical evidence for a protective role of n-3 PUFA in CHD.

In the more recent Lyon study investigating the effect of a Mediterranean type of diet in post-MI patients, there was a marked effect of dietary changes in preventing coronary deaths and coronary events (13). However, in this trial an increased intake of fish was only one of several dietary changes making it impossible to know what role the (marginally) increased intake of marine n-3 PUFA had in the outcome.

Coronary bypass surgery. In a study from Norway, 610 patients undergoing coronary bypass surgery were either supplemented with approximately 3.5 g of n-3 PUFA as fish oil capsules daily or assigned to a control group (14). The primary end point was graft patency assessed by angiography 1 yr after the bypass operation. There was a significant positive effect of fish oil on venous graft patency, but not on arterial (mammary) grafts.

Restenosis after percutaneous transluminal coronary angioplasty (PTCA). An unsatisfactorily high amount of patients treated with PTCA developed restenosis. The effect of

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Abbreviations: CHD, coronary heart disease; MI, myocardial infarction; PTCA, percutaneous transluminal coronary angioplasty; n-3 PUFA, n-3 polyunsaturated fatty acid.

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supplementation with n-3 PUFA in the prevention of restenosis was evaluated in several trials. The results were initially promising with significant beneficial effects reported in several randomized trials: an effect that was significant, when the data were subjected to a meta-analysis (15). However, recent larger trials set up to finally prove a beneficial effect of n-3 PUFA in these patients unfortunately failed to demonstrate this (16,17).

Cardiac transplant patients. It is not known if n-3 PUFA affect graft survival, development of coronary atherosclerosis, or prognosis in cardiac transplant recipients, but some studies showed positive biochemical effects (6).

Arrhythmias and sudden cardiac death. Animal studies (18), *in vitro* experiments (19), autopsy findings (20), and—however indirectly—diet and reinfarction trial (12) suggested an antiarrhythmic effect of n-3 PUFA. In line with this, a reduced risk for sudden cardiac death was reported in recent major case control and prospective cohort studies in fish consumers compared to those not eating fish (21,22). Furthermore, we showed (23) that n-3 PUFA beneficially influence heart rate variability, a powerful predictor of sudden cardiac death.

No effect on ventricular extrasystoles of 4 g n-3 PUFA was observed in a small cross-over study of patients with CHD (24). In a pilot study we randomized patients with previous serious ventricular arrhythmias to a supplement with 5.2 g n-3 PUFA daily or matching placebo for 16 wk (25). Those given n-3 PUFA experienced a large, but nonsignificant reduction in ventricular extrasystoles. Finally, in a larger study of healthy subjects with many extrasystoles, cod liver oil (2.4 g n-3 PUFA per day for 16 wk) had some effect on the number of ventricular extrasystoles (26).

SUGGESTIONS FOR FUTURE CLINICAL TRIALS

The area of primary prevention of CHD is an important, but difficult area to study. Prospective cohort studies seem today to be the only feasible way of studying this issue. The findings from DART in the secondary prevention of CHD need to be repeated. A study from Italy (GISSI) is currently investigating the effect of supplementation with fish oil capsules in post-MI patients, and the results are eagerly awaited.

It would be of interest to study the effect of n-3 PUFA in patients with acute coronary syndromes, not least if intravenous preparations with n-3 PUFA currently under investigation turn out to be safe.

At present there is no indication for the use of n-3 PUFA to prevent restenosis after PTCA. However, approximately two-thirds of patients are now treated with stents during PTCA, which has considerably reduced the risk of restenosis. The published studies with n-3 PUFA were all performed in the pre-stent era, and it would be worthwhile to study the effect of n-3 PUFA in stented patients.

The findings from Norway (14) in patients after bypass surgery call for further studies to prove or disprove an effect of n-3 PUFA in this setting.

One of the most promising areas to pursue may be the possible effect of n-3 PUFA on sudden cardiac death due to a protective effect against malignant arrhythmias. This may be studied in patients with implantable defibrillators, because such patients often develop serious arrhythmias, and documentation for the arrhythmias are stored on computer disks.

No matter what studies are undertaken, it is extremely important to be aware that only studies with hard end points (total mortality, mortality from CHD, mortality from cardiovascular disease, and perhaps also the number of revascularizations) are likely to have impact on the treatment modalities chosen by cardiologists. Such studies have to be randomized, controlled, and must include many patients. The studies are consequently going to be expensive, and funding is an obvious problem, due to the nonpatentability of the fish oil products.

FISH VS. FISH OIL CONCENTRATES

The message of the value of an increased intake of fish by the population was well-received by nutritional experts, in part because lean fish has a low content of saturated fat. It is also to some extent accepted by the general population, but the intake of marine n-3 PUFA is still low and below 1 g/wk in many Western countries. Several, but not all, studies showed that an intake of fish providing n-3 PUFA in this order of magnitude may offer protection against CHD (10–12,21,22,27). This is puzzling, because the many biochemical effects of n-3 PUFA in general are dose-dependent and only observed at doses well above what is obtained by this amount of fish (6).

When it comes to clinical trials, studies investigating the effects of an increased intake of fish are theoretically attractive, but with some important inborn methodological problems. Replacing other food sources (rich in saturated fat) with fish should in principle be better than adding fish oil supplements on top of the usual diet. However, only low amounts of n-3 PUFA are provided by this approach, and although some support from epidemiology exists, other well-known effects supposed to be beneficial in the reduction of CHD can only be obtained by higher doses of n-3 PUFA. Also, blinding of study participants in clinical trials is not possible. Practically, it is much easier to investigate the effect of a medication (fish oil supplements) than to provide a certain amount of fish regularly for an extended period of time. Another problem is that some people dislike seafood.

Despite these reservations, we still believe that clinical trials with an increased intake of fish in the diet are of major relevance and interest. However, studies with higher doses of fish oil concentrates are equally warranted, and in evaluation of the use of fish oil concentrates in the prevention and/or treatment of CHD the same standards of evidence for fish oil supplements should be required as for other medications.

CONCLUSIONS

Data from epidemiological studies, animal experiments, and clinical trials of biochemical effects in humans lend support for

a beneficial role of n-3 PUFA in CHD. So far only a few trials of n-3 PUFA with clinical endpoints have been published. Further clinical studies in patients with acute myocardial ischemia, post-MI patients, patients undergoing coronary bypass surgery or stenting during PTCA, and patients with arrhythmias should be undertaken. Only results from controlled clinical trials with hard end points can establish whether n-3 PUFA reduce the risk of CHD and will have an impact on patient treatment.

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Equal Antithrombotic and Triglyceride-Lowering Effectiveness of Eicosapentaenoic Acid-Rich and Docosahexaenoic Acid-Rich Fish Oil Supplements

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The relative proportions of eicosapentaenoic acid (EPA) and docosahexaenoic (DHA) acid in fish oil supplements vary between sources. With the awareness that certain health benefits of fish oil are attributable to effects of EPA on eicosanoid mechanisms, it is often assumed that EPA-rich oils are more beneficial than DHA-rich oils such as tuna oil, despite the recognized benefit of consuming DHA-rich fish. However, recent studies with purified EPA and DHA suggest that DHA may have a primary role in mediating some of the effects of fish oils. We have attempted to compare the relative efficacy of commercial DHA-rich and EPA-rich fish oil supplements on a range of health parameters.

In a 16-wk double-blind crossover trial, 32 middle-aged male volunteers were randomized to take eight 1 g capsules of either NuMega, a new DHA-rich tuna oil from Clover Corporation, Australia, providing 0.49 g EPA and 2.08 g DHA per day, or MaxEPA, providing 1.35 g EPA and 0.95 g DHA per day, for 6 wk. After a 4-wk washout period, they took the alternate oil for a further 6 wk. Initially and after 6 and 12 wk, we measured clinic blood pressure, 24-h ambulatory blood pressure, fasting plasma fatty acids, and lipids and platelet thromboxane production. Production of the inflammatory cytokines interleukin-1β (IL1β) and tumor necrosis factor-α (TNFα) was measured in mononuclear cells isolated from blood taken initially and after 6 wk.

Thirty subjects completed the trial. Their plasma EPA and DHA levels rose by 350 and 63%, respectively, with MaxEPA supplementation and by 140 and 130%, respectively, with NuMega. Despite these differences, the supplements had almost identical effects on platelet thromboxane production (reduced 40%) and on plasma lipid levels. Low density lipoprotein cholesterol rose 6%, but total and high density lipoprotein cholesterol were unaffected. Triglycerides fell 26% (from 1.96 to 1.45 mM) with both oils, the reductions being dependent on initial levels in each case. The overall increase of long chain n-3 fatty acids in plasma was less with NuMega (84%) than MaxEPA (138%), suggesting that, consistent with recent observations by Grimsgaard *et al.* and Hansen *et al.* (2) using highly purified EPA or DHA, DHA may be more efficacious than EPA in lowering both fasting and postprandial triglycerides. Conquer and Holub (3) and Nelson *et al.* (4) found that an EPA-free DHA supplement also lowered triglycerides but had no effect on thrombotic indices.

EPA and DHA may differentially affect mediators of inflammation, e.g., DHA appears to be more effective than EPA in inhibiting cytokine-induced expression of adhesion molecules (5,6). Fish oil supplementation also inhibits cytokine production in mononuclear cells, an effect which may be related to thromboxane inhibition (7). In our study, however, TNFα was unaffected, and IL 1β production fell only 29% on average, even though platelet thromboxane production was markedly reduced. MaxEPA appeared more effective than NuMega in suppressing IL1β (38 vs. 20%), but the difference was not significant.

Studies using typical EPA-rich oil supplements indicate that blood pressure can be lowered with an n-3 dose of ~3 g/d (8). If DHA is the antihypertensive mediator, we might have expected the supplementation with NuMega, providing >2 g/d of DHA, to be efficacious. However, there were no changes in clinic or ambulatory blood pressure with either MaxEPA or NuMega, even though our subjects were mildly hypertensive (150/91 mm Hg), suggesting that the increased intakes of EPA and DHA were insufficient to affect this cardiovascular risk factor.

In conclusion, it appears that various health parameters are differentially affected by DHA and EPA. Thus further comparative studies should be undertaken with EPA-rich and DHA-rich fish oils to determine appropriate supplementation strategies which will maximize specific health benefits.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; IL1β; interleukin 1β; TNFα, tumor necrosis factor α.

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Comparison of n-3 Polyunsaturated Fatty Acids from Vegetable Oils, Meat, and Fish in Raising Platelet Eicosapentaenoic Acid Levels in Humans

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Results from a recent observation study (1) showed that the n-3/n-6 ratio in plasma and platelet phospholipids (PL) was significantly lower in both ovolacto vegetarian and vegan groups compared with meat-eaters in the study population. Collagen- and ADP-stimulated whole blood platelet aggregation showed a significant opposite trend to both plasma and platelet PL n-3/n-6 ratios. Plasma 11-dehydro thromboxane B_2 (11-dehydro TXB_2), a stable metabolite of TXA_2, was found to be higher in ovolacto vegetarian and vegan groups compared with meat-eaters. That is, these data suggested that the vegetarians had a potentially greater thrombotic risk than the omnivore subjects. The aim of this study was to investigate whether male vegetarian subjects who consumed an increased dietary ratio of α-linolenic acid (ALA)/linoleic acid exhibit an increased n-3/n-6 polyunsaturated fatty acid (PUFA) ratio in platelet PL, plasma PL, and triacylglycerol (TAG), decreased plasma TX levels, and reduced platelet aggregability compared with their habitual diet. Seventeen healthy male vegetarian subjects aged 22–48 yr were recruited in Melbourne. During the study, all subjects maintained their habitual vegetarian diets, except a proportion of dietary fat was replaced with provided vegetable oils and oil-based margarines. All 17 subjects consumed a low n-3/n-6 ratio diet (safflower oil and safflower oil-based margarine) for 14 d, following which they consumed either a moderate n-3/n-6 ratio diet (canola oil and canola oil-based margarine) or a high n-3/n-6 ratio diet (linseed oil and linseed oil-based margarine) for 28 d. Subjects were requested to refrain from consuming fish during the 42 d of the intervention. Blood samples were collected at day 0 (baseline), day 14, and day 42. Collagen, ADP, and arachidonic acid (AA) stimulated whole blood platelet aggregation, plasma hemostatic factors, plasma lipid and lipoprotein lipids, plasma 11-dehydro TXB_2 and α-tocopherol, fatty acid composition of platelet and plasma PL, and plasma TAG were measured by standard methods.

Significant changes were seen in fatty acid profiles of platelet PL, plasma PL, and plasma TAG. Eicosapentaenoic acid (EPA) and total n-3 PUFA levels, and n-3/n-6 ratio were significantly increased, while the AA/EPA ratio decreased ($P < 0.05$), following either a canola or a linseed oil-based diet compared with a safflower oil-based diet. Docosapentaenoic acid was significantly increased following linseed oil-based diet compared with the safflower oil-based diet ($P < 0.05$). There was no significant increase in docosahexaenoic acid in these lipids. No statistically significant differences between the study diet period were observed on the various thrombotic risk factors. We compared these data with a previous study (2) in which 23 adult omnivore subjects consumed 350 g/d of lean red meat for 2 wk (EPA intake = 70 mg/d) or 133 g/d of Atlantic salmon for 2 wk (EPA intake = 847 mg/d) (2). These diets also led to significant changes in platelet EPA levels (0.5 to 0.7% on the red meat diet, $P < 0.001$, and 0.4 to 1.9% on the salmon diet, $P < 0.001$, as percentage of total platelet PL).

The results indicate that canola and linseed oils have similar effects on the fatty acid profile of platelet PL and plasma lipid. The greatest increases in n-3 PUFA occurred with linseed oil which had the highest ALA content. ALA from the vegetable oils (canola and linseed) used as a dietary fat for daily food preparation had a beneficial effect on alteration of fatty acid profiles of platelet PL and plasma lipids in vegetarian populations. Longer-term studies may be necessary to establish effects of plant source n-3 PUFA on hemostatic function in vegetarians. These data clearly show that dietary EPA alters platelet EPA levels more quickly and more effectively than *via* endogenous synthesis from dietary ALA. This study demonstrated that 70 mg/d of EPA was more effective than 3.7 g of ALA and almost as effective as 15.4 g/d of ALA in raising platelet EPA levels in short-term studies.

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Abbreviations: AA, arachidonic acid; ALA, α-linoleic acid; EPA, eicosapentaenoic acid; PL, phospholipids; PUFA, polyunsaturated fatty acid; TAG, triacylglycerol; TX, thromboxane.

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Intake of Small Amounts of n-3 Fatty Acids Decreases Platelet Lipid Peroxidation in Elderly People

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Free radical damage has long been believed to be a risk factor for the degenerative processes which accompany aging (1). Reactive oxygen species initiate a wide variety of oxidative reactions, including peroxidation of lipids, and contribute to the process of aging, as well as age-related pathology such as cardiovascular diseases. Moreover, platelet activation is believed to play a role in the development of such diseases, and an increased reactivity to most aggregating agents of platelets was reported in elderly people. An exacerbated arachidonic acid metabolism was also found, as well as a significant lowered vitamin E and glutathione peroxidase activity (2). On the other hand, epidemiologic studies suggest that consumption of n-3 polyunsaturated fatty acids (PUFA) may protect against thromboembolic diseases (3). Especially, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids could be responsible for such beneficial effects. However, an increase of n-3 PUFA in membrane phospholipids may enhance membrane susceptibility to lipid peroxidation and thus counteract these beneficial effects.

In this context, the purpose of the present study was to investigate the effects of low intake of n-3 PUFA on platelets from elderly people. In a randomized double-blind study, 20 healthy subjects (70–83 yr) were carefully selected. Ten subjects ingested one capsule of 600 mg (corresponding to 150 mg DHA and 30 mg EPA) of RO-PUFA triglyceride (Roche) per day and 10 others ingested one capsule of 600 mg sunflower oil as placebo during 42 d. RO-PUFA and placebo capsules contained 1900 and 600 ppm of α-tocopherol, respectively, representing at most 5% of the recommended daily allowance. Blood anticoagulated with ACD (acid/citrate/dextrose) was drawn before (day 0) and after oil supplementation (day 42). Platelets were isolated and several biochemical parameters were measured.

The low intake of n-3 PUFA modestly affected platelet aggregation. Indeed, a tendency to decrease the percentage of thrombin-induced aggregation in RO-PUFA group was observed. Although no modification in the composition of platelet total phospholipids appeared after the oil intake, a slight but significant increase in the proportion of DHA was found only in the phosphatidylethanolamine fraction after RO-PUFA intake. Concerning the antioxidant status, no significant changes of the platelet glutathione peroxidase activity nor of its quantity were observed. By contrast, a significant increase of both α- (+26%, from 0.85 to 1.07 nmol/10^9 platelets) and γ- (+58%, from 0.12 to 0.19 nmol/10^9 platelets) tocopherols occurred after RO-PUFA intake. Interestingly, the basal platelet thromboxane B_2 level, a marker of activation, tended to decrease (–25%). In the same way, a significant reduction of platelet malondialdehyde (–30%), a marker of the overall lipid peroxidation, was found in the RO-PUFA group. At the opposite, no significant changes for all these parameters were observed in the placebo group.

Although epidemiologic studies (3,4) indicate clearly that a low intake of fish may beneficially influence the coronary heart disease, only few experimental data are available. Our investigation indicates that platelet hyperactivity observed in elderly people could be reduced by intake of small amounts of n-3 PUFA. Such a modification was associated both with an increase of the major antioxidant vitamin E and a decrease of platelet lipid peroxidation. These data suggest that low doses of n-3 PUFA might behave as an antioxidant in a prethrombotic state as the elderly.

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; PUFA, polyunsaturated fatty acid.

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Docosahexaenoic Acid-Enriched Foods: Production and Effects on Blood Lipids

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Fish oil containing eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) is known to reduce human plasma triacylglycerol levels, but has little effect on high density lipoprotein (HDL)- or low density lipoprotein (LDL)-cholesterol levels (1). These changes were seen within 3 mon of beginning supplementation. The study included 26 subjects, including 15 with a low ratio of polyunsaturated to saturated fatty acids as is typical in Western diets. In this study, animal products enriched in n-3 polyunsaturated fatty acid (PUFA), primarily in DHA, were tested to determine if they have the same effects as fatty fish.

Healthy human volunteers in Taegu, Korea, consumed animal products for 4 wk. Boys, 14 yr of age, consumed 585 mL of milk per day. One group had generic milk with 3.4% fat, and the other group had Einstein milk, in which the fat contained 0.2% each of EPA and DHA. Each group contained 250 boys. The platelet aggregation values, with 5 μL of collagen (Sigma test kit), were 22.4 ± 2.1% after generic milk and 17.1 ± 1.8% after Einstein milk ($P < 0.05$). The values for DHA in the plasma fatty acids were 2.6 ± 0.1 mol% and 5.5 ± 0.2 mol%, respectively. No difference was found in the plasma cholesterol.

Women, 20 yr of age, were students at Kyungpook University (Taelgu, Korea). Groups of 100 consumed three generic eggs or three Edison eggs daily for 4 wk. Platelet aggregation values, with 5 μL of collagen) (Sigma test kit), were 26.3 ± 7.9% after generic eggs and 9.0 ± 4.3% after Edison eggs ($P < 0.05$). Total cholesterol values were 174.4 ± 11.3 mg/dL after generic eggs and 141.1 ± 9.1 mg/dL after Edison eggs ($P < 0.05$).

Groups of 20 women ate 200 g of chicken or 200 g of pork per day. Compared to values obtained before consuming the food, the subjects consuming DHA-enriched foods had significantly lower values for platelet aggregation and plasma total cholesterol. Before eating chicken, the women had 85.2 ± 6.4 mg/dL LDL-cholesterol. After 4 wk, they had 68.1 ± 4.4 mg/dL ($P < 0.05$). Before eating pork, the women had 110.0 ± 8.0 mg/dL LDL-cholesterol. After 4 wk, they had 91.8 ± 7.3 mg/dL ($P < 0.05$). They had no change in HDL-cholesterol, which was about 50 mg/dL for all subjects. The levels of plasma triacylglycerols were also lower by 30%, but this was not statistically significant. The proportions of DHA in plasma neutral and polar lipids were much higher after consuming the DHA-enriched foods than before.

Eggs enriched with n-3 PUFA are on the market in several countries. Edison 300™ eggs containing at least 300 mg DHA per 100 g of egg are now being produced in Korea by Woobang Science Co., Ltd. This company provided a feed supplement for laying hens containing fish oil, flaxseed, and a mixture that stimulates the conversion of n-3 PUFA to DHA. In tests at The Ohio State University with the feed supplement, the mole percentages of n-3 fatty acids in eggs from control and supplemented (3.75%) hens were: 0.4 and 1.2 for 18:3, 0 and 0.2 for 20:5, 0 and 0.2 for 22:5, and 0.6 and 3.0 for DHA. Thus, eggs can now be produced with relatively low increases in most n-3 fatty acids but with a fivefold increase in DHA.

Persons on a Western diet might also improve cardiovascular risk factors by consuming these DHA-enriched foods. Patients with combined hyperlipidemia were given DHA at dosages of 1.25 or 2.5 g/d or placebo. Serum triacylglycerols were reduced significantly. The LDL-cholesterol level was unchanged at the lower dose and increased at the higher dose (2). Further feeding experiments are necessary to determine whether the reduction of LDL-cholesterol levels seen with DHA-enriched foods in Korea will also be seen in subjects consuming a typical Western diet. All studies with dietary supplementation or enrichment in long-chain n-3 fatty acids showed marked reductions in serum triacylglycerol levels. The latter is an important risk factor for cardiovascular disease, so the addition of long-chain n-3 fatty acids to the diet may be clinically useful (3).

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDL, high density lipoprotein; LDL, low density lipoprotein; PUFA, polyunsaturated fatty acid.

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Free Radical-Scavenging Actions of Olive Oil Phenolics

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Exposure to environmental stress and ultraviolet light radiation enhances the synthesis of protective compounds, e.g., pigments and antioxidants, in fruits and vegetables. This results in the development of dark-colored fruits such as grapes and olives. Among the phenolic constituents of olives and extra virgin olive oil, of special interest for their biological activities are those sharing an artho-diphenolic moiety, namely hydroxytyrosol (HT) and oleuropein (OE): previous studies showed that these compounds potently inhibit low density lipoprotein oxidation. We also recently reported that OE increases nitric oxide production and inducible nitric oxide synthase expression in lipopolysaccharide-challenged murine macrophages. A net increase in nitric oxide levels may also be the result of a concomitant removal of superoxide anion, therefore preventing the formation of peroxynitrite. The present study aimed at investigating the scavenging properties of olive phenolics, that is OE and HT, with respect to superoxide anion and hypochlorous acid. The former was generated in a cell-free environment (xanthine/xanthine oxidase) and by phorbol 12-myristate 23-acetate-stimulated human neutrophils. While vitamin E was ineffective, the rate of formation of superoxide in the presence of HT and OE was greatly reduced. When superoxide was generated by the xanthine/xanthine oxidase system, EC_{50} were as follows: OE = 14.3 μM, HT = 91 μM, whereas in the case of stimulated polymorphonuclear cells they were 29.3 and 3.2 μM, respectively. A scavenging effect on physiologically feasible concentrations of hypochlorous acid was also demonstrated, as indicated by a protection of catalase integrity and by a reduced rate of oxidation of 5-thio-2-nitrobenzoic acid to 5,5′ dithiobis (2-nitrobenzoic) acid. Hypochlorous acid is produced at the site of inflammation, where it inactivates antioxidant enzymes such as catalase and glutathione peroxidase, and is a component of household bleach. These results indicate the potent *in vitro* scavenging effects of olive oil-derived compound on free radical generation. Although data on the absorption and disposition of micronutrients are still limited, there is evidence of protective effects toward oxidative stress in the laboratory animal. Also, analyses of diets rich in fruit and vegetables indicate that the daily consumption of such components reach "pharmacological" levels. Whenever demonstrated in humans, their properties would add further evidence to the observed beneficial effects of a diet rich in phenols and flavonoids, found in fresh fruits, vegetables, and olive oil.

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Abbreviations: HT, hydroxytyrosol; OE, oleuropein.

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Green Tea Extract Does Not Affect Urinary Markers of Lipid Peroxidation or Thromboxane or Nitric Oxide Synthesis During a High-Linoleic Acid Diet in Healthy Females

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Green tea contains polyphenolic catechins which are powerful antioxidants and cyclooxygenase inhibitors *in vitro*. We studied the effects of green tea extract on urinary 8-iso prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), which is a marker of *in vivo* lipid peroxidation; urinary 2,3-dinor thromboxane B_2 (2,3-dinor-TXB_2), a marker of thromboxane production; as well as urinary nitric oxide metabolites nitrate and nitrite ($NO_3 + NO_2$) during a controlled high-linoleic acid diet.

Twenty healthy, nonsmoking, normal weight females (23–50 yr) participated in the intervention. The study consisted of a 2-wk saturated fat-rich baseline diet which was followed by a 4-wk highly controlled experimental diet period. The experimental diet was rich in linoleic acid (9 en%) and contained fat, protein, and carbohydrates: 27, 14, and 59 en%. During the experimental period, the subjects were supplemented either with encapsulated green tea extract (3 g/d) or placebo in a double-blind manner. Fasting blood samples and five 24-h urines were collected before and at the end of the 4-wk experimental period. Samples from a control group of 10 healthy women who kept their habitual diet constant were also included in the analyses. Urinary 8-iso-$PGF_{2\alpha}$ was analyzed by radioimmunoassay (Dr. Basu, Uppsala University), 2,3-dinor-TXB_2 by enzymoimmunoassay (Cayman Chemicals, Ann Arbor, MI), and $NO_3 + NO_2$ using a colorimetric kit (Cayman Chemicals).

Serum cholesterol decreased, and vitamin E/cholesterol-ratio increased similarly in the two study groups, and both study groups differed from the control group in these respects. Also serum triglyceride fatty acids showed good compliance to the experimental diet. The treatments did not differ in their effects on urinary 8-iso-$PGF_{2\alpha}$, 2,3-dinor-TXB_2, or nitric oxide metabolites (Table 1).

When the differences and 95% confidence limits of the deltas in the different groups were compared, no differences between the groups were seen (data not shown). The results show that an amount of green tea extract which corresponds to 10 cups/day did not show specific antioxidative or cyclooxygenase inhibitive effects *in vivo* during a high-linoleic acid diet in healthy female subjects in comparison with placebo treatment.

TABLE 1
Treatment Effects on Urinary Markers

		8-iso-$PGF_{2\alpha}$[a] (ng/mmol crea)	2,3-dinor-TSB_2[b] (ng/mmol crea)	$NO_3 + NO_2$[c] (µmol/mmol crea)
Green tea	PRE[d]	554 (434)	16.7 (12.2)	236 (299)
	EXP	346 (185)	17.6 (11.5)	136 (102)
Placebo	PRE	319 (118)	17.0 (6.8)	119 (57)
	EXP	215 (51)[e]	13.4 (5.0)	95 (46)
Control	PRE	310 (122)	16.7 (6.3)	135 (55)
	EXP	266 (64)	19.2 (12.9)	84 (48)[e]

[a]Urinary 8-isoprostaglandin $F_{2\alpha}$.
[b]Urinary 2,3-dinor thromboxane B_2.
[c]Urinary nitric oxide metabolites nitrate and nitrite.
[d]PRE, pre-experimental period; EXP, experimental period.
[e]PRE vs. EXP within the group: $P < 0.05$, paired *t*-test.

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Abbreviations: 8-iso-$PGF_{2\alpha}$, 8-iso prostaglandin $F_{2\alpha}$; 2,3-dinor-TXB_2, 2,3-dinor thromboxane B_2; $NO_3 + NO_2$, nitric oxide metabolites nitrate and nitrite.

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Metabolism of Anandamide and 2-Arachidonoylglycerol: An Historical Overview and Some Recent Developments

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ABSTRACT: Anandamide (*N*-arachidonoylethanolamine) and 2-arachidonoylglycerol are the two endogenous agonists of cannabinoid receptors discovered to date. Like other eicosanoids, and unlike classical neuromodulators, these two compounds are synthesized by neurons on demand, i.e., their biosynthesis, rather than release, is stimulated by Ca^{2+} influx and cell membrane depolarization. Both endocannabinoids can be produced from membrane phosphoglycerides through the action of phospholipases, although *de novo* pathways have also been suggested. Once released by cells, the action of both anandamide and 2-arachidonoylglycerol is terminated—after their diffusion through the cell membrane—by the hydrolysis of the amide or ester bonds to yield arachidonic acid, which is then immediately reincorporated into phospholipids. One enzyme, fatty acid amide hydrolase, catalyzes the hydrolysis of both endocannabinoids in nervous and nonnervous cells. This enzyme also recognizes *N*-palmitoylethanolamine, an antiinflammatory congener of anandamide, with a catalytic efficiency that depends on the cell type under study. However, the existence of different isozymes with different affinity for anandamide and *N*-palmitoylethanolamine has not been investigated. Moreover, little work has been performed on the regulation of anandamide formation and breakdown, and several open questions remain as to the possible biosynthetic and degradative mechanisms of cannabimimetic 2-arachidonoylglycerol in nucleated blood cells such as macrophages. Finally, the co-existence of both endocannabinoids in invertebrates has not been fully established. Here we briefly review the state of the art, and present new data from our laboratory, on these four largely unexplored aspects of endocannabinoid metabolism.

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Abbreviations: AA, arachidonic acid; 2-AG, 2-arachidonoylglycerol; ATFMK, arachidonoyltrifluoromethane; DAG, diacylglycerol; FAAH, fatty acid amide hydrolase; GC–MS, gas chromatography–mass spectrometry; *p*-HMB, *p*-hydroxy-mercuribenzoate; HPLC, high-pressure liquid chromatography; 1-LG, 1(3)-linoleoylglycerol; MAFP, methylarachidonoylfluorophosphonate; NAE, *N*-acyl-ethanolamine; $C_{20:4}$ NAE, anandamide; NAPE, *N*-acylphosphatidylethanolamine; NArPE, *N*-arachidonoyl-phosphatidylethanolamine; NGF, nerve growth factor; PA, phosphatidic acid; PC, PE, and PI, phosphatidylcholine, -ethanolamine and -inositol; PG, phosphoglycerides; PLA_2, PLC, and PLD, phospholipases A_2, C, and D; RBL, rat basophilic leukemia; SDS-PAGE, SDS-polyacrylamide gel electrophoresis; TG, triglycerides.

The discovery that anandamide [*N*-arachidonoylethanolamine, $C_{20:4}$ NAE (1)]—a novel compound belonging to a well-known class of lipids, the *N*-acylethanolamines [NAE, (2)]—and 2-arachidonoylglycerol [2-AG (3,4)]—a widespread intermediate in tri-, di-, and phosphoglyceride metabolism—could bind and functionally activate one or both cannabinoid receptor subtypes present in mammalian tissues conclusively demonstrated the existence of an endogenous cannabinoid system (for a recent review see Ref. 5). Along with studies on the pharmacological and behavioral properties of the two compounds (recently reviewed in Ref. 6), much effort has been dedicated to the finding of the possible biochemical mechanisms underlying $C_{20:4}$ NAE and 2-AG biosynthesis and inactivation. This led to the identification of the metabolic pathways responsible for $C_{20:4}$ NAE and 2-AG biosynthesis in the central nervous system and to the understanding of the main catabolic routes for the two endocannabinoids. However, still very little is known on the regulation of these pathways or on their occurrence in peripheral cells or lower invertebrates. Progress on these subjects, with particular emphasis on recent data from our laboratory, is described here.

Biosynthesis and breakdown of $C_{20:4}$ NAE. Regulation during cell differentiation and possible isoforms of the hydrolytic enzyme. Recent evidence supports a phospholipid-mediated pathway for the biosynthesis of $C_{20:4}$ NAE, identical to that described in the 1980s for other NAE (7). According to this pathway, NAE, including the $C_{20:4}$ congener, are produced from the phospholipase D (PLD)-catalyzed hydrolysis of preformed phospholipids, the corresponding *N*-acylphosphatidylethanolamines (NAPE) (2,7). NAPE, including *N*-arachidonoylphosphatidylethanolamine (NArPE), are in turn produced from the transfer of a fatty acyl moiety from the *sn*-1 position of phospholipids to the amino group of phosphatidylethanolamine (PE) (2,8) (Fig. 1). This reaction is catalyzed by a Ca^{2+}-dependent *trans*-acylase, and it probably represents the rate-limiting step of NAE biosynthesis. This pathway explains why the formation/release of $C_{20:4}$ NAE is always accompanied by that of other more abundant NAE (7,9). In fact, very little arachidonic acid (AA) is present on the *sn*-1 position of phospholipids, and, consequently, only very little NArPE can be produced upon Ca^{2+}-influx into the cell and subsequent stimulation of the *trans*-acylase.

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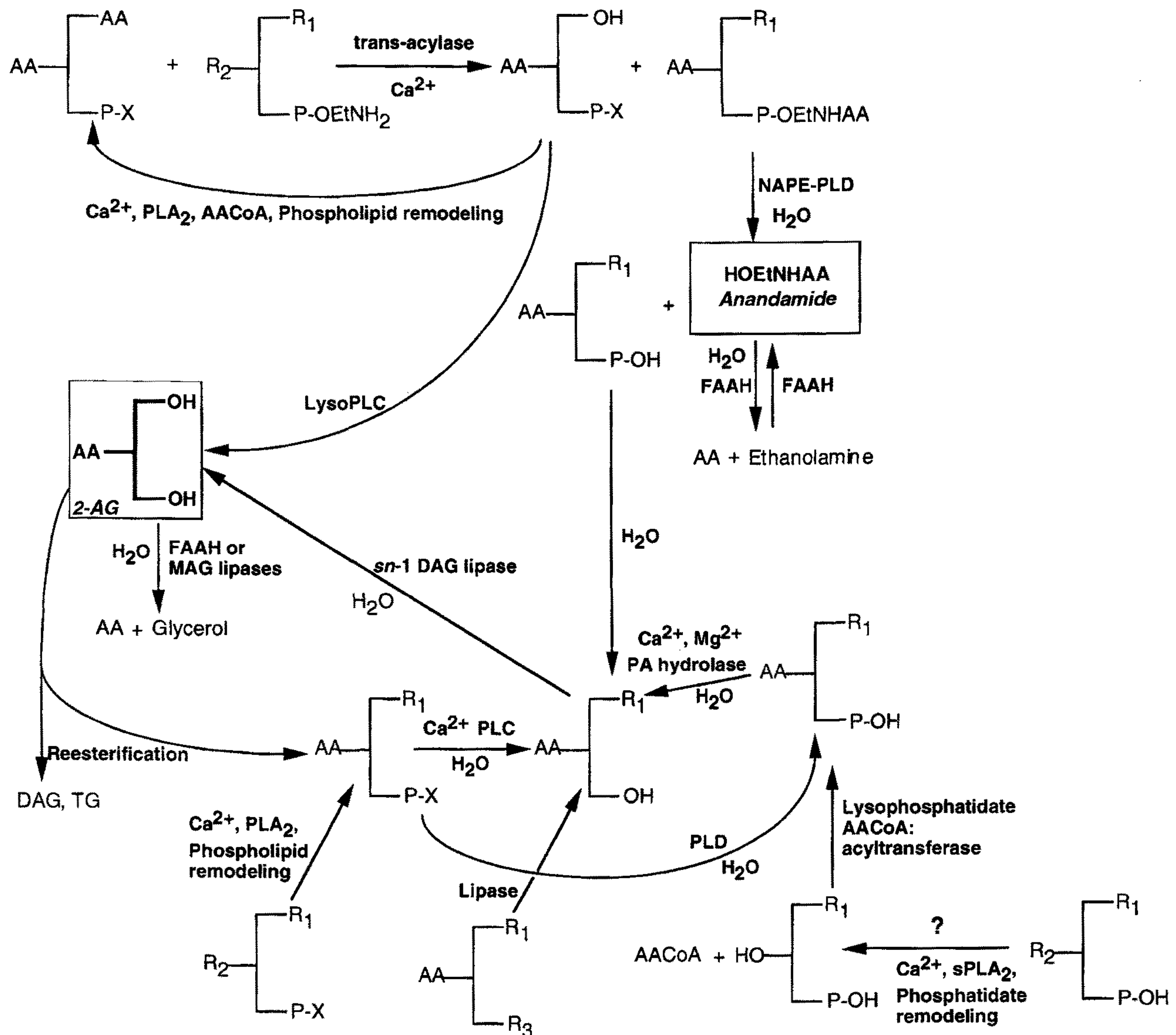

FIG. 1. Metabolic pathways for anandamide and 2-arachidonoylglycerol (2-AG) in mammalian cells. Calcium-activated phospholipid remodeling may play a role in the formation of precursors for both endocannabinoids. Data described here support the hypothesis of direct esterification of 2-AG into phospholipids and neutral lipids. AA, arachidonate; cPLA$_2$, sPLA$_2$, cytosolic or secretory phospholipase A$_2$; NAPE-PLD, phospholipase D selective for *N*-acylphosphatidylethanolamines; FAAH, fatty acid amide hydrolase; P, phosphate group; X, phospholipid base; PLC, phospholipase C; PA, phosphatidic acid; MAG, monoacylglycerol; DAG, diacylglycerols; TG, triglycerides.

There are, however, ways to stimulate the formation of NArPE over other NAPE through phospholipase A$_2$-mediated remodeling of membrane phospholipids (10). Moreover, the activity of the *trans*-acylase can be enhanced by adenylate cyclase-stimulating agonists such as the vasoactive intestinal peptide (11). We addressed the question of whether NAE and NAPE biosynthesis was upregulated following differentiation of peripheral cells, such as rat pheochromocytoma PC-12 cells, into sympathetic-like neurons. This transformation can be achieved by 2-wk treatment of PC-12 cells with the nerve growth factor (NGF). By using a gas chromatography–mass spectrometric (GC–MS) technique, we found that undifferentiated PC-12 cells contain measurable levels of $C_{20:4}$ NAE, $C_{16:0}$ NAE, and $C_{18:0}$ NAE and of the corresponding NAPE (data not shown). The production of these compounds cannot be increased by ionomycin treatment of cells or cell transformation into neurons (Fig. 2A). Moreover, transformed cells did not respond to ionomycin stimulation by producing more NAE and NAPE. Therefore, it is possible that the enzymes responsible for NAE and NAPE formation are not under control during NGF-induced peripheral cell differentiation into sympathetic-like neurons. Moreover, if NGF-transformed PC-12 cells can be taken as a model for sympathetic neurons, these data may suggest that depolarization of the latter cells may not contribute significantly to the formation of hypotensive $C_{20:4}$ NAE (see next section).

$C_{20:4}$ NAE inactivation occurs through facilitated diffusion into cells followed by enzymatic hydrolysis to AA and

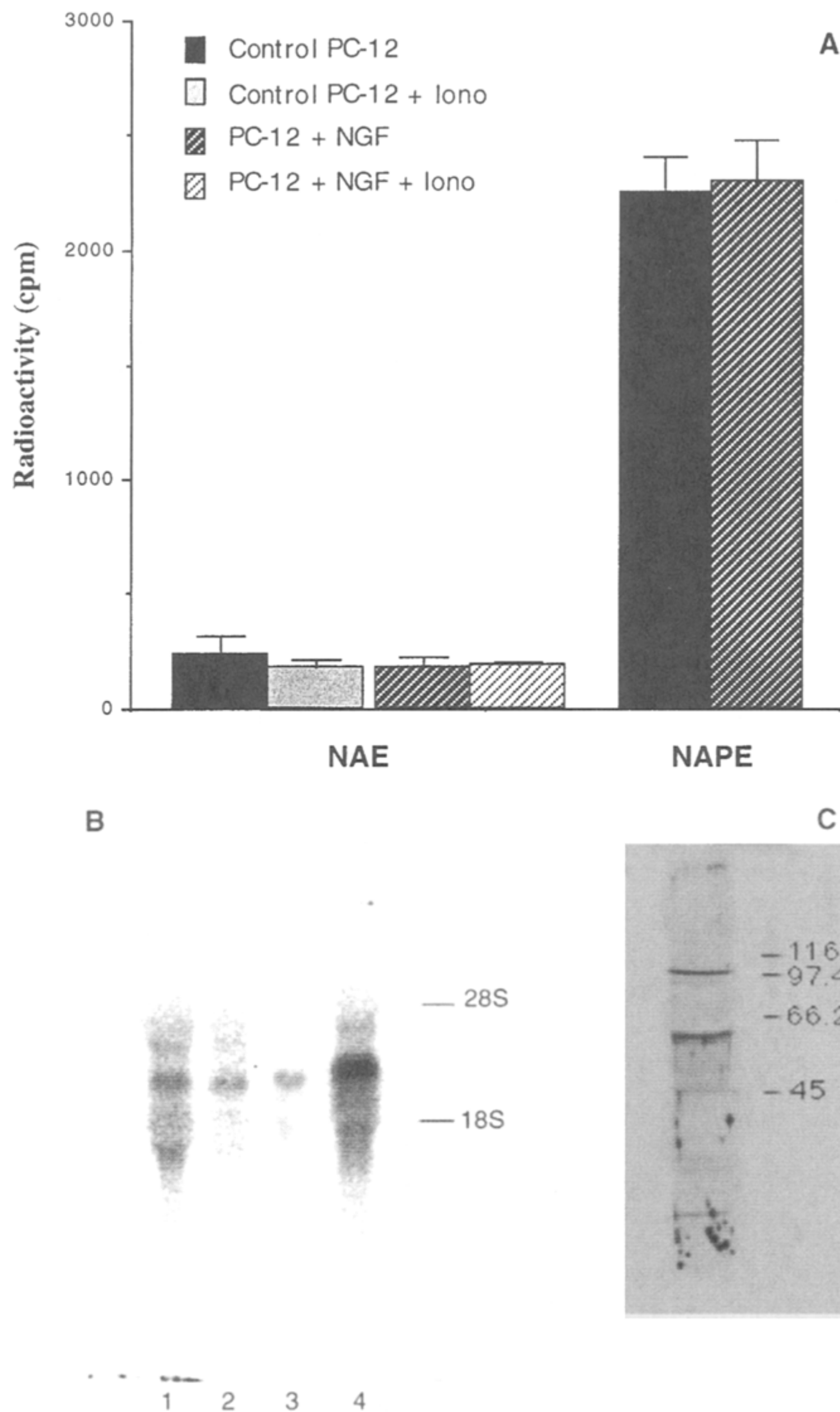

FIG. 2. Biosynthesis of *N*-acylethanolamines (NAE) and fatty acid amide hydrolase (FAAH) expression in phosphatidylcholine (PC-12) and rat basophilic leukemia (RBL)-1 cells. (A) PC-12 cells, either undifferentiated or transformed by 2-wk treatment with nerve growth factor (NGF) (50 ng/mL daily treatment), were labeled with [^{14}C]ethanolamine overnight and then stimulated with ionomycin (5 μM) or vehicle. Cells were then extracted and NAE and *N*-acylphosphatidylethanolamines (NAPE) purified as described previously (9). Their amounts are expressed as the radioactivity incorporated, measured by liquid scintillation counting of the purified compounds. The presence of $C_{20:4}$ NAE and *N*-arachidonoylphosphatidylethanolamine (NArPE) was assessed by gas chromatography–mass spectrometry (GC–MS) (9). (B) Northern blot analysis of 50 μg total RNA from either undifferentiated or differentiated PC-12 cells (lanes 1 and 2), RBL-1 cells (lane 3) and rat liver (lane 4). The blotting was performed according to the procedure described previously (24) by using an oligonucleotide probe designed from rat liver FAAH cDNA sequence. Lines indicate the mobility of 28S and 18S rRNA. Representative of four different blottings. The different band intensity in lanes 1 and 2 was not reproducible. (C) Western immunoblot analysis of proteins from RBL-1 cells with a polyclonal antibody against rat liver FAAH. Proteins were solubilized, purified by anion-exchange liquid chromatography and separated by SDS-polyacrylamide gel electrophoresis, all carried out as described previously (20). Lines indicate the mobility of molecular weight standards. Apart from the band at 62.8 kDa, another band (about 100 kDa) was also observed that did not peak with FAAH activity in chromatographic fractions.

ethanolamine (7) (Fig. 1). The enzyme responsible for $C_{20:4}$ NAE breakdown, originally named "anandamide amidohydrolase" (12), was recently cloned and expressed in COS-7 cells (13). The recombinant enzyme was found to catalyze also the hydrolysis of the sleep-inducing factor oleamide as well as other fatty acid amides and was renamed "fatty acid amide hydrolase" (FAAH) (13). This enzyme is widely distributed in membranes from rat tissues (liver > brain > gastrointestinal tract > lungs, testis > spleen) (13,24), and its expression in different brain regions fits with the distribution of the CB1 cannabinoid receptor subtype (14). Despite the fact that, of the enzymes participating in endocannabinoid metabolism, FAAH is undoubtedly the most studied one, the possibility of different FAAH isozymes and mechanisms for FAAH regulation have not been explored yet. In particular, the different substrate specificity of the enzyme from rat basophilic leukemia (RBL-1) cells as compared to the mouse neuronal enzyme (9) suggested the possible presence, in these cells, of a different FAAH isoform with a higher affinity for $C_{16:0}$ NAE. We analyzed, by Northern blotting of the total RNA from RBL-1 cells with an oligonucleotide probe obtained from the rat liver FAAH cDNA sequence, the size of FAAH mRNA in these cells as well as in PC-12 cells, either untransformed or differentiated by treatment with NGF. We found that FAAH mRNA in RBL-1 cells was undistinguishable in size from rat liver FAAH mRNA [which, in turn, is identical to rat brain FAAH mRNA (13)], and that PC-12 cell differentiation into sympathetic-like neurons does not significantly affect the levels of FAAH mRNA (Fig. 2B). Moreover, by using partially purified proteins from RBL-1 cells and a SDS-PAGE/Western immunoblotting technique exploiting a polyclonal antibody against rat liver FAAH, we analyzed the molecular size of FAAH in RBL-1 cells and found that the enzyme in these cells has an approximate molecular weight (62,800 ± 500 KDa, Fig. 2C) very similar to that calculated from rat liver FAAH amino acid sequence (i.e., 63,317 kDa). Taken together, these data do not support the presence of an FAAH isozyme in RBL-1 cells. However, the full molecular characterization of FAAH from these cells must be carried out before drawing a definitive conclusion on this issue.

Metabolism of 2-AG. Biosynthesis and catabolism in a macrophage cell line. The picture of 2-AG biosynthetic pathways is certainly more complicated than the one depicted above for $C_{20:4}$ NAE. Several routes can, in principle, contribute to 2-AG formation, all of which have as direct 2-AG precursors either *sn*-2-AA-containing diacylglycerols (DAG) or *sn*-1-lyso-2-arachidonoyl-phosphoglycerides. In the former case a *sn*-1 selective DAG lipase is responsible for 2-AG formation, whereas in the latter case a lysophospholipase C (lysoPLC) enzyme is required (Fig. 1). DAG, in turn, can be obtained through several pathways, i.e. (i) hydrolysis of triglycerides (TG) by lipases, (ii) hydrolysis of phosphatidic acid (PA) by PA hydrolase, (iii) hydrolysis of phosphatidylinositol or -choline (PI and PC) by PI- or PC-selective PLC. *sn*-2-AA-containing PA can be formed by either *de novo* synthesis and PA remodeling—through the action of lysophosphatidate AACoA:acyltransferases—or through the action of phospholipase D (PLD) and therefore also as a by-product of NAE formation. *sn*-1-Lyso-2-arachidonoyl-phosphoglycerides can be obtained from phospholipids through the action of phospholipase A_1 or as by-products of NAPE biosynthesis. In any case, there must be a Ca^{2+}-dependent, rate-limiting step since the biosynthesis and release of 2-AG, as for $C_{20:4}$ NAE, are triggered by Ca^{2+} influx into cells (15,16). Thus, in N18TG2 cells stimulated with ionomycin, DAG serving as precursors for 2-AG are probably obtained from PA formed from remodeling of nonpolyunsaturated species through pathways which may include the participation of secretory PLA_2 (15,25). Conversely, in ionomycin-stimulated rat cortical neurons and bradykinin-treated rat root dorsal ganglia, 2-AG is produced from DAG released by the action of PI-selective PLC (16,17). In platelets and other blood cells, PLC-derived intracellular 2-AG has always been regarded as the major source of AA for eicosanoid biosynthesis (18). Recently, however, platelet-derived extracellular 2-AG has been suggested to play a major role in lipopolysaccaride-induced and cannabinoid receptor-mediated hypotension (19). 2-AG was also found to be released by macrophage preparations, but since these were inevitably contaminated with platelets, it was not possible to ascertain that macrophages can produce 2-AG (19). We investigated whether a mouse macrophage/monocyte cell line, the J774 cells, once stimulated with ionomycin, could produce 2-AG. By using GC–MS for 2-AG quantitation, we found that, following such treatment, 2-AG levels in J774 macrophages can raise from 5 ± 5 mol up to 120 ± 29 pmol × 10^7 cells (about 1.1 nmol/g, means ± SD, $n = 2$) (Fig. 3A). These data provide strong evidence that stimulated macrophages can produce 2-AG.

Also 2-AG is inactivated by cells through the enzymatic hydrolysis of its ester bond, yielding AA and glycerol. Surprisingly, in both N18TG2 and RBL-2H3 cells, the enzyme mostly responsible for 2-AG hydrolysis was a protein with the same pH dependency, substrate specificity, sensitivity to inhibitors, and chromatographic behavior (after two purification steps) as FAAH (20). Conclusive evidence that this enzyme could efficiently catalyze 2-AG hydrolysis was provided by Goparaju *et al.* (21) by using recombinant FAAH overexpressed in COS-7 cells. We found that 2-AG, presumably after nonfacilitated diffusion through the plasma membrane, can also be esterified to membrane phospholipids in both N18TG2 and RBL-2H3 cells (20). We therefore decided to check whether these inactivating mechanisms also occur in living macrophages, a blood cell type whose production of vasodilatory 2-AG may play an important role during hemorrhagic- and septic shock-induced hypotension (19,22). We used again the J774 cell line and found that these macrophages can rapidly ($t_{1/2}$ = 28 min) sequester extracellular [^{3}H]2-AG from the incubation medium. Following this process, the radioactivity was time-dependently associated with 2-AG diffused into cells, free AA and DAG, TG, and membrane phospholipids (Fig. 3B). The fatty acylCoA transferase inhibitor thimerosal counteracted the formation of ra-

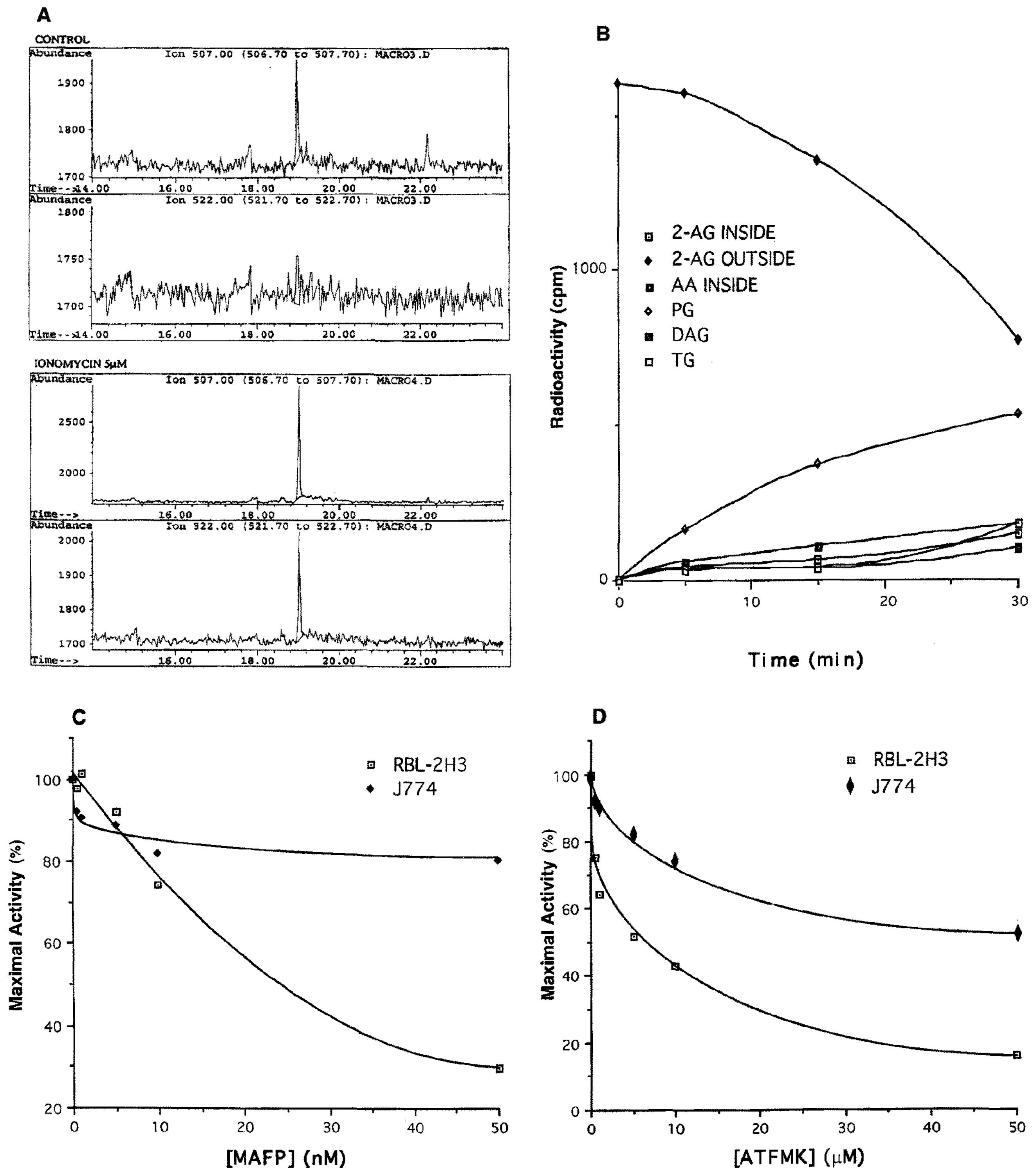

FIG. 3. Biosynthesis and catabolism of 2-AG in J774 macrophages. (A) Stimulation by ionomycin of 2-AG biosynthesis in intact J774 macrophages as detected by selected ion monitoring (SEM) GC–MS of the trimethylsilyl ether derivative. Confluent cells from twenty 100-mm Petri dishes (about 2×10^8 cells) were treated with either 5 μM ionomycin or vehicle for 20 min at 37°C. Cells plus media were then extracted in the presence of 2 nmol deuterated d_8-2-AG, and lipids processed as described previously (15). Normal-phase high-pressure liquid chromatography fractions with the same retention time as synthetic 2-AG were derivatized with 20 μL *N*-methyl-*N*-trimethylsilyl-trifluoroacetamide + 1% trimethylchlorosilane for 2 h at room temperature, prior to injection onto the GC–MS apparatus. GC was carried out as described previously (15), and selected ions monitored at $m/z = 522$ and 530 (molecular ion peaks) and $m/z = 507$ and 515 (−15, loss of methyl group). Only the fragmentograms (at $m/z = 522$ and 507) of endogenous, nondeuterated 2-AG from unstimulated (upper panel) and ionomycin-treated (lower panel) cells are shown. Representative of two experiments. (B) Catabolism of [^{3}H]2-AG in intact J774 cells. [^{3}H]2-AG (10,000 cpm/mL, 5 mCi/mmol) was added to the culture media of confluent cells in six-well dishes (about 1×10^6 cells/well) and incubated for 0, 5, 15, and 30 min. The radioactivity associated with 2-AG in the media, or with 2-AG and its metabolites in the cell extract was determined as described in the legend to Table 1. Data are means of triplicates and are representative of two separate experiments. AA, arachidonic acid; PG, phosphoglycerides . (C) and (D) Inhibition of [^{3}H]2-AG hydrolysis, catalyzed by membrane fractions from either RBL-2H3 basophils or J774 macrophages, by the two FAAH inhibitors methylarachidonoyl-fluorophosphonate (MAFP) and arachidonoyltrifluoromethane (ATFMK). Membranes were prepared and the hydrolysis of [^{3}H]2-AG (8 μM) assayed as described in (20). Data are means of triplicates and are representative of two separate experiments. See Figures 1 and 2 for other abbreviations.

diolabeled DAG, TG, and phosphoglycerides (PG) but not [^{3}H]2-AG hydrolysis to [^{3}H]AA. AA selectively inhibited the formation of [^{3}H]PG, whereas 1-linoleoylglycerol (1-LG) was also effective against [^{3}H]2-AG hydrolysis. By contrast, the other sulfhydryl group reagent, *p*-hydroxy-mercuribenzoate (*p*-HMB), inhibited [^{3}H]2-AG hydrolysis but not [^{3}H]PG formation (Table 1). These data suggest that to the formation of [^{3}H]PG contribute the re-acylation of both [^{3}H]2-AG (which is inhibited by thimerosal and 1-LG) and [^{3}H]AA (probably inhibited by AA and thimerosal), which in turn is produced from the hydrolysis of [^{3}H]2-AG (inhibited by 1-LG and *p*-HMB). Finally, we examined the 2-AG hydrolase activity in J774 cells, a cell line expressing very small amounts of FAAH (9). Unlike the enzyme in RBL-2H3 and N18TG2 cells (20), the hydrolase from J774 cells was different from FAAH in asmuch as it displayed little affinity for 2-AG (apparent K_m = 0.11 mM), and reduced sensitivity to the two FAAH inhibitors [reviewed in Ref. 6] arachidonoyltrifluoromethane (ATFMK) and methylarachidonoylfluorophosphonate (MAFP) (Fig. 3C,D). Nonetheless, the enzyme efficiently catalyzed 2-AG hydrolysis with an apparent V_{max} of 7.9 nmol min^{-1} · mg protein^{-1} at pH = 9.0. These data confirm our previous suggestion (20) that enzymes other than FAAH contribute to the inactivation of extracellular, cannabimimetic 2-AG, especially in those tissues and cells where very little FAAH is expressed, and suggest that a "cocktail" of inhibitors (for example thimerosal, *p*-HMB and ATFMK or MAFP) should always be used in biochemical and pharmacological studies on 2-AG.

Occurrence of $C_{20:4}$ NAE and 2-AG in lower invertebrates. The presence of $C_{20:4}$ NAE and NArPE in *Mytilus* and in the ovaries of sea urchins—two invertebrates which express cannabinoid receptors (6)—was previously established in our laboratory (6). However, no data exist on the presence of 2-AG in low invertebrates nor on the occurrence of the "anandamidergic" system in two widely used invertebrate models of neurobehavioral studies, i.e., *Hydra*, the first animal to have developed a neural network, and *Aplysia*. We analyzed by GC–MS (see legend to Fig. 3) the purified lipids extracted from *H. vulgaris* polyps and *Aplysia* ganglia and found that they contain measurable levels of endocannabinoids. $C_{20:4}$ NAE and NArPE contents were, respectively, 15.6 ± 1.5 and 32.4 ± 8.1 pmol/g in *Hydra*, and 10.5 ± 2.7 and 39.6 ± 7.1 pmol/g in *Aplysia* ganglia, whereas the amounts of 2-AG were 11.2 ± 1.9 and 5.7 ± 0.9 nmol/g, respectively (means ± SD, n = 2). Both endocannabinoids were accompanied by varying amounts of several congeners, including $C_{16:0}$ and $C_{18:1}$ NAE and $C_{16:0}$, $C_{18:0}$, $C_{18:1}$, $C_{20:3}$, $C_{20:5}$, $C_{22:5}$, and $C_{22:6}$ monoacylglycerols. Low levels of an FAAH-like activity were also found in both invertebrates. These data confirm that the endogenous cannabinoid system has been highly conserved during animal evolution and suggest that *Hydra* and *Aplysia* can be used for metabolic studies on $C_{20:4}$ NAE and 2-AG.

Conclusions. Although much progess has been made on the understanding of the mechanisms underlying endocannabinoid biosynthesis and degradation, still little is known on the regulation of these mechanisms, particularly in relation to some physiopathological conditions. Indeed, the two prototypical studies where a precise correlation between such conditions and $C_{20:4}$ NAE or 2-AG formation was established (19,22,23) dealt with peripheral tissues and posed further questions. Are the hypotensive endocannabinoids produced during hemorrhagic and septic shock (19,22) originated only from platelets and macrophages or also from sympathetic nervous fibers? Are they synthesized from the breakdown of phospholipid precursors? And how is the duration of their action regulated? How are produced the very high amounts of anandamide found in mouse uterus (20 nmol/g tissue, 1000 times higher than rat brain) when this organ is least receptive to embryo implantation (23)? From the phospholipid-mediated route depicted above—which can only sustain the formation of relatively low amounts of anandamide? Or through the enzymatic condensation between AA and ethanolamine (Fig. 1)—originally proposed to explain anandamide biosynthesis in cell-free systems, and then abandoned because of the high concentrations of substrates required for this reaction to occur (6)? A renewed effort from biochemists and physiologists will be required in order to provide an answer to these and other open questions on endocannabinoid metabolism.

TABLE 1
Effect of Various Inhibitors on [^{3}H]2-AG Catabolism in Intact J774 Macrophages[a]

Metabolite	[^{3}H]AA	[^{3}H]DAG	[^{3}H]TG	[^{3}H]PG
Substance				
None	100	100	100	100
p-HMB (100 μM)	39 ± 5	n.d.	n.d.	81 ± 10
MAFP (50 nM)	83 ± 4	n.d.	n.d.	84 ± 16
1-LG (100 μM)	49 ± 4	n.d.	n.d.	17 ± 7
AA (100 μM)	140 ± 6	n.d.	n.d.	23 ± 1
Thimerosal (100 μM)	89 ± 2	60 ± 9	31 ± 2	63 ± 4

[a]Intact J774 cells were incubated for 30 min at 37°C with [^{3}H]2-AG (10,000 cpm/mL, 5 mCi/mmol) in the presence of the various substances or vehicle. After the incubation, the media were removed, the cells extracted, and the lipids analyzed as previously descibed (15,20). The effect was measured as the percentage of the radioactivity found associated to each metabolite with no substance added (means ± SD, n = 3). 2-AG, 2-arachidonoylglycerol; AA, arachidonic acid; DAG diacylglycerols; TG, triglycerides; PG, phosphoglycerides; *p*-HMB, *p*-hydroxy-mercuribenzoate; MAFP, methylarachidonoylfluorophosphonate; 1-LG, 1(3)-linoleoylglycerol. The metabolites were separated by two-dimensional thin-layer chromatography using a first migration with iso-octane/ethyl acetate/water/acetic acid (50:110:100:20, by vol), to separate PG, 2-AG, and AA + DAG + TG, followed by a migration with diethyl ether/petroleum ether/ammonia (50:50:1, by vol), to separate AA, DAG, and TG (15).

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Formation of *N*-Acyl-phosphatidylethanolamine and *N*-Acylethanolamine (including anandamide) During Glutamate-Induced Neurotoxicity

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ABSTRACT: *N*-Acyl-phosphatidylethanolamine (NAPE) is present in very small amounts in mammalian tissues (less than 0.1% of total phospholipids). However, NAPE as well as its degradation product, *N*-acylethanolamine (NAE), can be formed in certain neuronal tissues in response to increased $[Ca^{2+}]_i$. A high $[Ca^{2+}]_i$ will activate the NAPE-forming *N*-acyltransferase using the *sn*-1 acyl group of a donor phospholipid as substrate in the transfer reaction. This membrane-bound enzyme seems to have no substrate specificity with respect to transfer of acyl groups; thus the fatty acids in the *N*-acyl group of NAPE are mainly 16:0 and 18:1, corresponding to the fatty acids in the *sn*-1 acyl group of the donor phospholipids. The NAPE-hydrolyzing phospholipase D also seems not to be acyl-group specific. In mouse neocortical neurons in primary culture, formation of NAPE and NAE is stimulated by glutamate *via* activation of the *N*-methyl-D-aspartate-receptor. Both NAPE and, to a lesser extent, NAE accumulate in a linear fashion for many hours while at the same time the neurons are dying. Likewise, in neurons prelabeled with ^{14}C-arachidonic acid, ^{14}C-arachidonic acid-labeled NAPE, and anandamide (= *N*-arachidonoylethanolamine) are accumulating. The formation of NAPE and NAE may represent a cytoprotective response in relation to various forms of neurotoxicity.

Formation of N-acyl-phosphatidylethanolamine (NAPE) and N-acylethanolamine (NAE). NAPE has for several decades been known to occur in a number of different plant seeds where its formation is believed to be a cytoprotective response to different stress stimuli (1). In mammals, NAPE can also be formed in certain tissues, e.g., in the brain (2). The formation is catalyzed by a membrane-associated *N*-acyltransferase that transfers an acyl group from the *sn*-1 position of a donor phospholipid to the amino group of phosphatidylethanolamine (PtdEtn) (Fig. 1). This enzyme is activated by calcium ions (ED_{50} = 0.2–0.5 mM) (2–4). The *N*-acyltransferase has been partially characterized, but it has not been purified (5). Since arachidonic acid in some cases is found in small amounts in the *sn*-1 position of phosphatidylcholine (6), formation of small amounts of *N*-arachidonoyl-PtdEtn (7–10) may occur. *N*-Arachidonoyl-PtdEtn is of special interest since it is the precursor for *N*-arachidonoylethanolamine, also called anandamide. This compound was found to be an endogenous ligand for the two types of cannabinoid receptors (11–13).

NAPE is found in very small amounts in mammalian tissues (less than 0.1% of the phospholipids), but it can accumulate during cell injury (2,5,9,10,14). This accumulation is probably caused by the activation of the *N*-acyltransferase, triggered by the increase in the concentration of intracellular Ca^{2+} that is associated with cell injury. NAPE can be hydrolyzed to phosphatidic acid plus NAE in both animal (2) and plant cells (15) by a phospholipase D (NAPE–PLD) that appears to be specific for NAPE. The mammalian NAPE–PLD does not seem to discriminate between different *N*-acyl groups (8,16,17), i.e., the acyl composition of the NAE formed appears to be identical to the *N*-acyl composition of NAPE. As opposed to the NAPE-forming *N*-acyltransferase, the NAE-forming NAPE–PLD seems to be unaffected by the calcium ion concentration (5). NAPE as well as NAE can accumulate during cell injury (2,14,18), and NAPE and NAE, including anandamide, have been found to accumulate postmortem (10,19,20).

Neuronal injury and formation of NAPE and NAE. We studied the formation of NAPE and NAE in mouse neocortical neurons in primary culture labeled with ^{14}C-ethanolamine for 22 h. In these cells, glutamate stimulated dose-dependently the formation of both NAPE and NAE *via* activation of the *N*-methyl-D-aspartate (NMDA)-subtype of glutamate receptors (14,18). The formation was linearly increasing within a time period of at least 20 h (18). However, glutamate is also known as an excitotoxic amino acid, and it can *via* the NMDA-receptor induce cell death in these cells (21). Another compound, sodium azide, which also very efficiently induces cell death *via* block of the respiratory chain, was found to be an even better stimulator of the formation of NAPE and NAE (18). The neurons were usually used after 6-7 d in culture at which developmental stage all-

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Abbreviations: NAE, *N*-acylethanolamine; NAPE, *N*-acyl-phosphatidylethanolamine; NMDA, *N*-methyl-D-aspartate; PLD., phospholipase D; PtdEtn, phosphatidylethanolamine.

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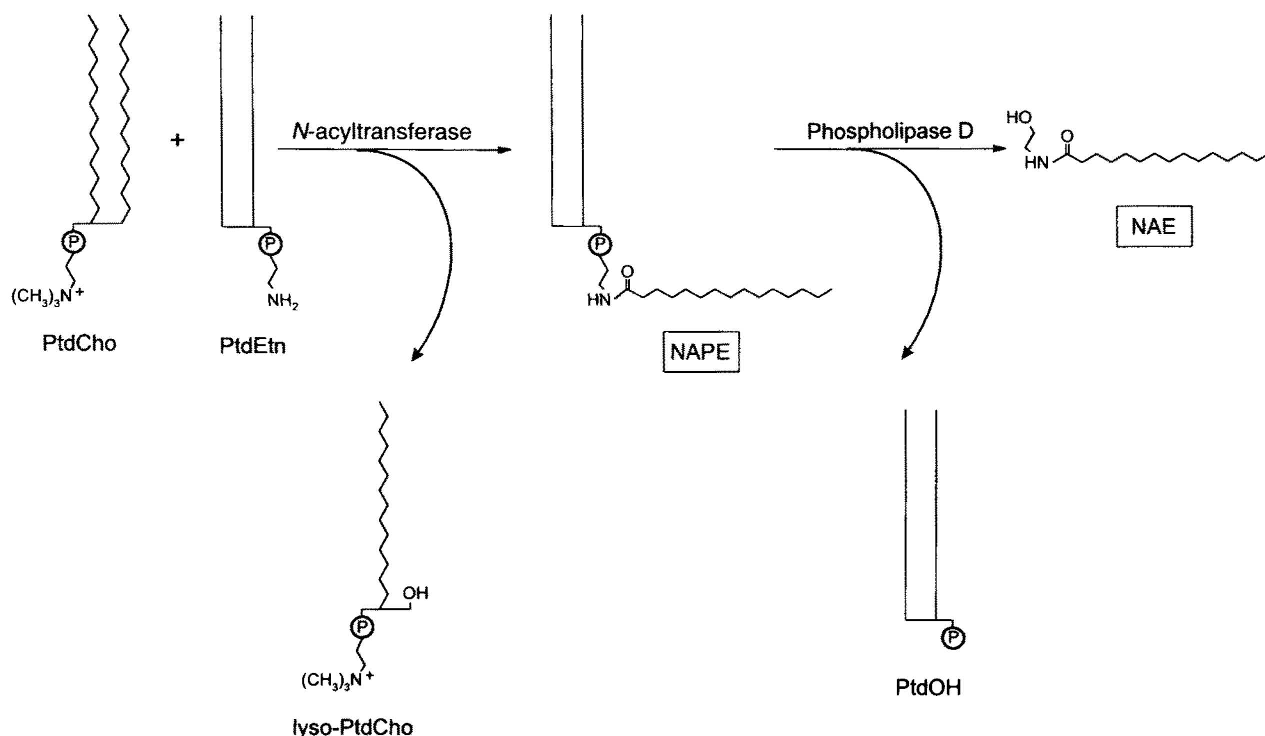

FIG. 1. The formation of *N*-acyl-phosphatidylethanolamine (NAPE) and *N*-acylethanolamine (NAE) catalyzed by the enzymes *N*-acyltransferase and NAPE-hydrolyzing phospholipase D. PtdCho, phosphatidylcholine; PtdOH, phosphatidic acid; PtdEtn, phosphatidylethanolamine.

glutamate receptor subtypes are present (22). However, as can be seen from Figure 2, the background level (= control value) of ^{14}C-ethanolamine-NAPE and ^{14}C-ethanolamine-NAE increased with time in culture, maybe indicating that the number of injured/aged cells increased with time in culture. In order to test whether ^{14}C-arachidonoylethanolamine (= anandamide) could also be formed in the neocortical neurons, the neurons were prelabeled for 22 h with ^{14}C-arachidonic acid, and then exposed to 100 μM NMDA. Figure 3 shows that ^{14}C-anandamide as well as ^{14}C-arachidonoyl-NAPE accumulated in a linear fashion with time for up to 24 h. This confirms the results of Di Marzo *et al.* (7) and extends these studies by showing that the NMDA-receptor-stimulated formation of anandamide is steadily increasing for a rather long period of time as also found for NAE in ^{14}C-ethanolamine-labeled neurons (18). These results, as well as others, (2,19,23,24) suggest that NAPE and NAE, including anandamide, are formed in response to cell injury and that anandamide accounts for less than 5–10% of the NAE formed, i.e., a rather nonspecific formation for a putative signaling molecule. This leads to the assumption that the other endogenous ligand for the cannabinoid receptors, 2-arachidonoyl-glycerol, may turn out to be a more physiologically relevant ligand for the cannabinoid receptors than is anandamide (5,24–30).

Proposed biological function of NAPE and NAE in neurotoxicity. In agreement with the original proposal of Schmid *et al.* (2) that NAPE is formed in response to cell injury, we have proposed that in neuronal tissue NAPE and NAE (including a minor amount of anandamide) are formed in response to neuronal injury and that this formation in the brain may be an indication of neurotoxicity (5,14,18). Formation of these two compounds may have a cytoprotective function in the brain. A number of the reported effects of both NAPE and NAE may be recognized as being cytoprotective, e.g., (i) NAPE can stabilize synthetic phospholipid membranes (31,32); (ii) *N*-oleoylethanolamine protects against increased calcium ion permeability of isolated damaged mitochondria (33), and *N*-palmitoylethanolamine was reported in some special cases to protect cerebellar granule neurons against excitotoxic death (34); (iii) the saturated NAE have antiinflammatory properties (2,35); (iv) anandamide can inhibit synaptic release of glutamate *via* activation of a cannabinoid receptor (36); and (v) *N*-oleoylethanolamine can potentiate staurosporine-induced apoptosis in embryonic chick neuronal cultures (37). All of these effects of NAPE and different species of NAE tend to reduce the impact of a local injury within the brain, whether acting directly on the injured neurons or by acting on neighboring cells, thereby inhibiting the spread of a local injury.

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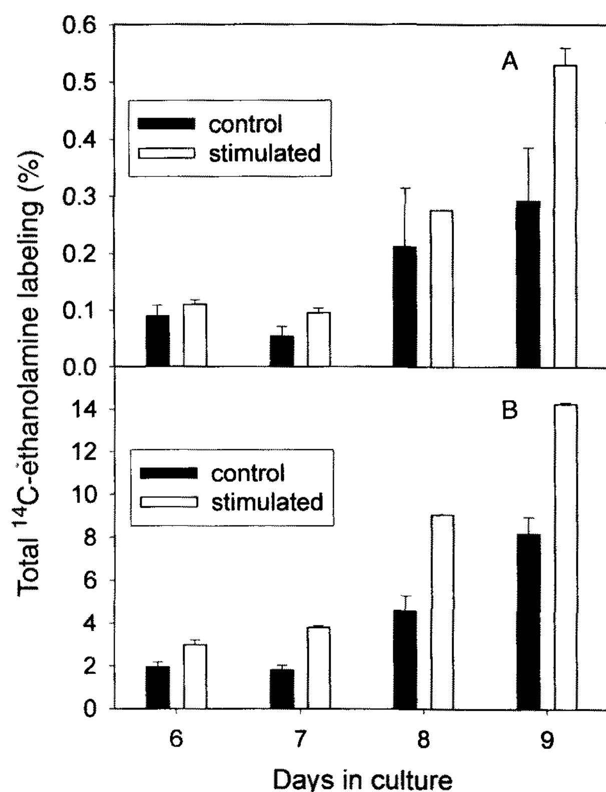

FIG. 2. N-Methyl-D-aspartate (NMDA)-induced formation of (A) ^{14}C-ethanolamine-NAE and (B) ^{14}C-ethanolamine-NAPE in neocortical neurons maintained in primary culture for different periods of time. The neurons were prelabeled with ^{14}C-ethanolamine for 22 h and then exposed to 100 μM NMDA for 2 h. Lipids were extracted and separated on thin-layer chromatography (TLC) as previously described. Results are from one of two experiments, each performed in duplicate. See Figure 1 for other abbreviations.

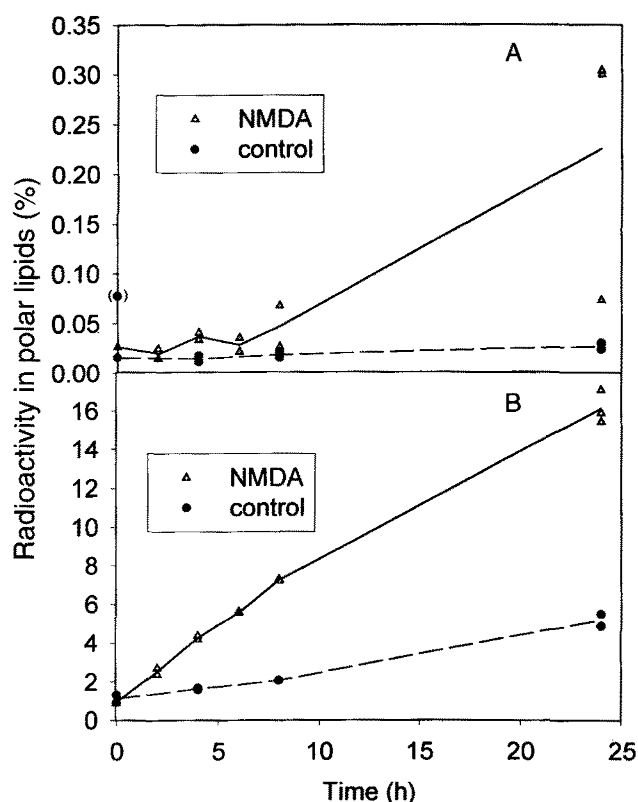

FIG. 3. NMDA-induced formation of (A) ^{14}C-anandamide and (B) ^{14}C-arachidonoyl-NAPE in neocortical neurons, day 7 in culture. The neurons were prelabeled with 1.5 μCi ^{14}C-arachidonic acid per 6×10^6 cells for 22 h and then exposed to 100 μM NMDA for various periods of time. Lipids were extracted and separated on two-dimensional TLC as previously described (14,18). Results are from one of two experiments, each performed in duplicate or triplicate. See Figures 1 and 2 for abbreviations.

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The Pool of Fatty Acids Covalently Bound to Platelet Proteins by Thioester Linkages Can Be Altered by Exogenously Supplied Fatty Acids

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ABSTRACT: The goals of this investigation were, first, to develop a chemical strategy to identify and quantitate the mass of fatty acid which is covalently bound to proteins by thioester linkage in unactivated platelets, and, second, to determine whether exogeneously added fatty acids can alter the fatty acid composition of thioester bound fatty acids. Studies with radiolabeled fatty acids cannot identify and quantitate the actual fatty acids bound to proteins because they permit analysis of only the radiolabeled fatty acids added and their metabolites. Therefore, in the absence of metabolic labeling by radiolabeled fatty acids, we isolated the thioester-linked fatty acids from platelet proteins using hydroxylamine at neutral pH to form fatty acid hydroxamates. The hydroxamates were subsequently converted to fatty acid methyl esters by acid methanolysis for quantitation by gas chromatography–mass spectrometry. Using platelet specimens from 14 subjects, 74% of the fatty acid recovered from the unactivated platelet proteins as thioester linked was palmitate. Importantly, however, 22% was stearic acid, and oleate was 4% of the total thioester bound fatty acid. There was minimal variability (2.6-fold at maximum) between the subjects in the amount of the thioester-linked palmitate and thioester-linked stearate. However, there was substantial variability (>100-fold at maximum) between subjects in the amount of thioester-linked oleate. We also demonstrated that incubation of platelets with exogenous fatty acids can alter the profile of fatty acids bound to platelet proteins by thioester linkages. Incubation of platelets with 100 μM palmitate for 3 h increased the amount of thioester-linked palmitate by up to 26%, and incubation of platelets with 100 μM stearate increased the amount of thioester-linked stearate up to 30%. In support of the observation that radiolabeled fatty acids other than palmitate were shown to be capable of binding to platelet proteins by thioester linkage, our results indicate that the fatty acids actually bound to unactivated platelet proteins include a significant amount of stearate, and variable amounts of oleate, as well as palmitate. In addition, the data show that palmitate and stearate can be increased, as a percentage of total protein-bound fatty acid, by incubation with exogenous palmitate and stearate, respectively.

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Abbreviations: GC–MS, gas chromatography—mass spectrometry; GPI, glycosylphosphatidylinositol; FAME, fatty acid methyl ester; PGE_1, prostaglandin E_1.

Fatty acids can become covalently bound to proteins by three different types of linkages (reviewed in Refs. 1–4). First, fatty acids can be linked to proteins by way of an amide linkage, and the fatty acid found in this type of linkage is almost exclusively myristate (14:0). Second, fatty acids can become bound to proteins by way of thioester linkages. Fatty acids able to bind with this linkage include myristate (5), palmitate (16:0) (the most commonly studied fatty acid in thioester linkage (1–4)), stearate (18:0) (1–4), oleate (18:1) (6), arachidonate (20:4n-6) (7), docosatetraenoate (22:4n-6) (7), eicosapentaenoate (20:5n-3) (7), and docosahexaenoate (22:6n-3) (7). Fatty acids may also be bound covalently to proteins indirectly in glycosylphosphatidylinositol (GPI) anchor proteins (reviewed in Refs. 1–4). In this situation, the fatty acid is associated by an O-ester linkage with a phospholipid moiety which is linked through a variety of sugars to a protein. Although the fatty acid is not directly bound to the protein, in the isolation of total protein from the cells, the fatty acid is recovered. In trypanosomes, the common fatty acid in O-ester linkage in GPI anchor proteins is myristate (8). However, in other organisms the fatty acids in O-ester linkages of GPI anchor proteins are highly varied (3,4).

Nearly all previous studies on fatty acid acylation of proteins identified which fatty acids can become covalently bound to proteins and not the actual fatty acids that are bound to proteins in the native state. In studies with radiolabeled fatty acids, the fatty acids identified as protein-bound will only be those radiolabeled fatty acids added to the cells and their metabolites. If the radiolabeled fatty acids incorporated by the cells do not represent the fatty acids actually bound to protein, then an incorrect conclusion will be drawn concerning the identity of the fatty acids bound to the proteins. This

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is a particularly important question with regard to thioester linked fatty acids because so many different fatty acids have been shown to be capable of binding to proteins in thioester linkage. If 16:0 is added to platelets, with covalent binding of the fatty acid and its metabolites to proteins, the fatty acids detectable will include 16:0, 18:0, and 18:1. This is a very limited number of fatty acids relative to the number of naturally occurring fatty acids. If 14:0 is added to platelets, the fatty acids detectable will include primarily 14:0 and 16:0. If 20:4n-6 is added to the cells, 20:4n-6 and 22:4n-6 may be detected, and if 20:5n-3 is added to the cells, 20:5n-3 and 22:6n-3 are detectable.

There are two major limitations to identifying and quantitating the fatty acids bound to cellular proteins by thioester-linkage in cells in the native state. First, the small mass of thioester-linked fatty acid presents a major limitation to identifying and quantitating the fatty acids. A second obstacle is the isolation of thioester-linked fatty acids away from O-ester-linked fatty acids in GPI anchor proteins. A common means to liberate thioester-linked fatty acids from proteins is alkaline methanolysis, which removes both thioester-linked and O-ester-linked fatty acids (1–4). Thus, this method cannot be used to selectively evaluate the thioester-linked fatty acid pool. To selectively remove thioester-linked fatty acids from proteins and allow O-ester-linked fatty acids to remain bound, it is necessary to use hydroxylamine at neutral pH. Hydroxylamine at neutral pH will specifically liberate thioester-linked fatty acids from protein (9), but it does not lead to formation of fatty acid methyl esters (FAME) which are detectable and quantifiable by gas chromatography (GC). Instead fatty acid hydroxamates are formed. Thus, the efficient chemical conversion of a very small mass of fatty acid hydroxamates to FAME is necessary for detection and quantitation of the fatty acids bound to platelet proteins in thioester linkage. We performed this analysis in the present studies, creating fatty acid hydroxamates and then FAME from the thioester-linked fatty acids bound to platelet proteins. We determined that the fatty acids actually covalently bound to platelet proteins by thioester linkage include stearate with highly variable amounts of oleate, as well as palmitate. Most importantly, however, we showed that the percentages of palmitate and stearate thioester linked to platelet proteins can be altered by incubation of the platelets with palmitate or stearate. In model membranes an increase of two carbons in fatty acid chain length greatly increases the time required for flip-flop and greatly decreases (by approximately 10-fold) the rate of dissociation of the fatty acids from the bilayer (10). This raises the possibility that proteins with the same primary amino acid sequence may be bound to membranes with different affinities because of differences in thioester-bound fatty acids which influence membrane binding.

MATERIALS AND METHODS

Isolation of the washed platelet suspension. Blood (6 mL) was collected from fasting subjects in acid citrate dextrose. Then 0.28 mM prostaglandin E_1 (PGE_1) was added to the blood, and the sample was centrifuged at 137 × *g* for 15 min at 37°C. The platelet-rich plasma (PRP) was aspirated from a total of eight samples, pooled, and subsequently centrifuged at 1230 × *g* for 15 min at 37°C. The supernatant from the centrifugation was aspirated, and the platelet pellet was resuspended in 10 ml of washing solution [36.0 mg fatty acid free bovine serum albumin (BSA) in 10 mL solution A] (10 mM HEPES, 0.14 M NaCl, 2.5 mM KCl, 0.1 mM $MgCl_2$, 0.1% glucose, 0.5 mM NaH_2P0_4, 10 mM $NaHC0_3$, pH 7.4), containing 0.28 mM PGE_1 supplemented with 50 µL 200 U/mL apyrase.). The resuspended platelet pellet was then centrifuged at 1000 × *g* for 15 min at 37°C. The supernatant from this centrifugation was aspirated, and the platelet pellet was washed an additional two times using the washing solution without bovine serum albumin.

Incubation of platelets with fatty acids. In some experiments, the platelets were incubated for 3 h at 37°C at this point with either 100 µM palmitate or 100 µM stearate in the albumin-containing washing solution described above. An aliquot was removed for a platelet count. The final pellet was resuspended in 0.5 ml SDS buffer (2.0% SDS, 62.5 mM Tris-HCl, pH 6.8). The platelet suspension in SDS was heated in boiling water for 5 min.

Removal of noncovalently bound fatty acids. To precipitate the platelet proteins and remove the lipids noncovalently associated with the proteins, the denatured platelet solution was mixed with 15 mL of $CHCl_3$/MEOH (2:1, vol/vol). The mixture was vortexed and incubated for 30 min at room temperature. The sample was vortexed again for 1 min and centrifuged at 4,468 × *g* at room temperature for 15 min. The supernatant was aspirated and discarded. At that point, 15 mL of $CHCl_3$/MEOH (2:1, vol/vol) was added to the pellet with vortexing for 1 min. The mixture was incubated for 5 min at room temperature, after which the sample was centrifuged at 4,468 × *g* at room temperature for 15 min. The supernatant was aspirated and discarded. The extraction sequence described above was repeated using 15 mL of $CHCl_3$/MEOH (2:1), $CHCl_3$/MEOH (1:2), $CHCl_3$/MEOH/H_2O (1:1:0.3) and MEOH. The final precipitate was dissolved in 1.0 mL guanidine HCl (6M) containing 20 mM TRIS-HCl and 0.02% Na_2 EDTA. An aliquot was removed for protein determination.

Release of protein-bound fatty acids and extraction of released fatty acid hydroxamates. To release the thioester-linked fatty acids from the platelet proteins by neutral hydroxylamine treatment and extract fatty acid hydroxamates, the protein precipitate from the platelets was divided into two aliquots. To one aliquot of platelets was added hydroxylamine and 4 nmol of 17:0 acyl-CoA as the thioester-linked internal standard. This was denoted as the "sample." To the second aliquot of platelet protein precipitate, 4 nmol of 17:0 acyl-CoA without hydroxylamine was added. This was denoted as the "sample blank." In place of hydroxylamine, 1 mL of 2 M TRIS-HCl was added. There were two additional blanks which contained no platelets. One blank included 1 mL of hydroxylamine with 1.0 mL of 6 M quanidine HCl to account

for any contamination introduced by hydroxylamine (the "hydroxylamine blank"). The final blank (the "TRIS blank") included 1 mL of TRIS buffer with 1.0 mL of 6 M guanidine HCl to account for any contaminating fatty acids introduced into the "sample blank" which was dissolved in 2 M TRIS-HCl rather than hydroxylamine. All tubes were vortexed gently and incubated for 18 h at room temperature. To the sample and the three blanks was added 2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol). The four tubes were then vortexed for 1 min and centrifuged at 4,468 × *g* for 15 min at room temperature. This resulted in the formation of two phases. The infranatant was removed and saved, and 2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol) was added to the remaining supernatant. The sample was vortexed for 1 min and then centrifuged at 4,468 × *g* for 15 min at room temperature. The infranatant was again collected. This process was repeated one additional time, such that for each tube, three infranatants were collected and dried completely under nitrogen. The combined dried infranatants from each tube were resuspended in 0.2 mL $CHCl_3$/ethyl acetate (1:1, vol/vol) and vortexed vigorously.

Conversion of fatty acid hydroxamates into FAME by acid methanolysis. The tube was centrifuged at 1,115 × *g* for 5 min at room temperature. The 0.2 mL supernatant was transferred to a hydrolysis tube. The original centrifuge tube was then rinsed with another 0.2 mL of $CHCl_3$/ethyl acetate (1:1, vol/vol), vortexed, and centrifuged again at 1,115 × *g* for 5 min at room temperature. The 0.2 mL rinse was added to the hydrolysis tube. The combined contents of the hydrolysis tube were dried completely under nitrogen. At that point, 1 mL of 3 M methanolic HCl was added to the hydrolysis tube which was then closed and gently agitated. The tube was then incubated at 100°C for 24 h. After cooling to room temperature, the contents of the tube were transferred to a vial for extraction three times with 1.0 mL hexane. In each 1.0 mL-extraction with hexane, the tube was vortexed vigorously. The upper hexane phase was aspirated each time and the three extracts were pooled. To these extracts was added 4 nmol of methyl 15:0 as a second internal standard to account for evaporation losses. Each specimen was then dried completely under nitrogen and resuspended in 10 μL hexane, and 1 μL of the 10 μL was then injected into a Hewlett-Packard 5890 series II model gas chromatograph-mass spectrometer (GC–MS) with a Hewlett-Packard 5971 mass spectrometer (Hewlett-Packard, Wilmington, DE). The column was a Supelcowax 10 capillary column (Supelco, Bellefonte, PA). The injector temperature was 260°C with a detector temperature of 280°C. The flow rate of the carrier gas was 0.7 ml per minute, and the carrier gas was helium. To elute the FAME from column, the GC was programmed to initiate elution at 150°C for 2 min. At that point the temperature was increased 10°C per minute to 200°C where it was held for 4 min. At that time, the temperature was increased at 5°C per minute to 240°C and held for 3 min. Finally, the temperature was ramped at 10°C per minute to 270°C where it was held for 6 min. This temperature program eluted all of the FAME. For each of the analyses, single ion monitoring using ions 55, 67, 69, 74, 79, and 91 was performed. Total ion scans were also run at 50–500 AMU. The fatty acids in thioester linkage were quantitated by determining (the pmol fatty acid in the sample) minus (the pmol fatty acid in the hydroxylamine blank) minus (the pmol fatty acid in the sample blank) minus (the pmol fatty acid in the Tris blank)/mg platelet protein. The 15:0 methyl ester blank was included to account for changes in FAME concentration as a result of evaporation, but because the recovery of the 15:0 methyl ester was found to be greater than 95% in all cases, it had a neglible impact on the calculations. The 17:0 fatty acyl-CoA, therefore, which contains a fatty acid thioester linked to CoA, was the internal standard used to account for essentially all losses in the procedure.

RESULTS

Figure 1 shows the electron-impact tracing of the GC–MS peaks which had the expected retention times for methyl palmitate and methyl stearate. The mass spectra of authentic standards of methyl palmitate and methyl stearate are shown for comparison with the fatty acid peaks eluting at the appropriate times for methyl palmitate and methyl stearate. The major ion peaks characteristic of FAME and ion peaks unique to methyl palmitate and methyl stearate were found in the methyl palmitate and methyl stearate peaks, respectively. The ions unique to methyl palmitate and methyl stearate were the molecular ions of 270 (M^+) for methyl palmitate and 298 (M^+) for methyl stearate.

Because the amount of fatty acid covalently bound to proteins by thioester linkage was expectedly low, it was important to establish the reproducibility of the measurement for thioester-linked fatty acid. To assess the precision of the analytical method, we performed five analyses on one sample of pooled platelets. For the quantitation of thioester-bound palmitate, the mean value for the sample tested was 796 pmol/mg with a standard deviation of 62 pmol/mg. The coefficient of variation was calculated to be 7.8%. A similar result was obtained for the quantitation of thioester-linked stearate. The mean value was 267 pmol/mg, and the standard deviation was 18 pmol/mg, resulting in a coefficient of variation of 7.0%. Thus, the measurement showed excellent reproducibility, and, therefore, major differences in values between subjects most likely reflect biological variability rather than analytical variability in the measurement.

The fundamental question addressed in these studies was the identity and quantitation of the mass of fatty acids covalently bound to platelet proteins by thioester linkage. Table 1 shows the results of this quantitation from 14 normal healthy subjects in pmol fatty acid/mg platelet protein, and as percentage of total fatty acid recovered. As shown in the table, the three fatty acids detected were 16:0, 18:0, and 18:1. Although palmitate was the predominant fatty acid, there was a significant amount of stearate which was thioester-linked to platelet proteins. In some subjects, oleate was also recovered, but it represented on average, less than 5% of total fatty acid. Particularly noteworthy, stearate represents a significant

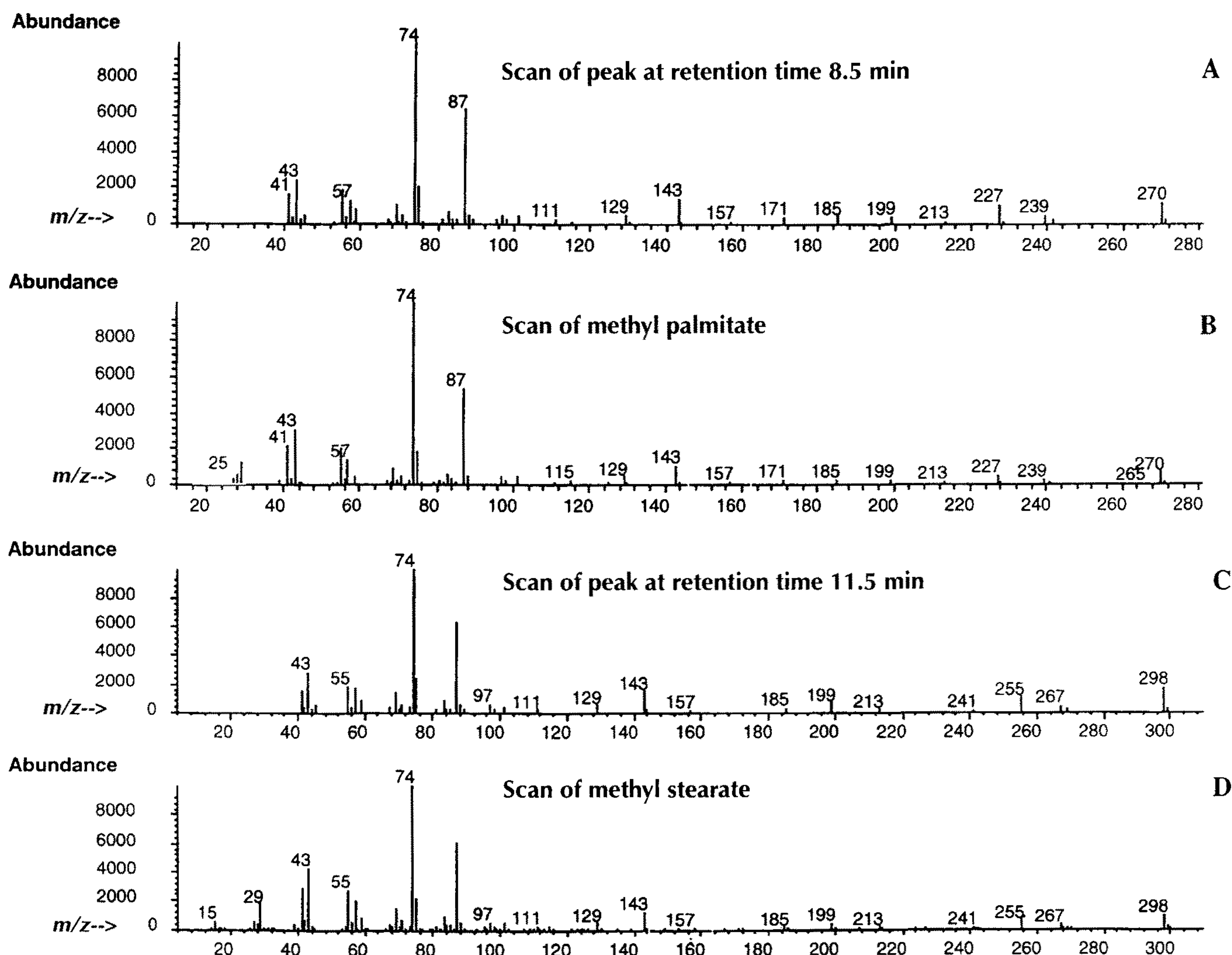

FIG. 1. Gas chromatography—mass spectrometry scans (GC–MS). Electron impact scan from GC–MS of (**A**) peak with retention time of methyl palmitate (8.5 min); (**B**) methyl palmitate standard; (**C**) peak with retention time of methyl stearate; (**D**) methyl stearate standard.

quantity of the fatty acid which is thioester-linked to protein. In studies with radiolabeled fatty acids using radiolabeled palmitate, where stearate generation is dependent on an elongase enzyme activity which converts palmitate to stearate, only trace amounts of stearate, far less than 22% of thioester-linked fatty acid, were found to be protein bound.

Table 1 also shows that there was a relatively modest amount of biological variability in the percentage of thioester-linked palmitate, and even less variability in stearate among the individual subjects. The amount of protein-bound oleate in several of the subjects (numbers 1, 3, 4, 10, and 11) was notably higher than in the others, while some subjects had undetectable levels of oleate. Although it was not possible to determine the basis for this disparity, this finding raised the possibility that the ingestion of dietary fat with a high amount of oleate, such as olive oil, may be responsible for this variability between individuals in fatty acid composition. We therefore asked whether the fatty acid composition bound to platelet proteins by thioester linkage could be modified by exogenously provided fatty acids.

To address this question, platelets were incubated for 3 h with 100 μM palmitate or 100 μM stearate to determine whether incubation with these fatty acids could alter the fatty acid composition of the thioester-linked fatty acids. Figure 2 shows the increase in thioester-linked palmitate after incubation with palmitate, and Figure 3 shows the increase in thioester-linked stearate after incubation with stearate. In each of the three samples, platelets incubated with palmitate showed an increase in the amount of covalently bound palmitate. It should also be noted, although not shown in the figure, that subjects 2 and 3 showed significant increases in the amount of protein-bound stearic acid after incubation with palmitate. This is not unexpected since platelets are able to elongate palmitate to stearate (11). Subject 1 did not show an increase in protein-bound stearate after incubation with palmitate, possibly because the platelets were less effective at elongating palmitate to stearate. The increase in stearate in subjects 2 and 3 after incubation with palmitate was, in absolute amount, less than the increase in palmitate. The increases in thioester-linked stearate after incubation of the

TABLE 1
Quantitation of Fatty Acids Covalently Bound to Platelet Proteins Through Thioester Linkage[a]

	pmol fatty acid/mg protein					
Subject	16:0	(%)	18:0	(%)	18:1	(%)
1	444	(67)	149	(23)	67	(10)
2	453	(77)	135	(23)	n.d.	
3	355	(69)	128	(25)	33	(6)
4	489	(67)	185	(25)	54	(7)
5	731	(78)	192	(21)	11	(1)
6	928	(82)	210	(18)	n.d.	
7	564	(79)	141	(20)	6	(1)
8	860	(78)	232	(21)	7	(1)
9	709	(74)	250	(26)	n.d.	
10	678	(67)	218	(22)	116	(11)
11	779	(63)	277	(22)	178	(14)
12	651	(78)	185	(22)	n.d.	
13	669	(78)	191	(22)	n.d.	
14	565	(77)	173	(23)	n.d.	
MEAN + SEM	634 ± 44	(74 ± 2)	190 ± 12	(22 ± 1)	34 ± 14	(4 ± 1)

[a]n.d., none detected.

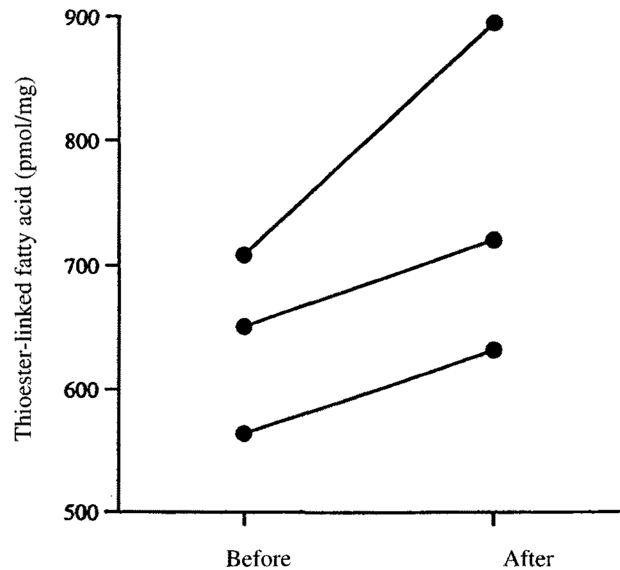

FIG. 2. Effect of palmitate (16:0) incubation on platelet protein fatty acid composition. Change in mass of thioester-linked palmitate after incubation of platelets from three subjects *in vitro* with 100 μM palmitate for 3 h at 37°C.

platelets with 100 μM stearate ranged from 10 to 30% for the three subjects. There was a decrease or no change in the amount of thioester-linked palmitate when the platelets were incubated with stearate. This was expected since incubation of platelets with stearate is unlikely to generate palmitate by retroconversion in the platelet. In some of the GC–MS tracings, arachidonate was identified as a thioester-linked fatty acid. However, the amount detected was too low to quantitate in these studies with unactivated platelets. In previous studies with radiolabeled arachidonate, we showed that arachidonate can become covalently bound to proteins by way of thioester linkages (7). These results raise the possibility that arachidonate becomes covalently bound to proteins only when the platelets are activated. In support of this hypothesis, arachidonate is liberated in substantial quantity from phospholipids upon cell activation, and the free arachidonate concentration in the platelet increases significantly at that time.

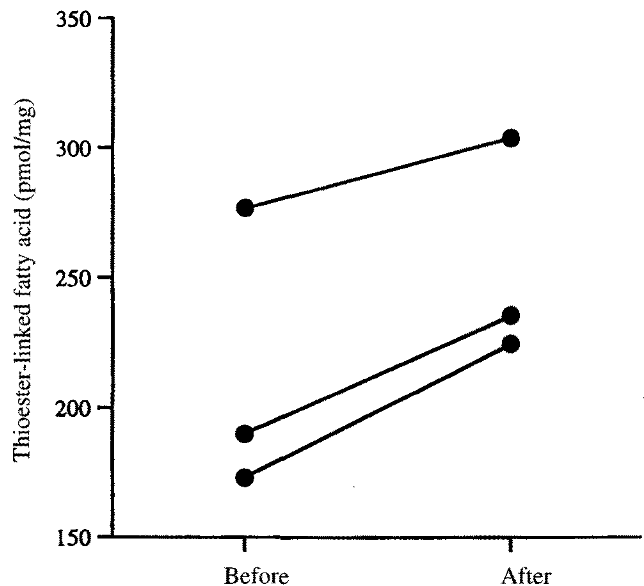

FIG. 3. Effect of stearate (18:0) incubation on platelet protein fatty acid composition. Change in mass of thioester-linked stearate after incubation of platelets from three subjects *in vitro* with 100 μM stearate for 3 h at 37°C.

DISCUSSION

The elaborate methods used in our studies, to identify and quantitate the fatty acids thioesterified to platelet proteins, were required to remove the O-ester-linked fatty acids associated with the GPI anchor proteins from the thioester-bound fatty acids. Amide-linked fatty acids do not represent a potential confounding variable because platelets have little, if any, capacity for protein synthesis, and amide-linked fatty acids are added cotranslationally. In addition, alkaline hydrolysis will remove and methylate the O-ester bound fatty acids from the GPI-linked proteins as well as thioester-bound fatty acids, but not liberate the amide-linked fatty acids (12). In addition to identifying the relative amounts of fatty acids thioester-linked to platelet proteins, these data provide a quantitation in pmol fatty acid/mg platelet protein of the actual amount of fatty acid covalently bound to platelet proteins in thioester linkages. This number is not obtainable in studies with radiolabeled fatty acids because upon incorporation into the cell, the specific activity of the radiolabeled fatty acid is decreased to an undeterminable value by dilution with an unknown amount of unlabeled precursor fatty acid already in the cell. The amount of unlabeled precursor fatty acid is unknown because the precursor fatty acid pool for thioester-linked fatty acids in the cell has not been identified. The data also permit an estimation of the stoichiometry between fatty

acids and platelet proteins. Assuming an average molecular weight of 65,000 daltons for a platelet protein, with the data from our studies showing 858 pmol of thioester-linked (palmitate + stearate + oleate)/mg platelet protein, on average 0.056 molecules of fatty acid are thioester-linked to protein if a single fatty acid is bound to a single protein. Therefore, if a single fatty acid becomes bound to a single protein molecule, approximately one out of every 20 protein molecules in the platelet would be fatty acid-acylated with a thioester linkage. It is quite possible, however, that at any given time, certain proteins are acylated with more than one fatty acid. Thus, one out of 20 protein molecules acylated with a thioester-linked fatty acid represents a maximal estimate.

The results of our studies indicate that the fatty acids thioester-linked to platelet proteins in the unactivated state include stearate and oleate as well as palmitate. The studies also demonstrate that exogenously presented fatty acids can augment the mass of thioester-linked fatty acid. There is evidence that differences in fatty acid chain length and unsaturation affect the ability of fatty acids to promote protein binding to membranes (13–15). The significance of stearate as a fatty acid bound in thioester linkage to protein is that the additional two carbons on the saturated chain relative to palmitate may enhance its ability to promote protein binding to membranes. In studies evaluating the dissociation of fatty acids from phospholipid bilayers, the rate constants (Koff $(s)^{-1}$) for fatty acid desorption from bilayers were 39.0 for myristate, 8.2 for palmitate, and 0.5 for stearate (10). Thus, there is a 16.4-fold difference in the strength of the association for the phospholipid bilayer between palmitate and stearate. Accordingly, a change from palmitate to stearate or palmitate to oleate may significantly impact the function of a protein without any alteration in the primary amino acid sequence of the protein molecule.

There are at least three studies which support our finding that stearate is not a trace fatty acid among thioester-linked fatty acids. In a study involving a single platelet protein, P-selectin was isolated from human platelets, and by GC–MS it was shown that stearate as well as palmitate was thioester-linked to the isolated P-selectin molecule (16). In work by Zeng *et al.* (17), it was demonstrated that asialoglycoprotein receptor subunits contained both thioester-linked palmitate and stearate. The relative amounts of palmitate and stearate covalently bound in thioester linkage to the asialoglycoprotein receptor could not be determined, but the data suggest that stearate is unlikely to be a trace fatty acid. Although these results were obtained with individual proteins, they are consistent with the results of this investigation which identified and quantitated the fatty acids bound to all platelet proteins. In a third study, Berthiaume and Resh (18) reported on the biochemical characterization of a palmitoyl acyltransferase activity that palmitoylates myristylated proteins. They evaluated the fatty acyl-CoA specificity of the palmitoyl acyltransferase enzyme preparation. The investigators found, as expected, that unlabeled palmitate was the most effective fatty acid at inhibiting the acylation of radiolabeled palmitate to their substrate. Importantly, however, they showed that stearate was also effective in inhibiting the acylation of radiolabeled palmitate to their substrate, indicating that stearate is an acceptable substrate for palmitoyl acyltransferase. Taken together, our results indicate that stearate, as well as palmitate, is covalently bound in thioester linkage to platelet proteins and that exogenously presented fatty acids can alter the fatty acid composition of the thioester-linked fatty acid pool.

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The Influence of a Novel Antioxidant Fatty Acid on the Development of Stenosis After Balloon Injury

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The implantation of coronary stents reduces restenosis significantly, but it is still about 20%. Despite many studies, no pharmacological agent was found to reduce the occurrence of restenosis. Here we report that a sulfur-containing fatty acid analog tetradecylthioacetic acid (TTA) [CH_3-$(CH_2)_{13}$-S-CH_2-COOH], which has triglyceride and cholesterol-lowering effects and itself acts as an antioxidant, is effectively reducing the development of restenosis in the rabbit iliac artery after balloon injury (overstretching). Twelve rabbits in each group were re-examined 6 wk after balloon injury. Angiography showed 43% stenosis in the placebo group against 23% for the TAA-treated group. The iliac artery dilated by balloon angioplasty showed a stronger response in intima hyperplasia for the TTA group. The mean of minimal luminal diameter (MLD) in the placebo group was 1.08 whereas in the TTA group the MLD was 1.60.

We examined whether this could be a direct effect on smooth muscle cells and endothelial cells by TTA. Human smooth muscle cells and human endothelial cells were cultured in the presence and absence of TTA. We find that TTA inhibits the proliferation rate of smooth muscle cells, but not endothelial cells *in vitro.*

The data show that TTA inhibits restenosis, and this may be due to reduced proliferation of smooth muscle cells. Evidently TTA may be of potential interest for a new therapeutic approach to atherosclerosis and restenosis, as it is an antioxidant itself and influences the antioxidant dependence status.

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Abbreviations: MLD, minimal luminal diameter; TTA, tetradecylthioacetic acid.

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Effects of β-Oxa and β-Thia Polyunsaturated Fatty Acids on Agonist-Induced Increase in Endothelial Cell Adhesion Molecules

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Fish oils contain an abundance of n-3 fatty acids derived from linolenic acid (18:3n-3), in particular eicosapentaenoic (20:5n-3) and docosahexaenoic acids (22:6n-3). Both of these fatty acids are generally found in low concentrations in nonmarine animals although 22:6n-3 is found in high concentrations in certain specialized tissues such as retina and spermatozoa. There is increasing evidence that the beneficial effects of fish diets are related to the presence of n-3 fatty acids. Thus the ingestion of these fatty acids is thought to be responsible for the lower incidence of cardiovascular disease in certain human populations (1). It has been suggested that they may exert their antiinflammatory effects by inhibiting the 5-lipoxygenase pathway in neutrophils and monocytes. However, some fatty acids, notably *cis* monounsaturated (e.g., oleic acid) and n-6 fatty acids (arachidonic acid), stimulate superoxide production in these cells and could therefore be expected to increase the inflammatory response and cardiovascular homeostasis, resulting in myocardial injury.

Recent findings from our laboratory showed that, unlike the n-3 and n-6 polyunsaturated fatty acids (PUFA), the 15-hydroperoxy derivatives such as HPETE were much more active than the parent fatty acids in depressing tumor necrosis factor (TNF) α-induced increase in the expression of adhesion molecules in endothelial cells (2). This is interesting and of potential therapeutic importance in the treatment of intravascular inflammation since HPETE lacked leukocyte-activating properties, i.e., ability to stimulate neutrophils and macrophages to adhere to the endothelium, release oxygen radicals, and release lysosomal enzymes (3). As such these are devoid of some of the proinflammatory activities of the free fatty acids such as 20:4n-6, 20:5n-3, and 22:6n-3. However the instability of HPETE would preclude its use in any form of therapy.

We have now been able to achieve the synthesis of some compounds (4,5) which have the advantage in that they are not readily β-oxidized and show high levels of intracellular stability. Some of these have similar biological characteristics as the HPETE, such as inhibition of cytokine production and inability to stimulate neutrophil oxygen radical response. This group of novel PUFA has an oxygen atom in the β-position: β-oxa-derivatives; β-oxa-23:4n-6, β-oxa-21:3n-3, β-oxa-21:3n-6 or a sulfur atom in the β-position: β-thia-derivatives; β-thia-23:4n-6, β-thia-21:3n-2, β-thia-21:3n-6. These were examined for antiinflammatory effects in relation to activation of endothelial cell adhesion activity.

Pretreatment of human umbilical vein endothelial cells (HUVEC) with these engineered PUFA markedly reduced their ability to be stimulated by TNF-α, lipopolysaccharide (LPS), or phorbal myristate acetate for enhanced neutrophil adhesion in a time- and concentration-dependent manner, with the β-oxa-PUFA being more potent than the β-thia-PUFA. In contrast, 20:4n-6, 20:5n-3, and 22:6n-3 acids had no significant effect on the stimulus-induced adhesion of leukocytes to HUVEC. The cells remained viable throughout incubations with all fatty acids and recovered from these inhibitory effects. β-Oxa-23:4n-6 caused the greatest attenuation of TNF-α or LPS-induced neutrophil-HUVEC adhesion and was therefore used in subsequent studies. Derivatives of β-oxa-23:4n-6 (including saturated and methyl ester forms) had no inhibitory effect on TNF-α-stimulated adhesion, highlighting the importance of the parent β-oxa-23:4n-6 structure. The β-oxa-23:4n-6 inhibitory activity on adhesion was reflected in the ability of this β-oxa-PUFA to decrease the TNF-α-induced expression of the following endothelial cell adhesion molecules: intercellular adhesion molecule-1, E-selectin, and vascular cell adhesion molecule-1. TNF-α-induced increased expression of E-selectin mRNA was found to be significantly depressed by β-oxa-23:4n-6 to the three oxygenated metabolites, one being tentatively identified as a hydroxylated derivative of β-oxa-23:4n-6. β-Oxa-23:4n-6 inhibition of TNF-α-enhanced neutrophil adherence to HUVEC was abolished when the cells were preincubated with nordihydroguaiaretic acid (lipoxygen-ase inhibitor) but not with indomethacin (cyclooxygenase inhibitor). This suggests that conversion of β-oxa-23:4n-6 to an oxygenated product(s) *via*

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Abbreviations: HUVEC, human umbilical vein endothelial cells; LPS, lipopolysaccharide; PUFA, polyunsaturated fatty acid; TNF-α, tumor necrosis factor-α.

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the lipoxygenase pathway mediates its action. We are continuing to investigate the inhibitory mechanism(s) of β-oxa-23:4n-6 on leukocyte-HUVEC adhesion.

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Possible Mechanisms for the Putative Antiatherogenic and Antitumorigenic Effects of Conjugated Polyenoic Fatty Acids

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Conjugated polyenoic fatty acids (CPFA) are derived from the parent polyenoic acid by oxidation or hydrogenation reactions *in vivo* or *in vitro*. They occur naturally in ruminant-derived foods, due to bacterial hydrogenation in the rumen, and in chemically hydrogenated vegetable fats. The most common CPFA are those derived from linoleic acid (CLA) which occur as a mixture of *cis,trans* and *trans,cis* 9,11 and 10,12 isomers; derivatives of other PFA also occur. Originally regarded as detrimental lipid oxidation products, CLA were recently attributed with antiatherogenic and antitumorigenic properties (1). The cell mechanisms underlying these effects are not known. Our finding that CLA induced glutathione peroxidase (GPx) activity in human umbilical vein endothelial cells (HUVEC) could be important (2). This study highlights other possible beneficial mechanisms elicited by CLA in HUVEC and cancer cells.

Methods and results. HUVEC and human prostate cancer cells (PC3) were treated with 0–150 mM CLA (9, 11 and 10, 12 mix) for 24 h. mRNA expression of GPx, phospholipid GPx (PHGPx) and the adhesion molecules ICAM-1 and E-Selectin was assessed by Northern analysis; PHGPx activity by spectral analysis using an NADPH-coupled assay, and diacylglycerol (DAG) by thin-layer chromatography after incubation with ^{14}C arachidonic acid.

In HUVEC, GPx and PHGPx mRNA were increased *circa* twofold with increasing CLA concentrations although enzyme activity was unchanged. CLA (10 mM) inhibited cytokine-induced adhesion molecules (ICAM-1 and E-Selectin) by 50%. In PC3 cells, CLA increased GPx mRNA by about 50% and reduced DAG to *circa* 50% of control at 100 mM.

Conclusions. Induction of intrinsic antioxidant enzyme mRNA and suppression of adhesion molecule mRNA by CLA in vascular and/or cancer cells suggests these fatty acids are potent modulators of gene expression. These findings also emphasize the possibility that such gene modulations are important mechanisms whereby CLA elicit their reported antiatherogenic and antitumorigenic effects. Increased redox enzyme activity protects against free radical-induced cell damage and is implicated in attenuation of stress protein expression like adhesion molecules, upregulation of which are implicated in increased vascular disease. Enhanced DAG production by malignant prostate tissue was regarded as a tumor-promoting factor through protein kinase C activation similar to phorbol ester action. Inhibition of DAG in PC3 cells by CLA could explain, at least in part, their anticancer effects.

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Abbreviations: CLA, conjugated linoleic acid; CPFA, conjugated polyenoic fatty acids; DAG, diacylglycerol; GPx, glutathione peroxidase; HUVEC, human umbilical vein endothelial cells; PC3, prostate cancer cells; PHGPx, phospholipid glutathione peroxidase.

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Docosahexaenoic Acid Supplementation Improves the Moderately Severe Dementia from Thrombotic Cerebrovascular Diseases

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In Japan, the prevalence of dementia of thrombotic cerebrovascular disorders (CVD) is almost the same as that of the Alzheimer type. Supplementation of n-3 polyunsaturated fatty acids (PUFA) improves hemorrheological properties (1) and endothelial function and also might protect neuronal cells, and may result in the improvement of dementia of CVD. Docosahexaenoic acid (DHA), one of the n-3 PUFA, is rich in brain tissue and plays an antithrombotic effect. We administered DHA to the elderly with senile dementia of CVD and evaluated the effect of DHA on dementia using psychometric tests.

Methods. Male and female elderly people (average 83 yr) living in the home for the elderly with mild to moderate dementia of CVD were entered into the study. The controller randomly divided the 20 elderly in two groups according to age, baseline scores of psychometric tests [Mini-Mental State examination (MMSE) and Hasegawa's Dementia rating scale (HDS-R)] and serum fatty acid composition. All the elderly presented MMSE and HDS-R scores of 15–22. Ten elderly received 6 DHA capsules containing 0.72 g of DHA daily for 1 yr (DHA group), and other 10 elderly were registered as a control group. They ate the same food every day and lived together in the same home. Nursing staff gave DHA capsules directly to the elderly three times a day just after meals and ascertained swallowing. Prestudy assessments included clinical history, complete physical and neurological examination, standard laboratory tests, and CT or MRI of brain. All patients were assessed at baseline, and then after 3, 6, and 12 mon of continuous treatment. Assessments were based on psychometric tests (MMSE and HDS-R), as well as clinical evaluation. Serum lipid profiles, red blood cell (RBC) deformability, whole blood viscosity, RBC fragility, platelet aggregation, and fatty acid composition were also monitored after the supplementation of DHA. Informed consent was obtained from each elderly person and his or her family. For statistical analysis, F-test, paired t-test, unpaired t-test, and Wilcoxon signed-ranks tests were used.

Results. No significant pretreatment difference between the two groups emerged for scores of two psychometric tests, age, serum lipid profile, and serum fatty acid composition. In DHA group, scores of HDS-R and MMSE scale improved but in the control group, scores were unchanged or lowered as shown in Table 1 (HDS-R) and Table 2 (MMSE). There was significant difference in the dementia scores 3 to 6 mo after DHA supplementation ($P < 0.05$). In DHA group, the content of DHA and eicosapentaenoic acid (EPA) increased without altering other fatty acids compositions including arachidonic acid (AA). (DHA; before: 5.3 mol%, 3 mon: 6.8, 6 mon: 6.1, 12 mon: 9.5. EPA; before: 3.0, 3 mon: 4.2, 6 mon: 3.9, 12 mon: 5.1. DHA/AA; before: 0.96, 3 mon: 1.19, 6 mon: 1.04, 12 mon: 1.33), but no change of fatty acids compositions was observed in the control group. RBC deformability significantly improved by DHA supplementation ($P < 0.01$). Platelet aggregation by ADP and collagen did not change in both groups after treatment with DHA. There was positive correlation between serum DHA/AA and dementia scores of HDS-R ($n = 32$, $r = 0.44$, $P < 0.05$).

Discussion. In the elderly with moderately severe dementia from thrombotic cerebrovascular disorder, DHA supplementation improved the dementia scores, and this improvement was accompanied with the increase in the content of DHA. This might be mainly derived from the improvement of RBC function, and direct protective effect of DHA on neu-

TABLE 1
Change of Scores of HDS-R in DHA-Supplemented Group [DHA (+)] and in the Control Group [DHA (–)] for 12 Mon

	Before	After 3 M	After 6 M	After 12 M
DHA (+)	17.2 (5.9)	20.6 (6.0) *	19.9 (5.1) *	20.2 (5.6)
DHA (–)	16.3 (6.2)	16.7 (7.5)	16.7 (7.3)	15.3 (7.1)

[a]HDS-R, Hasegawa's Dementia rating scale; DHA, docosahexaenoic acid; M, months. Paired t-test, mean (SD), $*P < 0.05$.

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Abbreviations: AA, arachidonic acid; CVD, cerebrovascular disorders; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; HDS-R, Hasegawa's Dementia rating scale; MMSE, Mini-Mental State Examination; RBC, red blood cell.

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TABLE 2
Change of Mini-Mental State Examination Scale in DHA-Supplemented Group [DHA (+)] and in the Control Group [DHA (–)] for 12 M

	Before	After 3 M	After 6 M	After 12 M
DHA (+)	20.1 (5.6)	21.3 (6.2)	22.2 (5.0) *	21.9 (6.6)
DHA (–)	19.7 (7.1)	19.4 (7.7)	19.6 (5.8)	19.1 (7.5)

[a]See Table 1 for abbreviations. Paired *t*-test, mean (SD), $^*P < 0.05$.

ronal cell death might be one of the other mechanisms. This study is a pilot study. Further study is necessary to clarify whether DHA acts on dementia of CVD in a double-blind manner and also the effect of DHA on other types of dementia from Alzheimer's disease should be evaluated.

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Dietary 18:3n-3 and 22:6n-3 as Sources of 22:6n-3 Accretion in Neonatal Baboon Brain and Associated Organs

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ABSTRACT: The bioequivalence of dietary linolenic acid (LNA) and docosahexaenoic acid (DHA) for brain DHA accretion was measured in neonatal baboons at 4–6 wk of age using stable isotope tracers. Neonates consumed a conventional U.S. term-infant formula devoid of long chain polyunsaturates and with an n-6/n-3 ratio of about 10:1. At 4 wk of age, neonates were dosed with either ^{13}C LNA or ^{13}C DHA. At 6 wk of age, neonate brain, retina, and other organs were harvested for fatty acid and isotopic analyses. The relative accretion of labeled DHA was 7-fold greater as a percentage of dose for the DHA-dosed animals compared to the LNA-dosed animals. The baboon is an omnivore that regularly consumes meat and insects; its plasma lipid profile responds similarly to humans in response to changes in feeding and living habits. These observations suggest that the baboon is a suitable model for human unsaturated fatty acid studies.

It has long been known that n-3 fatty acids cannot be synthesized *de novo* by mammals and must be acquired from the diet. From studies in rats (1,2), primates (3–6), and human infants (7–9), it is now well established that docosahexaenoic acid (DHA) is required for proper function of the brain and retina. The predominant n-3 fatty acid in terrestrial foods is α-linolenic acid (LNA), which can be converted to the functionally important long chain polyunsaturated fatty acids (LCP) eicosapentaenoic acid (EPA), and DHA primarily by a series of alternating desaturations and elongations. DHA can be acquired in the diet from marine foods and is present in all human breast milks. Only recently have commercial infant formulas begun to include preformed DHA, as it has been implicitly assumed that all infants could meet their needs for DHA through biosynthesis from LNA. However, there are no direct measurements of the bioequivalence of dietary LNA as a substrate for DHA accretion compared to dietary preformed DHA in infants or in any primate species.

Dietary fatty acids rapidly affect expression of one of the desaturases, the stearoyl-CoA desaturase (Δ-9), in the mouse (10). In addition, dietary fatty acids have specific and direct effects on gene expression (11–13). Thus, it is likely that the metabolism of tissue fatty acids depends on the mix of dietary fatty acids. Quantitative studies of fatty acid interconversion are best conducted under conditions where the diet of the subjects is controlled. There are no data available on the modulation of desaturases directly relevant to n-6 or n-3 fatty acid metabolism. In the absence of such information, it is reasonable to assume that synthesis of DHA from LNA is downregulated when DHA is present in the diet, which in turn implies that the organism's synthesis of DHA is maximal when DHA is absent from the diet. Further, the bioequivalence under conditions of dietary LNA or DHA excess is of much less practical importance than when DHA is absent and LNA is at marginal levels. It is of most interest to determine the amount of dietary DHA that could replace dietary LNA when LNA levels are low.

In part because the primate brain is more than twice as massive relative to body weight than rodents and other common laboratory species, we sought to study brain DHA accretion directly in primates. Our previous measurements in pregnant baboons showed that doses of [U-^{13}C]-LNA (LNA*) or [U-^{13}C]-DHA (DHA*) result in fetal brain DHA accretion that plateaus at about 1 4 d post-dose (14). Doses directly to a neonate are expected to equilibrate at least as quickly since the tracer enters the neonate bloodstream directly. Also, the apparent plateau observed in fetuses coupled with common experience that DHA deficiency is very difficult to produce in adult animals suggests that DHA is avidly retained by the brain.

The goal of these studies was to determine the bioequivalence of LNA and DHA as substrates for brain DHA accretion in infant primates. Commercially available infant formula, de-

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Abbreviations: DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acids; LCP, long chain polyunsaturated fatty acids; LNA, α-linolenic acid.

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void of LCP and with an n-6/n-3 ratio of about 10:1, was used as a basal diet, and ^{13}C-fatty acids were used to characterize total accretion in brain and other organs.

METHODS

All procedures were approved by the Cornell Institutional Review Board on Animal Use. Six pregnant baboons (*Papio cynocephalus*) were flown from a colony at San Antonio, Texas, to Ithaca and were immediately placed on specially prepared diets balanced in all nutrients with an n-6/n-3 ratio of 10:1 and devoid of LCP, as described in detail previously (14). Pregnant baboons remained on the diets for at least 8 wk prior to birth. Near term (182 d), a caesarean section was performed to avoid nursing, and neonates were placed in a temperature- and humidity-controlled nursery. Neonates consumed a commercial human infant formula obtained at a local supermarket. At 4 wk of age, neonates received oral doses of [U-^{13}C]-labeled fatty acids, LNA* or DHA*, loaded into formula during normal preparation. At 6 wk, brain (occipital cortex), retina, retinal pigment epithelium (RPE), liver, erythrocytes, and plasma were obtained at necropsy, and fatty acids (FA) were extracted by standard methods. Fatty acid methyl esters were prepared and analyzed by gas chromatography and by high-precision mass spectrometry as described in detail elsewhere (15,16).

Isotope ratios and quantitative profiles were used to calculate percentage of dose (dose%) and percentage of total label found in a pool at a particular time point (total%). Units of dose% are useful as normalized measures of isotope tracer relative to tracee. As long as the tracer dose does not interfere with normal physiology (i.e., it is a true tracer), dose% is interpreted as the percent of tracee that appears in a particular pool at a particular time point in a particular form. For instance, a finding that dose 1.6% appears in brain DHA after an LNA dose means that 1.6 of 100 mmol dietary LNA molecules were converted to DHA and appeared in the brain.

RESULTS AND DISCUSSION

No significant differences were found in brain and liver weights between LNA*- or DHA*-dosed neonatal baboons. Brain weight averaged about 8% of total body weight in these 6-wk-old baboons.

No statistically significant differences in FA profiles between LNA* and DHA*-dosed neonates were detected. The brain DHA composition was 9% of total FA, similar to previous findings in fetuses (8.2%) , and consistent with the figure of 7% in human infants . Retinal DHA levels were 16% of total FA again in agreement with human infant data; this tissue was the highest in DHA concentration of those investigated. DHA constituted 1 to 4% of total FA in RPE, liver, plasma, and erythrocyte.

Brain tissue had more than twice the DHA concentration per gram wet tissue (1.35 mg) than liver (0.6 mg). Erythrocytes and plasma had 0.02 and 0.05 mg DHA per mL, respectively, and there were 0.23 and 0.03 mg of DHA per retina and RPE, respectively.

Figure 1 shows the distribution of labeled FA in liver at 2 wk post-dose. DHA* was the predominant labeled FA resulting from both doses. However, the total amount of dose retained was about 2.4% for the DHA* dose compared to about 0.13% due to the LNA* dose. DHA* was overwhelmingly the bulk of the label found due to the preformed DHA* dose, while from the LNA* dose about 30% of the label was detected as 22:5n-3. The ratio of DHA* due to DHA* or LNA* doses was 27:1 in liver.

Figure 2 shows the distribution of labeled FA in brain. Preformed DHA* dose was detected at 1.71%, while 0.23% of the LNA* dose detected as DHA*. Thus preformed DHA was 7-fold more effective than LNA-derived DHA as a source for DHA accretion. The distribution of labeled FA was determined in each tissue. DHA* was >95% of labeled FA in all tissues examined, although a small amount of retroconversion to labeled docosapentaenoic acid (DPA*) and EPA* was ob-

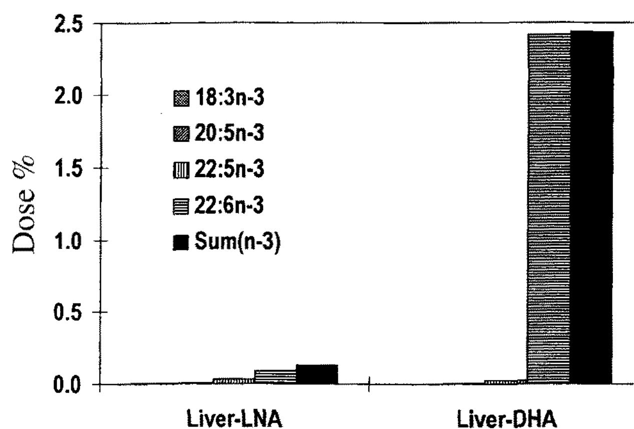

FIG. 1. Liver distribution of labeled fatty acids at 2 wk post-dose, showing (DHA) to be the predominant fatty acid due to either (LNA) or DHA dose, and that 2.4% of the DHA was retained compared to 0.13% of the LNA dose.

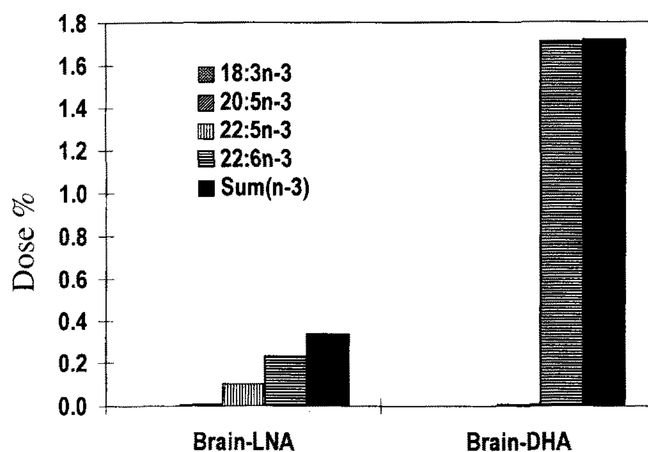

FIG. 2. Brain distribution of labeled fatty acids at 2 wk post-dose showing DHA at 1.7 and 0.23% of the DHA and LAN doses, respectively. See Figure 1 for abbreviations.

served. For the LNA*-dosed animals, the distributions of labeled FA metabolites for LNA*-dosed animals were in the order DHA>DPA>EPA>LNA, except for erythrocytes, where the order was DPA>DHA>EPA>LNA. In LNA*-dosed animals, DHA* was greater than 60% of labeled FA in tissues except erythrocytes, where DPA was 55%.

From these data we have estimated that brain DHA turnover is <5% per week between 4 and 6 wk of age . For retina and RPE, respectively, preformed DHA was found at 12- and 15-fold greater concentration than LNA-derived DHA. Liver, plasma, and erythrocytes ratios were 27, 29, and 51, respectively, showing that blood pools do not parallel tissue handling of a single dose of n-3 FA. These are the first direct measurements of the bioequivalence of DHA and LNA in neonatal primates.

There is considerable species variation in desaturation activity. Rodents are thought to be prodigious desaturators, while cats have long been known to have poor capability to synthesize 22:6 from precursors (19). In cats, this limitation is manifest in abnormal electroretinograms when young animals consume diets devoid of n-3 LCP, even though arachidonate levels are maintained (20). Presumably, this is related to the normal feline diet of meat and organs, which is rich in LCP. A trend thus emerges suggesting that biosynthesis of LCP from C_{18} precursors is species-specific and is related to the natural diet, with herbivores capable of rapid desaturation and carnivores less capable. The human is an omnivore whose conversion capability is expected to be between these extremes.

Baboons have been observed extensively in the wild. They are omnivorous primates whose diet includes insects and vertebrate small animals such as hares, birds and eggs, rodents, and newborn mammals (21,22). Baboons blood lipids, primarily plasma cholesterol and high density lipoprotein cholesterol, are known to be related to their incidence of atherosclerosis, and their responses to diets of differing FA composition parallel humans (23,24). Their feeding behaviors and endocrine responses are related to social dominance and appear to reflect social stress (25–31). Baboons frequenting a human dumpsite and consuming discarded human processed foods (including meats and high-energy desserts), gained weight rapidly, became lethargic, and reached puberty early (32). They also contracted bovine tuberculosis from infected meat (33). All these parallels to human physiology suggest that the baboon may be a highly relevant model for physiological processes that are related to dietary and environmental factors.

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